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The PtdIns3-phosphatase MTMR3 interacts with mTORC1 and suppresses its activity

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Macroautophagy is a major intracellular degradation system. We previously reported that overexpression of phosphatase-deficient MTMR3, a member of the myotubularin phosphatidylinositol (PI) 3-phosphatase family, leads to induction of autophagy. In this study, we found that MTMR3 interacted with mTORC1, an evolutionarily conserved serine/threonine kinase complex, which regulates cell growth and autophagy in response to environmental stimuli. Furthermore, overexpression of MTMR3 inhibited mTORC1 activity. The N-terminal half of MTMR3, including the PH-G and phosphatase domains, was necessary and sufficient for these effects. Phosphatase-deficient MTMR3 provided more robust suppression of mTORC1 activity than wild-type MTMR3. Furthermore, phosphatase-deficient full length MTMR3 and the phosphatase domain alone were localized to the Golgi. These results suggest a new regulatory mechanism of mTORC1 in association with PI3P.

Keywords: autophagy; MTMR3; mTOR; mTOR complex1; PI3P; Ptdlns3P phosphatase

Macroautophagy (hereafter referred to as autophagy) is a major intracellular degradation system that depends on lysosomes. It responds to multiple extracellular and intracellular stimuli, and the main axis is regulated by mTORC1 [1–3]. The mechanistic target of rapamycin (mTOR, also called mammalian target of rapamycin) is an evolutionarily conserved serine/threonine kinase that regulates cell growth and metabolism in mammals. mTOR forms two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [1,2,4,5]. mTORC1 is a master regulator of cell growth, proliferation, and protein synthesis. By contrast, mTORC2 is responsible for control of cell survival, metabolism, and cytoskeletal organization [2,5–9]. In mammals, mTORC1 also contains regulatory-associated protein of mTOR (Raptor), G protein β-subunit-like protein (GβL), and two non-core components, proline-rich Akt substrate 40 kDa (PRAS40) and DEP domain containing mTOR-interacting protein (DEPTOR) [1,5,7]. As a critical hub of signaling, mTORC1 is activated or inactivated by changes in the intracellular and extracellular environment, such as levels of amino acids, growth factors, energy, and stress [10,11]. Atg13 and ULK1, the target of mTORC1 in the autophagic pathway, are phosphorylated and inactivated by mTORC1 under

Abbreviations
DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FYVE, Fab1, YGLO23, Vps27, and EEA1; MEF, mouse embryonic fibroblast; MTMR3, myotubularin-related phosphatase 3; mTORC1, mTOR complex 1; mTOR, the mechanistic target of rapamycin; PH-GRAM, Pleckstrin homology-glucosyltransferase, rab-like GTPase activator and myotubularins; PI3P, phosphatidylinositol 3-phosphate; PTP, phosphatase; Raptor, regulatory-associated protein of mTOR; S6K1, ribosomal protein S6 kinase 1.

nutrient-rich conditions. Because the ULK1–Atg13 complex is required for the initial step of autophagy [12], inhibition of mTORC1 by deprivation of amino acids and/or growth factors induces autophagy [13,14]. It is well established that the two major stimuli of cell growth and proliferation, growth factors and amino acids, activate mTORC1 via different mechanisms. Growth factors activate mTORC1 primarily via stimulation of class I phosphatidylinositol-3-kinase (PI3K)-Akt signaling cascade [15–17]. By contrast, mTORC1 is activated in response to amino acids by the Ragulator–Rag complex [18–20]. Although these two mechanisms are distinct, activation of mTORC1 in both contexts was previously believed to occur on the lysosomes. However, recent work showed that mTORC1 can be activated even independent of lysosomes [21,22]. Jewell *et al*. demonstrated that glutamine can activate mTORC1 in RagA/B-deficient cells through the small GTPase Arf1, which is localized on the Golgi apparatus and plays a role in intra-Golgi transport. In addition, Thomas *et al*. showed that Rab1A, another small GTPase that regulates Golgi membrane trafficking, tethers mTORC1 to the Golgi apparatus to promote activation. Activated mTORC1 evokes cellular events related to proliferation by phosphorylating its substrates, including S6K1 (ribosomal protein S6 kinase 1, referred to as S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [10,23].

In addition to mTORC1, autophagy is also regulated by metabolism of phosphatidylinositol (PI), especially of PI3P [24]. A class III PI3K, Vps34, is essential for autophagy; consistent with this, treatment with PI3K inhibitors, such as wortmannin and 3-methyladenine, or knockout of Atg14L, a specific subunit of the autophagy-specific PI 3-kinase complex, suppresses autophagy [25–27]. We have also shown that recruitment of the autophagy-specific PI 3-kinase complex to the site of autophagosome formation, the omegasome, is necessary for initiation of autophagy [25,28].

The myotubularin family is a class of PI 3-phosphatases that regulate several physiological and pathophysiological phenomena including endosomal trafficking, apoptosis, autophagy, and muscle development [29–32]. We showed that one of these proteins, myotubularin-related phosphatase 3 (MTMR3) [33], plays a negative role in the initiation stage of autophagy in mammalian cells [31]. Over-expression of phosphatase-deficient MTMR3 induces autophagy irrespective of other environmental inducers. This observation could be explained by the fact that a local increase in the PI3P level recruits WIPI-1, the PI3P effector protein involved in autophagy, thereby promoting initiation of autophagy. However, the drawback of this interpretation is that some components of the autophagy-executing machinery, such as ULK1, are believed to function upstream of the PI3P-dependent step. Specifically, these proteins can be recruited to the autophagosome formation site even when the cell is treated with wortmannin [12]. Although there is an additional report that the PI3P itself may create a positive feedback loop to reinforce the recruitment of the ULK1 complex for further stimulating PI3P synthesis [34], there still remains a missing link between MTMR3 function and autophagy regulation.

In this study, we demonstrated that MTMR3 interacts with mTORC1 and inhibits its activity. Our results suggest that MTMR3 regulates autophagy via its effect on mTORC1 activity, in addition to its phosphatase activity.

