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The Ca^{2+} channel TRPML3 specifically interacts with the mammalian ATG8 homologue GATE16 to regulate autophagy

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ABSTRACT

TRPML3 is a Ca^{2+} permeable cation channel expressed in multiple intracellular compartments. Although TRPML3 is implicated in autophagy, how TRPML3 can regulate autophagy is not understood. To search interacting proteins with TRPML3 in autophagy, we performed split-ubiquitin membrane yeast two-hybrid (MY2H) screening with TRPML3-loop as a bait and identified GATE16, a mammalian ATG8 homologue. GST pull-down assay revealed that TRPML3 and TRPML3-loop specifically bind to GATE16, not to LC3B. Co-immunoprecipitation (co-IP) experiments showed that TRPML3 and TRPML3-loop pull down only the lipidated form of GATE16, indicating that the interaction occurs exclusively at the organellar membrane. The interaction of TRPML3 with GATE16 and GATE16-positive vesicle formation were increased in starvation induced autophagy, suggesting that the interaction facilitates the function of GATE16 in autophagosome formation. However, GATE16 was not required for TRPML3 trafficking to autophagosomes. Experiments using dominant-negative (DN) TRPML3(D458K) showed that GATE16 is localized not only in autophagosomes but also in extra-autophagosomal compartments, by contrast with LC3B. Since GATE16 acts at a later stage of the autophagosome biogenesis, our results suggest that TRPML3 plays a role in autophagosome maturation through the interaction with GATE16, by providing Ca^{2+} in the fusion process.

1. Introduction

TRPML3 is a member of TRPML subfamily in TRP channel superfamily [1]. Like other TRP channels, TRPML3 is a 6 transmembrane domain (TMD)-containing protein with a putative pore region between TMD5 and TMD6 and cytosolic N- and C-termini (Fig. 1A). One exceptional feature of TRPML3 is a large extracytosolic loop (aa 84-283 in TRPML3, see Fig. 1A) between TMD1 and TMD2, which is believed to play important roles in its function because of the unusually lengthy size. Although our previous study showed that a H^+ regulatory domain exists in the loop and regulates pH dependency of TRPML3 [2], no other information is available about the physiological role of the large extracytosolic loop of TRPML3.

TRPML3 is a Ca^{2+} permeable cation channel that is expressed in plasma membranes and multiple organelles such as endosomes, lysosomes and autophagosomes [3]. We and others have also reported that TRPML3 regulates membrane trafficking and autophagy, perhaps by controlling Ca^{2+} in the vicinity of organelles [4,5]. In this study, we showed that overexpression of TRPML3 induces autophagy while knock-down (KD) inhibits it and that TRPML3 is massively recruited to autophagosomes upon induction of autophagy. However, it is not clear how TRPML3 regulates autophagy and is translocated to autophagosomes, largely due to lack of information about the interacting proteins especially in the autophagic process. The hydrophobicity of TRPML3 and its organellar localization made it difficult to apply the conventional screening methods. Despite the importance of the TRPML3 function in autophagy, so far, only 7 proteins are known to interact with TRPML3 [6] and among them only one, HSC70 is suggested in autophagy [7].

In this study, to find interacting proteins of TRPML3 in autophagy we applied the split-ubiquitin MY2H system, which is specially designed for the membrane proteins to detect interactions inside cellular membranes [8]. We screened a mouse adult brain cDNA library using TRPML3-loop as the bait and identified a mammalian ATG8 homologue GATE16 as a novel interacting protein of TRPML3.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

The cDNA fragment encoding the loop of TRPML3 (aa 1-312, TRPML3-loop) was amplified by PCR and cloned into SfiI restriction

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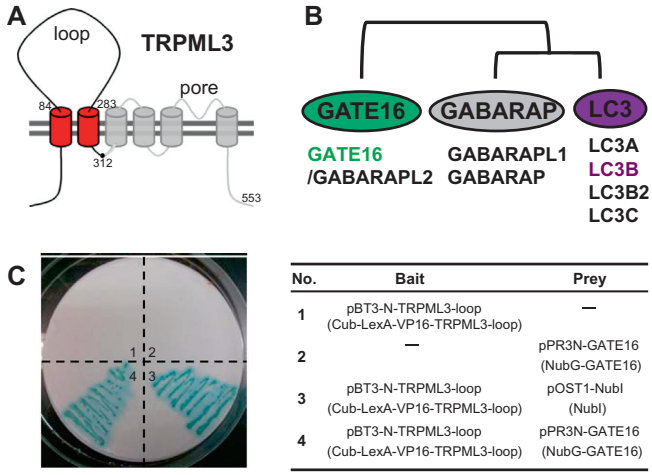

Fig. 1. Split-ubiquitin MY2H assay for TRPML3–GATE16 interaction. (A) Schematic model of TRPML3. Only aa 1–312 of TRPML3 containing a large extracytosolic loop (TRPML3-loop) was used as bait for the screening. (B) Mammalian ATG8 homologs. Note that GATE16 belongs to a different subfamily from LC3B. (C) Interaction between TRPML3-loop and GATE16 was determined by colony-lift filter assay. Yeast cells were co-transformed with indicated bait-prey pairs and streaked on selective medium (SD/-Leu/-Trp/-His+1 mM 3-AT). The colonies were replica transferred to filter paper and tested for β-gal activity. pOst1-NubI was used as a positive control prey. The pictures were captured after 6 h incubation at 30 °C in the presence of X-gal.

sites of the pBT3-N plasmid (Dualsystems). The full-length coding sequence of human TRPML3 was inserted into the mammalian expression plasmid pEGFPC1 or pCMV-HA (Clontech). pEGFPC1-TRPML3-loop (aa 1-311) was generated by site-directed mutagenesis using QuikChange mutagenesis kit (Stratagene). mCherrry-TRPML3(D458K) was generated by site-directed mutagenesis using QuikChange mutagenesis kit using mCherry-TRPML3 [4] as a template. The full-length coding sequence of GATE16 was amplified from the mouse adult brain cDNA library (Dualsystems) and subcloned into the p3XFLAG-CMV-7.1 plasmid for co-IP and into the pEGFPC1 plasmid for confocal imaging. The coding sequence of GATE16 and LC3B was fused with frame into pGEX-4T1 plasmid (Amersham Biosciences) using EcoRI and SalI sites for glutathione S-transferase (GST)-fusion protein production (GST-GATE16 and GST-LC3B).

2.2. Cell culture and transfection

HEK293T or HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified incubator at 37 °C and 5% CO_2/air. For plasmid DNA transfection, cells were plated 1 day before transfection and transfected with Lipofectamine2000 (Invitrogen) according to the manufacturer's instruction. siRNA for GATE16 (M-006853-02) and non-targeting control siRNA (D-001206-14)were purchased from Dharmacon and also transfected with Lipofectamine2000 according to the manufacturer's instruction.

2.3. Split-ubiquitin MY2H analysis

The screening of TRPML3 with mouse adult cDNA library was carried out by using split-ubiquitin MY2H system of Dualsystems Biotech according to the manufacturer's instruction. The bait construct, pBT3-N-TRPML3-loop was co-transformed with mouse adult brain cDNA library in pNubGx plasmid into NMY51 yeast strain and plated on selective medium (SD-Trp/-His/-Ade/-Leu containing 1 mM 3-Amino-1,2,4-triazole(3-AT)). β-galactosidase (β-gal) activity was tested using the colony-lift filter assay to identify the positive colonies. All positive clones were confirmed by plasmid extraction and sequencing.

2.4. GST pull-down assays

GST-GATE16 or GST-LC3B was expressed in BL21 *Escherichia coli* strain. Production of the fusion proteins was initiated by adding 100 nM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the bacterial cultures grown to an A600 of 0.6. Bacteria were lysed by three cycles of rapid freezing/thawing and the insoluble material was removed by centrifugation for 10 min at 14,000 rpm. The supernatant was then incubated with glutathione-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. After five washes with PBS, bound proteins were released by incubating the beads in 5× sample buffer for 5 min at 60 °C. Elutes were analyzed on 12% SDS–PAGE and stained with Coomassie blue. HEK293T cells expressing GFP-TRPML3 or GFP-TRPML3-loop were lysed 24 h after transfection. Samples (150 μl of Triton-solubilized proteins) were incubated for 12 h at 4 °C with glutathione-Sepharose beads charged with GST-GATE16 or GST-LC3B. The beads were then harvested by centrifugation and washed five times with PBS. Bound proteins were eluted with 5× sample buffer and analyzed by SDS–PAGE and immunoblot.