Material and methods

Plasmid construction

The open reading frames (ORF) of human MTMR3 and the C413S mutant were prepared as described previously [31]. The MTMR3 ORF and mutant were transferred to the pCAG-OSF tag mammalian expression vector using the following oligonucleotides with artificial *Bsi*WI and *Sal*I sites (underlined): 5′-AACGTACGATGGATGAAGAGACTCGGC-3′ and 5′-TAAGTCGACTCAGTTGGAAGTGGCAGC-3′. The *Kpn*I and *Xho*I sites of the OSF vector were destroyed after insertion of MTMR3. The MTMR3 fragments were then subcloned into the pCAG-OSF tag vector using the same method and the following oligonucleotides: 5′-AACGTACGATGGATGAAGAGACTCGGC-3′ and 5′-TAAGTCGACTCATGGGCAGGGCAGGTA-3′ (OSF-MTMR3-N), 5′-AACGTACGCCACCTGCTAAAATA-3′ and 5′-TAAGTCGACTCAGTTGGAAGTGGCAGC-3′ (OSF-MTMR3-ΔPH-G) 5′-AACGTACGTCCCCAACCACCCCT-3′ and 5′-TAAGTCGACTCAGTTGGAAGTGGCAGC-3′ (OSF-MTMR3-C), 5′-AACGTACGATGGATGAAGAGACTCGGC-3′ and 5′-TAAGTCGACTCAATAGACCTCCATGCA-3′ (OSF-MTMR3-PH-G), 5′-AACGTACGCCACCTGCTAAAATA-3′ and TAAGTCGACTCATGGGCAGGGCAGGTA-3′ (OSF-MTMR3-PTP). The following oligonucleotides with artificial *Eco*RI and *Sal*I sites (underlined) were used to prepare the GFP-tagged MTMR3 and MTMR3 fragments in vector pEGFP-C2: 5′-TTTCGAATTCATGGATGAAGAGACTCGGCACAG-3′ and 5′-TAAGTCGACTCAGTTGGAAGTGGCAGC-3′ (GFP-MTMR3), 5′-TTTCGAATTCATGGATGAAGAGACTCGGCACAG-3′ and 5′-TAAGTCGACTCATGGGCAGGGCAGGTA-3′ (GFP-MTMR3-N), 5′-AAAGAATTCTCCCCAACCACCCCT-3′ and TAAGTCGACTCAGTTGGAAGTGGCAGC-3′ (GFP-MTMR3-C), 5′-TTTCGAATTCATGGATGAAGAGACTCGGCACAG-3′ and 5′-TAA

GTCGACTCAATAGACCTCCATGCA-3′ (GFP-MTMR 3-PH-G), 5′-AAGAATTCCCACCTGCTAAAATA-3′ and 5′-TAAGTCGACTCATGGGCAGGGCAGGTA-3′ (GFP-MTMR3-PTP). Myc-Raptor (Addgene plasmid # 1859) [8] and pRK7-HA-S6K1-WT (Addgene plasmid # 8984) [35], originally developed by Dr. David Sabatini and Dr. John Blenis, respectively, were obtained from Addgene (Cambridge, MA, USA).

Cell culture, transient transfections, and drug treatment

HEK293T and mouse embryonic fibroblast (MEF) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) containing 10% fetal bovine serum and antibiotics in a 5% CO_2 incubator at 37 °C. For nutrient starvation, cells were washed once with Eisen's balanced salt solution (EBSS) (Sigma-Aldrich, St. Louis, MO, USA) and cultured in EBSS for 50 min. For amino-acid stimulation, cells were cultured in DMEM for 10 min after starvation treatment for 50 min. Transient transfections of HEK293T cells were performed using linear polyethylenimine (PEI) (MW 25 000; Polysciences, Warrington, PA, USA), which was prepared as a 1 mg·mL^{-1} solution. Plasmid DNA was mixed with PEI solution (1 : 3) diluted in Opti-MEM (Gibco, Waltham, MA, USA) and incubated for 10 min at room temperature before being dropped onto cells. For MEF cells, transient transfections were performed using the Lipofectamine 2000 reagent (Invitrogen, Waltham, MA, USA). Plasmid DNA was mixed with Lipofectamine 2000 (1 : 2.5) diluted in Opti-MEM and incubated for 5 min at room temperature before being dropped onto cells. For knockdown of MTMR3 expression, the control siRNA (12935-112, Invitrogen) and the MTMR3 siRNA (5'-UGUUGAAUGCCG AGAUAUAdTdT-3', sense) was mixed with Lipofectamine RNAiMAX (Invitrogen) (10 pmol: 1 μL) diluted in Opti-MEM and incubated for 5 min at room temperature before being dropped onto cells. To inhibit mTORC1 activity, cells were treated with 20 nM rapamycin (Sigma-Aldrich), diluted from a 1 mM stock solution in DMSO, and incubated for 24 h. For the wortmannin treatment, the cells were treated with 200 nM wortmannin (Sigma-Aldrich), diluted from a 100 μM stock solution in DMSO, and incubated for 1 h before fixation.

Identification of OSF-MTMR3 binding proteins

HEK293T cells transiently expressing OSF-MTMR3 or OSF-MTMR3C413S were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, complete protease inhibitor cocktail (Roche, Basel, Switzerland)]. Lysates were cleared by centrifugation at 20 400 ***g*** (MX201, TOMY, Tokyo, Japan) for 10 min at 4 °C. Supernatants were incubated with Strep-Tactin Sepharose (IBA, Goettingen, Germany) for 4 h at 4 °C. The beads were washed four times with washing buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100] and eluted in 25 μL of 2× sample buffer (1×: 2% SDS, 100 mM DTT, 60 mM Tris-HCl [pH 6.8], 10% glycerol, 0.001% bromophenol blue). After boiling for 5 min, the samples were subjected to SDS/polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie Brilliant Blue (CBB) R-250 staining. The gels were digested with trypsin, and the resultant peptide mixtures were analyzed by liquid chromatography/electrospray ionization linear ion trap quadrupole-Orbitrap-mass spectrometry (LC/ESI-LTQ-Orbitrap-MS) (Thermo Fisher Scientific, Waltham, MA, USA). All MS/MS spectra were searched against the non-redundant protein sequence database using the MASCOT software (Matrix Science, Boston, MA, USA).

Immunoprecipitation

HEK293T cells transiently expressing the indicated plasmids were lysed in lysis buffer. Lysates were cleared by centrifugation at 20 400 ***g*** for 10 min at 4 °C. Supernatants (200 μL) were incubated with 30 μL of Strep-Tactin Sepharose for 2 h at 4 °C. The beads were washed four times with washing buffer and eluted in 30 μL of 2× sample buffer. After boiling for 5 min, the samples were subjected to western blotting.

Analysis of mTOR activity

HEK293T cells transiently expressing HA-S6K with the indicated plasmids were lysed in lysis buffer. Lysates were subjected to centrifugation at 20 400 ***g*** for 10 min at 4 °C. Supernatants (200 μL) were incubated with 1 μL of anti-HA mouse monoclonal antibody (BioLegend, San Diego, CA, USA) for 2 h at 4 °C. Next, 10 μL of Protein G-Sepharose 4FF (GE Healthcare, Little Chalfont, UK) was added to lysates and incubated for 1 h at 4 °C. The beads were washed four times with washing buffer and eluted in 30 μL of 2× sample buffer. After boiling for 5 min, the samples were subjected to western blotting.

Western blotting

Proteins were subjected to SDS/PAGE and then transferred to PVDF membranes (GE Healthcare) using transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol) at 120 V for 1 h. The transferred membrane was blocked for 1 h at room temperature in 5% skim milk in TBS-T (25 mM Tris base, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, adjust pH to 7.4). After blocking, the membrane was incubated overnight with appropriate dilutions of primary antibody in blocking buffer at 4 °C. The following primary antibodies were obtained from the indicated suppliers: anti-mTOR (7C10) rabbit (1 : 1000), anti-Raptor (24C12) Rab-

bit (1 : 1000), anti-GβL (86B8) Rabbit (1 : 1000), anti-Rictor (53A2) Rabbit (1 : 1000), anti-p70 S6 kinase (49D7) Rabbit (1 : 1000), anti-phospho-p70 S6 kinase (Thr389) rabbit (1 : 1000) and anti-MTMR3 rabbit (1 : 1000) antibodies, Cell Signaling Technology (Danvers, MA, USA); anti-FLAG (M2) (1 : 2000) and anti-α-tubulin mouse monoclonal antibody (1 : 2000), Sigma-Aldrich; anti-c-Myc (9E10) mouse monoclonal antibody (1 : 1000), Santa Cruz Biotechnology (Dallas, TX, USA); and anti-HA mouse monoclonal antibody (1 : 2000), BioLegend. The membrane was washed three times in TBS-T and incubated at room temperature for 30 min with a 1 : 5000 dilution of HRP-conjugated secondary antibody (Cell Signaling Technology) in blocking buffer. The membrane was washed three times visualized using the Luminata Forte Western HRP Substrate (Merck Millipore, Darmstadt, Germany) on a Gene Gnome-5 chemiluminescence detector (Syngene, Cambridge, UK). Quantification of band intensity was performed using the IMAGEJ software (National Institutes of Health, Bethesda, MD, USA). Statistical analysis was performed using R software (version 3.2.1, Free Software, https://www.r-project.org).

Fluorescence microscopy

Mouse embryonic fibroblast cells were cultured on coverslips and transiently transfected with GFP-MTMR3 or GFP tagged MTMR3 fragments, as indicated, using Lipofectamine 2000. After 24 h of transfection, the cells were fixed for 15 min with 4% paraformaldehyde in PBS. For immunofluorescence, the following primary antibodies were used and obtained from the indicated suppliers: anti-GM130 mouse monoclonal (1 : 1000), anti-γ adaptin mouse monoclonal (1 : 500) antibodies, BD Transduction Laboratories (Lexington, KY, USA); anti-mTOR (7C10) rabbit antibody (1 : 400), Cell Signaling Technology; anti-LAMP-1 (1D48) rat antibody (1 : 1000), Santa Cruz Biotechnology. The cells were permeabilized for 10 min with 50 $\mu g \cdot mL^{-1}$ digitonin in 0.2% gelatin-PBS. The coverslips were incubated with indicated primary antibodies diluted in blocking buffer (0.2% gelatin-PBS) for 1 h at room temperature, washed three times in blocking buffer, incubated for 40 min with Alexa Fluor 568 or 633–conjugated secondary antibodies (Invitrogen) diluted 1 : 1000 in blocking buffer, and washed three times in blocking buffer. The samples were mounted using Slow Fade Gold (Invitrogen) and observed on a confocal laser scanning microscope (TCS SP8; HC PL APO 63X/1.4 OIL Objective, Leica, Wetzlar, Germany).

Live-cell imaging

Cells were plated onto a glass bottom dish and transiently transfected with GFP-MTMR3 by using lipofectamine 2000. After 24 h of transfection, the DMEM was replaced by DMEM without Phenol Red and L-glutamine (Wako) with addition of L-glutamine. The glass bottom dish was mounted in a chamber (TOKAI HIT, Fujinomiya, Japan) for live-cell recording (TCS SP8; HC PL APO 63X/1.4 OIL Objective, Leica) and the conditions maintained with 5% CO_2 at 37 °C by a digital gas mixer (TOKAI HIT). Data were analyzed using LAS X software (Leica).

Results

MTMR3 binds to mTOR independent of its phosphatase activity

We previously reported that MTMR3 negatively regulates autophagy, via PI3P metabolism and a mechanism that remains to be determined [31]. To understand this novel function of MTMR3, we searched for proteins that interact with it. To this end, we transfected OSF-tagged wild-type MTMR3 (OSF-MTMR3) and a point mutant (C413S) that disables the phosphatase activity (OSF-MTMR3-C413S, Fig. 1A) into HEK293T cells and purified the MTMR3 proteins using the Strep-Tactin purification system [36]. Over 100 potential binding candidates were co-purified with MTMR3 and subsequently identified by LC-MS/MS analysis. MTMR4, a bona fide MTMR3 binding protein [37], appeared in both OSF-MTMR3 and OSF-C413S co-purified pools with a high MASCOT search score, which is associated with binding probability and affinity (WT: 336, C413S: 283). mTOR had a higher MASCOT search score (1100) than MTMR4 in OSF-MTMR3-C413S co-purified pools. This result prompted us to further investigate the relationship between MTMR3 and mTOR.

As this kind of screening sometimes brought about false positive or negative results, we confirmed the MTMR3-mTOR interaction by co-immunoprecipitation (co-IP) in HEK293T cells transfected with OSF-MTMR3, OSF-MTMR3-C413S, or only OSF as a control. Endogenous mTOR was efficiently coimmunoprecipitated not only with OSF-MTMR3-C413S but also with wild-type OSF-MTMR3 (Fig. 1B, lanes 5 and 6 of upper panel). As nutrient conditions affect the stability of the mTOR–Raptor association [38], we next investigated whether nutrient conditions would affect the interaction between MTMR3 and mTOR. As shown in Fig. 1B, MTMR3 interacted with mTOR under starvation conditions (right half, lanes 11 and 12), as well as under nutrient-rich conditions (left half, lanes 5 and 6), indicating that the interaction between MTMR3 and mTOR was independent of nutrient conditions and phosphatase activity of MTMR3.

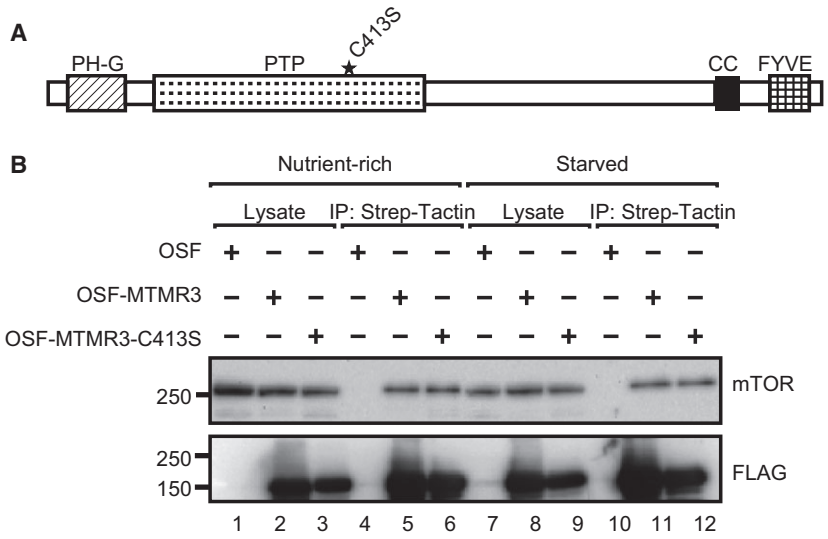

Fig. 1. MTMR3 interacts with mTOR. (A) Schematic model of MTMR3. The point mutation C413S disables the phosphatase activity. PH-G, PH-GRAM; PTP, phosphatase; CC, coiled-coil; FYVE, FYVE zinc finger domain. (B) HEK293T cells were transfected with OSF alone, OSF-MTMR3, or OSF-MTMR3-C413S. After 48 h of transfection, cells were shifted into fresh Dulbecco's modified Eagle's medium (nutrient-rich conditions, nutrient-rich) or Eisen's balanced salt solution (starved conditions, starved) for 2 h. Lysates from those cells were incubated with Strep-Tactin Sepharose, and proteins bound to the Sepharose were analyzed by western blotting using anti-mTOR (upper panel) and anti-FLAG (lower panel) antibodies.