2.5. Co-immunoprecipitation

Transfected cells were collected and lysed using 350 μl lysis buffer (50 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1 tablet of protease inhibitor cocktail and with 1% Triton X-100 for whole cell lysate or without for membrane fraction). Membrane fractions were made by ultracentrifugation (55,000 rpm for 30 min at 4 °C) and then pellets were lysed in lysis buffer with 1% Triton X-100 (v/v). The lysates were sonicated and any insoluble material was cleared by centrifugation at 14,000 rpm for 20 min. For co-IP, anti-GFP antibody was added to each whole cell lysate and incubated for 1 h at 4 °C. Then, 100 μl of a 1:1 slurry of protein A agarose bead was added to the lysate-antibody mixture and incubated for 12 h at 4 °C. Beads were washed five times with ice-cold binding buffer and eluted in 5× sample buffer at 60 °C. Samples were resolved by SDS–PAGE, transferred to PVDF membrane, blotted with appropriated antibodies.

2.6. Confocal microscopy

HeLa cells transfected with indicated constructs were grown on glass coverslips and fixed by 4% paraformaldehyde at RT for 20 min. After wash, coverslips were mounted on glass slides and analyzed using Zeiss LSM 510 confocal microscope. The images were analyzed off-line using NIH ImageJ™ software.

3. Results and discussion

3.1. GATE16 was identified as a novel interacting protein of TRPML3 by split-ubiquitin MY2H screen

To identify interacting proteins involved in TRPML3 function in autophagy, we used the split-ubiquitin MY2H system and screened a mouse adult brain cDNA library. For the bait construct, we cloned the mouse cDNA encoding TRPML3-loop (aa 1-312) (Fig. 1A) into yeast-expression pBT3-N vector fused with the Cub and LexA-VP16 transcription factor at the N-terminus. After an initial screening using a colony-lift filter assay, all clones were confirmed by plasmid extraction and sequencing. As a result, we found 53 independent interactors for TRPML3 and identified GATE16, as an autophagy-related protein.

As shown in Fig. 1B, GATE16 (also known as GABARAPL2) is one of the seven mammalian homologues of the yeast ATG8 protein [9]. To date only LC3B has been mainly studied and other members are not fully understood yet. GATE16 was initially characterized as intracellular trafficking factor [10] and later was revealed to be localized to autophagosomes under starvation condition [11]. All mammalian Atg8 homologues undergo lipidation, a unique ubiquitin-like conjugation to phosphatidylethanolamine to associate with autophagosomal membrane as the form II [11] and this process is required for autophagosome formation [12,13]. A recent study has shown that both LC3B and GATE16 are essential for autophagosome biogenesis but act at different stages [14].

To verify the interaction between TRPML3-loop and GATE16, first we performed *in vivo* binding assay in yeast. Yeast cells expressing either Cub-VP16-LexA-TRPML3-loop or NubG-GATE16 were not grown on selective medium (Fig. 1C, Nos. 1 and 2). By contrast, yeast cells co-transformed with Cub-VP16-LexA-TRPML3-loop and NubG-GATE16 or NubI (a positive control prey) showed robust growth on selective medium and β-gal activity in the presence of X-gal, confirming the interaction in yeast (Fig. 1C, Nos. 3 and 4).