The N-terminal half of MTMR3 is necessary and sufficient for the interaction with mTORC1

Figure 2A shows the domain organization of MTMR3 [39]. The phosphatase (PTP) domain is a conserved myotubularin phosphatase domain. The FYVE domain is generally regarded as specifically binding to PtdIns 3P; however, a mutation in the FYVE domain did not alter the localization of MTMR3 [39]. Instead, the PH-G domain of MTMR3 is required for binding to PI3P and PI3,5P_2 [39]. The function of the coiled-coil domain remains unknown. To investigate the roles of these domain/region in the interaction of MTMR3 with mTOR, we transfected several OSF-tagged MTMR3 fragments (Fig. 2A) or full-length MTMR3 into HEK293T cells. As shown in Fig. 2B (top panel), the N-terminal half of MTMR3 (MTMR3-N, amino acids 1–580), which contains the PH-G and PTP domains, interacted with mTOR almost as well as full-length MTMR3, whereas the C-terminal half (MTMR3-C, amino acids 581–1198), which contains the coiled-coil and FYVE domains, did not. However, neither the PH-G domain (amino acids 1–140) nor the PTP domain alone (amino acids 120–580) interacted with mTOR (Fig. 2C, top panel). These results indicate that both the PH-G and PTP domains of MTMR3 are necessary and sufficient for the interaction with mTOR.

We next investigated whether these interactions were affected when mTORC1 was specifically inhibited by rapamycin. After 24 h treatment, the mTORC1 substrate S6K was dephosphorylated (Fig. 2D, second panel from the bottom). However, the interactions between MTMR3 and the N-terminal half of the protein with mTOR were unaffected by this treatment (Fig. 2D, top panel). This result suggests that the N-terminal half of MTMR3 binds to mTOR independent of mTORC1 activity.

We further investigated if the other mTORC1 subunits are pulled down by MTMR3 [1,38]. As shown in Fig. 3A, transfected Myc-tagged Raptor (myc-Raptor) co-IPed with both OSF-MTMR3 and OSF-MTMR3-C413S. Furthermore, the endogenous Raptor and GβL could be pulled down by both full-length MTMR3 and N-terminal half of MTMR3, but not by the MTMR3 lacking the PH-G domain (Fig. 3B). mTOR also forms mTORC2 with two core components, Rictor and GβL, however, Rictor was not coimmunoprecipitated with MTMR3 (Fig. 3C). Taken together, MTMR3 interacts with mTORC1 but not with mTORC2.

MTMR3 suppresses mTORC1 activity

We next asked whether the interaction between MTMR3 and mTOR is associated with mTORC1

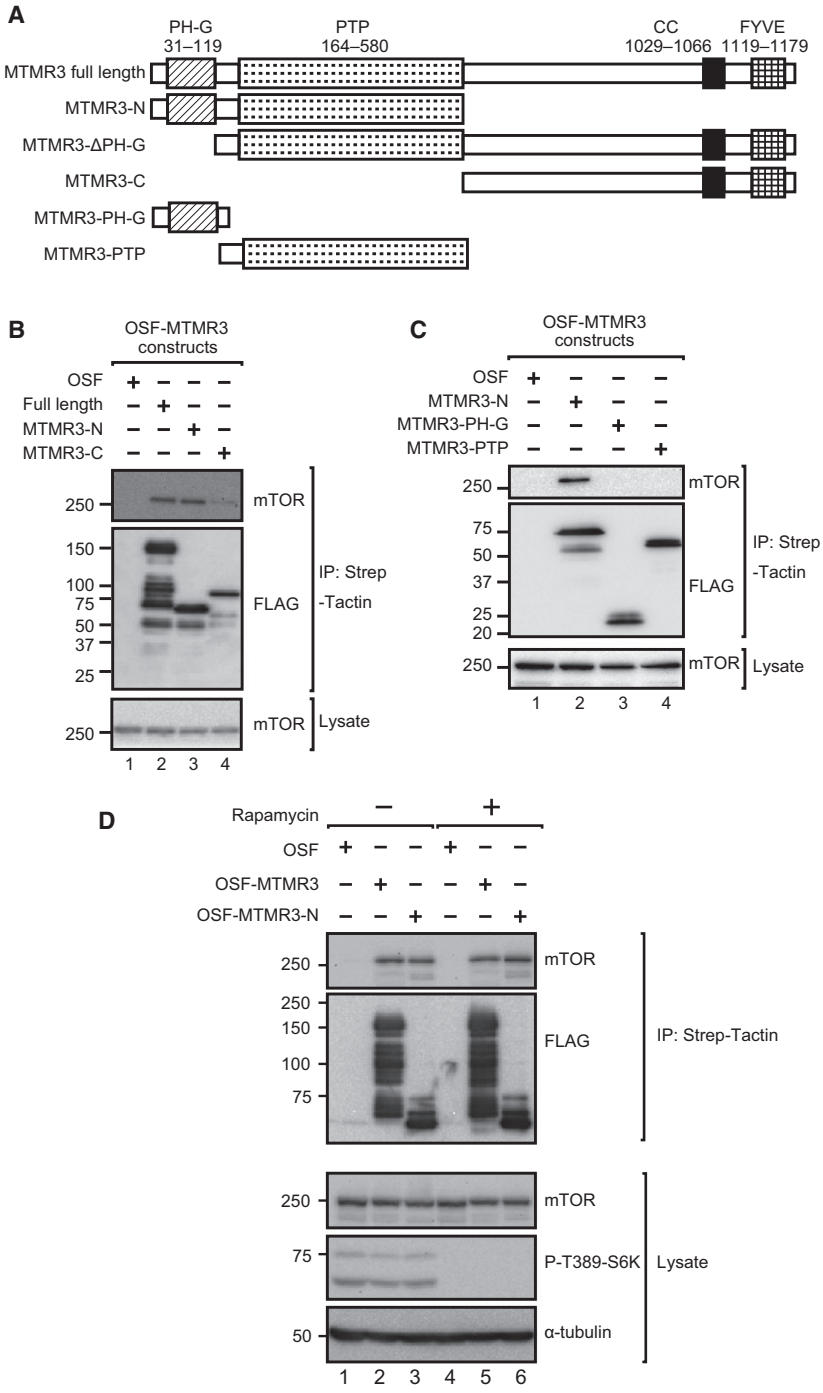

Fig. 2. MTMR3 interacts with mTOR through its N-terminal half. (A) Schematic models of truncated MTMR3. (B) HEK293T cells were transfected with OSF alone or the indicated OSF tagged MTMR3 constructs. IP experiment was performed as in Fig. 1B. (C) HEK293T cells were transfected with OSF alone or the indicated OSF-tagged MTMR3 constructs. IP experiment was performed as in Fig. 1B. (D) HEK293T cells were transfected with OSF alone, OSF-MTMR3, or OSF-MTMR3-N for 48 h and treated with rapamycin (20 nM) for 24 h. To detect proteins, anti-mTOR and anti-FLAG antibodies were used on immunoprecipitates (IP, upper two panels). To confirm the effect of rapamycin, anti-phospho-T389-S6K antibody was used on total lysate (Lysate, the second panel from the bottom). The anti-α-tubulin antibody was used on total lysate as internal control (Lysate, the first panel from the bottom).

activity. To detect mTORC1 activity derived only from cells transfected with MTMR3 and its derivatives, we co-transfected plasmids encoding HA-tagged S6K (HA-S6K) with OSF-MTMR3 and observed the phosphorylation of immunoprecipitated HA-S6K [19]. Over-expression of wild-type MTMR3 reduced the amount of phosphorylated HA-S6K both under nutrient-rich conditions and nutrient replenished conditions following starvation (Fig. 4A, the top panel, 4B). Conversely, knockdown of MTMR3 expression by siRNA enhanced mTORC1 activity (Fig. 4C). The S6K phosphorylation was mostly doubled under replenished conditions in every three independent experiments, although the increment seems to be marginal under nutrient-rich conditions. These observations suggest that MTMR3 suppresses mTORC1

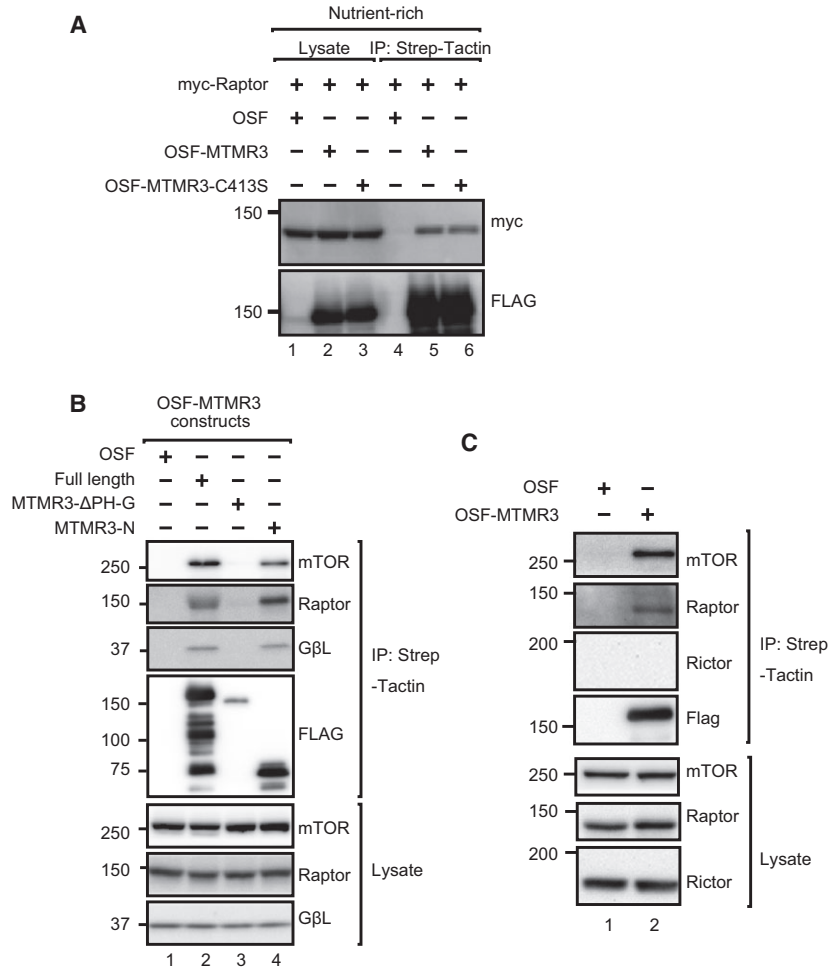

Fig. 3. MTMR3 binds to mTORC1. (A) HEK293T cells were cotransfected with myc-Raptor and OSF alone, OSF-MTMR3, or OSF-MTMR3-C413S. The cells were cultured in Dulbecco's modified Eagle's medium (nutrient-rich) and treated as described in Fig. 1B. Proteins were detected using anti-Myc (upper panel) and anti-FLAG (lower panel) antibodies. (B) HEK293T cells were transfected with OSF alone or the indicated OSF-tagged MTMR3 constructs. IP experiment was performed as in Fig. 1B. Proteins were detected using anti-mTOR, anti-Raptor, anti-G protein β-subunit-like protein (GβL) and anti-FLAG antibodies. (C) HEK293T cells were transfected with OSF alone or OSF-MTMR3. IP experiment was performed as in Fig. 1B. Proteins were detected using anti-mTOR, anti-Raptor, anti-Rictor and anti-FLAG antibodies.

activity. Furthermore, phosphatase-deficient MTMR3 provided more robust suppression than wild-type MTMR3 (Fig. 4A, lanes 2, 3 and lines 8, 9, 4B).

The N-terminal half of MTMR3, which was bound to mTOR (Fig. 2B), significantly suppressed mTORC1 activity under nutrient-rich condition, whereas MTMR3-C did not (Fig. 5A, the top panel, 5B). However, overexpression of either fragment of the N-terminal half, i.e., the PTP or the PH-G domain alone, did not suppress mTORC1 activity either nutrient-rich or replenished conditions (Fig. 5C, the top panel, 5D). Taken together, these observations indicate that MTMR3 inhibits mTORC1 via an interaction between its N-terminal half (PH-G and PTP) with mTORC1.

MTMR3-PTP domain is sufficient for localization to the Golgi

Wild-type MTMR3 is largely distributed throughout the cytosol (Fig. 6A, top panel), whereas the phosphatase-deficient MTMR3 is localized on the autophagosome [31]. Interestingly, a portion of GFP-MTMR3-C413S exhibited the colocalization with the cis-Golgi marker GM130 (Fig. 6A, third panel from the top). GFP-MTMR3–N, even that of the wild-type protein, also exhibited the same localization (Fig. 6A, fourth panel from the top), whereas the MTMR3-C did not (Fig. 6A, third panel from the bottom). The GFP-MTMR3-C413S and GFP-MTMR3-N appeared more closely to the GM130 compared with trans Golgi network maker, γ adaptin, which indicates that both GFP-MTMR3-C413S and GFP-MTMR3-N are likely localized to the cis-Golgi (Fig. S1). We then examined if MTMR3 affects the mTOR localization, which leads to the suppression of mTORC1 activity. However, we could not observe significant alteration of mTOR localization, namely, the lysosomal localization (Fig. S2).

Within the N-terminal half, the GFP-MTMR3-PTP domain alone was localized to the Golgi, whereas GFP-MTMR3-PH-G domain was not (Fig. 6A, first and second panels from the bottom). Thus, the PTP domain of MTMR3 is sufficient for Golgi localization, but the

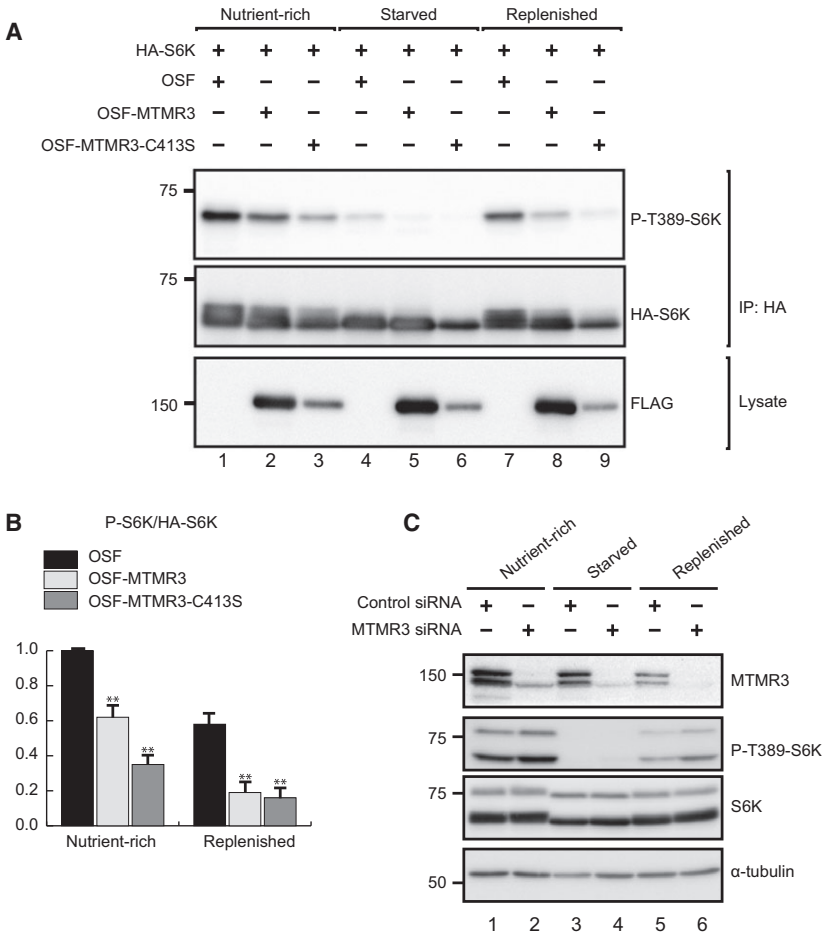

Fig. 4. MTMR3 suppresses mTORC1 activity. (A) HEK293T cells were cotransfected with HA-S6K and OSF alone, OSF-MTMR3, or OSF-MTMR3-C413S. After 48 h of transfection, the cells were cultured in fresh Dulbecco's modified Eagle's medium (DMEM) (nutrient-rich conditions, nutrient-rich), in Eisen's balanced salt solution (EBSS) for 50 min (starved conditions: starved), or in EBSS for 50 min followed by in DMEM for 10 min (replenished conditions: replenished). Lysates from each condition were incubated with anti-HA antibody and protein G–Sepharose. Precipitated HA-S6K proteins were analyzed by western blotting using anti-HA and anti-phospho-T389-S6K antibodies (upper two panels). Expression levels of OSF-MTMR3 wild-type and mutant were detected using anti-FLAG antibody (the bottom panel). (B) Quantification of the ratio of P-S6K/HA-S6K from nutrient-rich and replenished conditions described in (A). Bars represent the means ± SE from three independent experiments (compared with the control under the same conditions). $^{**}P < 0.01$, one-way ANOVA, Tukey's *post hoc* test. (C) HEK293T cells were transfected with control siRNA or MTMR3 siRNA. After 48 h of transfection, cells were cultured in nutrient-rich, starved, or replenished conditions as described in (A). The cells were lysed and analyzed by western blotting using indicated antibodies. The knockdown of MTMR3 was confirmed by anti-MTMR3 antibody. The other proteins were detected using anti-phospho-T389-S6K, anti-S6K and anti-α-tubulin antibodies.

C-terminal half has a negative impact on Golgi localization. We also noticed that only in a small fraction of cells (9.1 ± 0.8%), wild-type GFP-MTMR3 was also colocalized with GM130 at the perinuclear region (Fig. 6A, second panel from the top). Live-cell imaging of GFP-MTMR3 expressing cells showed that it is transiently localized at Golgi and eventually detached from there (Fig. 6B and Movie S1). This Golgi localization of wild-type MTMR3 was not affected when the cells were treated with wortmannin (9.5 ± 0.7%) or under starved (9.4 ± 0.7%) and replenished conditions (10.6 ± 0.8%) (Fig. S3). Thus, MTMR3 may exist in a dynamic equilibrium between the cytosolic pool and smaller pools at the Golgi (and other organelles), and the phosphatase activity and the C-terminal half may be involved in the dissociation from the Golgi.

Discussion

In this study, we uncovered an unexpected connection between MTMR3 and mTORC1. MTMR3 can, at least transiently, associate with mTORC1, although

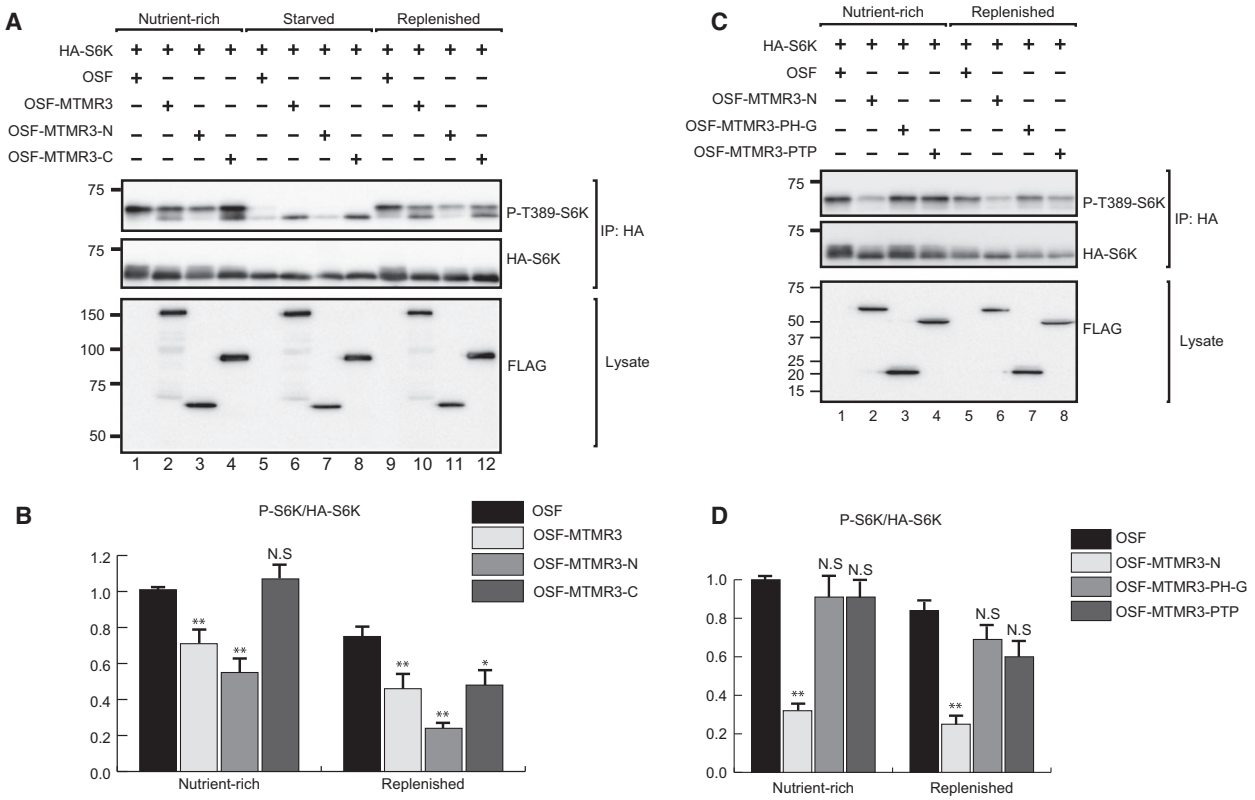

Fig. 5. N-terminal half of MTMR3 suppresses the mTORC1 activity. (A) HEK293T cells were cotransfected with HA-S6K and OSF alone or the indicated OSF-tagged MTMR3 constructs. After 48 h of transfection, cells were cultured in nutrient-rich, starved, or replenished conditions as described in Fig. 4A. Purification of HA-S6K proteins and western blotting analysis were performed as described in Fig. 4A. (B) Quantification analysis was performed under nutrient-rich and replenished conditions as described in (A). Bars represent the means ± SE from three independent experiments (compared with the control under the same conditions). $^{**}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA, Tukey's *post hoc* test. (C) HEK293T cells were cotransfected with HA-S6K and OSF alone or the indicated OSF-tagged MTMR3 constructs. After 48 h of transfection, cells were cultured in nutrient and replenished conditions as described in Fig. 4A. Purification of HA-S6K proteins and western blotting analysis were performed as described in Fig. 4A. (D) Quantification analysis was performed under nutrient-rich and replenished conditions as described in (C). Bars represent the means ± SE from three independent experiments (compared with the control under the same conditions). $^{**}P < 0.01$, one-way ANOVA, Tukey's *post hoc* test.

they would not constitute a stable complex. Our results indicate that MTMR3 affects mTORC1 activity in a bimodal way. First, MTMR3 binding suppresses mTORC1 activity in a phosphatase activity–independent manner (Figs 1B and 4). Second, MTMR3 phosphatase activity negatively influences the inhibitory effects on mTORC1 activity (Fig. 4A). Thus, our previous finding that overexpression of phosphatase-deficient MTMR3 can induce autophagy [31] can be attributed at least partly to the suppression of mTORC1 activity. A local increase in the PI3P level should further accelerate the progression of autophagy [24]. By contrast, overexpression of wild-type MTMR3 does not upregulate autophagy [31], although our data indicate that even overexpression of the wild-type MTMR3 also inhibits mTOR activity. This is because overexpression of MTMR3 reduces the level of PI3P, which is required for the formation of autophagosomes. It is possible that autophagy may be induced, but that overall activity is too low; consistent with this idea, many autophagosomes/autolysosomes are unusually small in MTMR3-overexpressing cells [31].

Previous work showed that overexpression of hVps34, a Class III PI 3-kinase, activates mTORC1, suggesting that PI3P plays some role in mTORC1 regulation [40–43]. We also found that MTMR3 phosphatase activity negatively impacts the suppression of mTORC1 activity (Fig. 4A). Thus, the detailed mechanism by which the PI3P affect remains enigmatic. The most well-established site of mTORC1 function is the lysosome, although the complex is also active in the cytosol, plasma membrane, and nucleus [44]. On

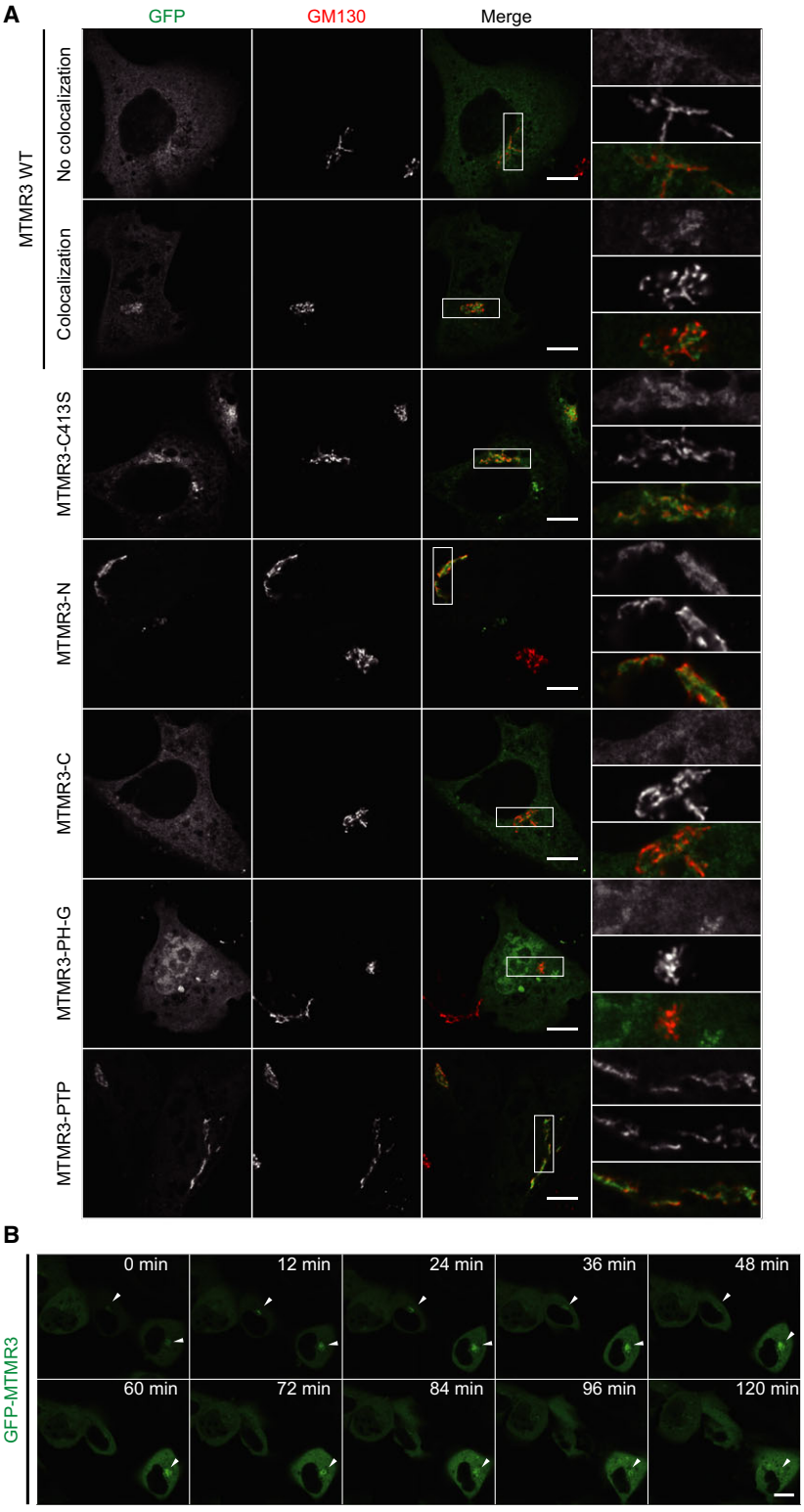

Fig. 6. Localization of MTMR3. (A) mouse embryonic fibroblast (MEF) cells transiently expressing full-length or the indicated mutants of GFP-MTMR3 (left row) were fixed and stained with anti-GM130 antibody (second row from left). The signal color in the merged images (second row from right) is indicated by the color of the typeface. The right row shows magnified views of the boxed area. Scale bars, 10 μm. (B) Selected frames from Movie S1, which is live-cell imaging of MEF cells transiently expressing GFP-MTMR3 wild-type. After 24 h of transfection, the cells were imaged starting from 30 min after the Dulbecco's modified Eagle's medium (DMEM) was replaced by DMEM without Phenol Red. Golgi-like localization of GFP-MTMR3 is indicated by arrowheads. Scale bars, 10 μm.

the other hand, sites of PI3P localization, such as the endosome and the site of autophagosome formation on the endoplasmic reticulum, have never been investigated in this regard. The identification of PI3P effector protein that is also associated with mTORC1 might resolve this enigma. As it binds to PI3P via its PH-G

domain, MTMR3 is an attractive candidate for participation in mTORC1 regulation [39]; moreover, we have revealed that MTMR3 can bind to mTORC1 and regulate its activity. Furthermore, the MTMR3 N-terminal half, which contains the PH-G and phosphatase domains, is required for binding and suppression of mTORC1 activity (Figs 2, 3B and 5A,B). MTMR3-C–overexpressing cells also exhibited a mild inhibitory effect, like those expressing full-length MTMR3, which was weaker than the effect observed in cells expressing the N-terminal half (Fig. 5A,B). The ultimate cause of this effect remains to be determined, but the MTMR3 binding partner MTMR4 should be investigated in this regard. Collectively, our data show that MTMR3 is a negative regulator of mTORC1, although many mechanistic details remain to be elucidated.

How does MTMR3 function as a negative regulator of mTORC1? In a previous report, we proposed that MTMR3 acts as a negative regulator of autophagy [31]. In general, the endoplasmic reticulum is devoid of PI3P, but when autophagy is induced, the autophagy-specific PI3-kinase complex is recruited to the ER, where it generates PI3P in a limited area within the ER called the omegasome [3]. We propose that MTMR3 is needed to maintain an adequate amount of PI3P within the omegasome, and that dysfunctional MTMR3 leads to massive generation of PI3P [31]. In a similar manner, MTMR3 may also act to deplete unnecessary PI3P and inactivate unnecessary mTORC1. Moreover, a fraction of MTMR3-C413S exhibited Golgi localization (Fig. 6A, third panel from top), and a similar observation was also reported by Lorenzo *et al*. [39]. Wild-type MTMR3 exhibited a mostly cytosolic localization, but a small portion is transiently localized at the Golgi (Fig. 6A,B and Movie S1). Similarly, we showed that the MTMR3-C413S mutant and wild-type PTP domain of MTMR3 were localized at the Golgi. Lorenzo *et al*. showed that MTMR3 lacking PH-G was localized to the Golgi, implying that the PH-G domain may also exert some inhibitory effect on the Golgi localization [39]. The PH-G and C413S mutation affect phosphatase activity [39], and it is possible that lack of C-terminal region also reduces phosphatase activity, resulting in Golgi localization. We did not observe an apparent alteration of Golgi morphology by overexpression of MTMR3-C413S, as demonstrated by Lorenzo *et al*. [39], possibly due to differences in the cell lines and expression systems used in each study. However, the Golgi is usually devoid of PI3P, and the chance presence of PI3P would lead to alteration of Golgi function; thus, MTMR3 may contribute to cleaning up PI3P mislocalized at the Golgi. Recent studies shed light on the role of the Golgi in the regulation of mTORC1 [21,22]. The Golgi localization and the binding of MTMR3 to mTOR prompted us to hypothesize that MTMR3 is involved in the mTORC1 regulation at the Golgi. Although we failed to detect mTOR at the Golgi in our microscopic system, more sensitive techniques may elucidate such a Golgi-associated function in a future study.

In conclusion, our study suggests that MTMR3 has multiple functions in the regulation of autophagy: inhibition of mTORC1 and reduction in local PI3P level. As inhibition of mTORC1 induces autophagy and reduction in PI3P inhibits autophagy, these functions have opposite effects on autophagic flux. Furthermore, MTM3 in *Caenorhabditis elegans* shows a positive role during autophagosome maturation suggests an additional function of MTMR3 in mammalian cells [45]. Therefore, we concluded that regulation of autophagy by MTMR3 is multi-modal, and that further investigation is necessary to uncover the physiological significance of the MTMR3–mTORC1 connection.

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Author contributions

FH conducted most of the experiments, analyzed the results, and wrote most of the paper. TI conceived the idea for the project, provided overall experimental instruction, and critically read the manuscript. EM conducted the search for MTMR3 binding partners, with FH. KSN conducted experiments involving OSF-MTMR3 plasmid construction and provided technical support. TY engaged in critical discussions with FH. TN conceived the idea for the project, engaged in discussions, and wrote the paper.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:
Fig. S1. The localization of MTMR3 and the TGN marker, γ adaptin.
Fig. S2. Overexpression of MTMR3 does not affect the mTORC1 localization to lysosome.
Fig. S3. The localization of GFP-MTMR3 wild-type.
Movie S1. The localization of MTMR3 in live-cell imaging.