3.2. TRPML3 specifically binds to GATE16 in vitro

Many autophagosomal proteins are recruited to autophagosomes through specific binding with ATG8 proteins [9]. For example, recruitment of p62 into autophagosomes specifically depends on LC3B, not on GATE16 [15]. Since TRPML3 is known to be translocated to autophagosomes in cell stress condition [4], not only biding to GATE16 but also specificity of the interaction with other ATG8 homologues should be examined. To do this, we investigated the interaction of TRPML3 with LC3B or GATE16 *in vitro* using GST pull-down assay. GST, GST-LC3B or GST-GATE16 fusion proteins were expressed in *E. coli* and purified on glutathione beads. The beads were then incubated with whole cell lysates from HEK293T cells expressing GFP-TRPML3-loop or GFP-TRPML3. Consistent with the previous result in yeast, GST pull-down assay confirmed the binding of GATE16 to TRPML3-loop (Fig. 2A, left panel). Neither GST alone nor GST-LC3B could pull down TRPML3-loop, indicating the binding is specific for GATE16. GST-LC3B could not pull down even full-length TRPML3 (Fig. 2A, right panel), suggesting that TRPML3 does not contain a LC3B binding site. These results indicate that TRPML3 specifically and directly binds to GATE16 via its loop region, although the possibility of binding through TRPML3 N-terminus cannot be ruled out.

3.3. The interaction of TRPML3 and GATE16 depends on the status of autophagy

To examine whether TRPML3–GATE16 interaction occurs in the context of mammalian cell, we carried out co-IP experiments. As shown in Fig. 2B, both TRPML3 and TRPML3-loop co-immunoprecipitated GATE16, but stronger interaction was observed with TRPML3. The size of co-immunoprecipitated GATE16 was lower than that of the input, suggesting that only the lipidated form of GATE16 participates in the interaction with TRPML3, as previously reported in LC3B-p62 binding [15]. To test the hypothesis, we prepared the membrane fraction to reduce the amount of soluble form (type-I) of GATE16. Fig. 2C clearly shows that expression of GATE16 only resulted in soluble type-I form, whereas co-expression with TRPML3 caused marked increase of lipidated type-II form of GATE16 leading to increased co-IP. As expected, only the lipidated type-II GATE16 was co-immunoprecipitated, indicating that the interaction between TRPML3 and GATE16 takes place at the membrane. Interestingly, co-expression of TRPML3-loop also caused the increased level of type-II GATE16 and more interaction as well. As TRPML3-loop does not contain a pore region (Fig. 1A), this result implies that channel function of TRPML3 is not essential for the binding but it can rather induce more autophagy and that the interaction of TRPML3-loop with GATE16 itself can induce some degree of autophagy. This raises the possibility that the interaction of

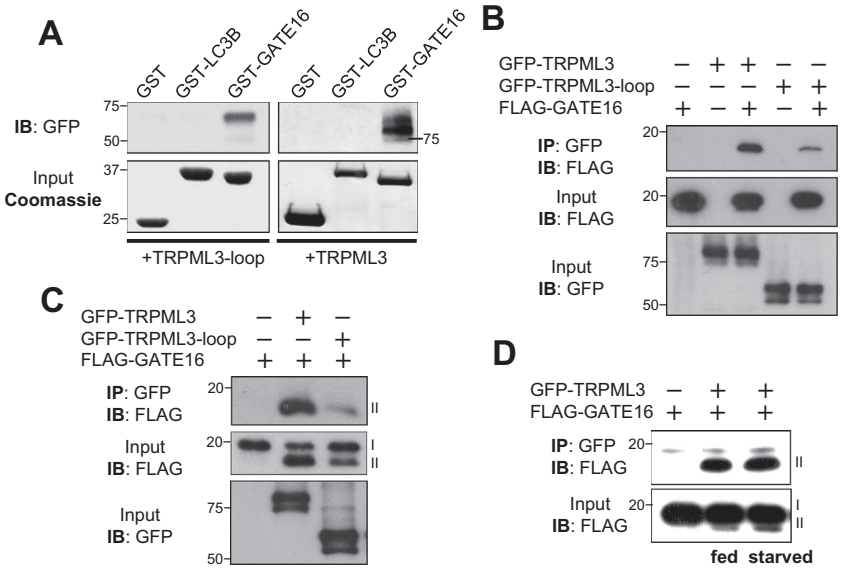

Fig. 2. GST pull-down and co-immunoprecipitation of TRPML3 and GATE16. (A) GST, GST-LC3B or GST-GATE16 coupled to glutathione Sepharose beads were incubated with whole cell lysate from HEK293T cells expressing GFP-TRPML3-loop or GFP-TRPML3. Elutes were subjected to SDS–PAGE, and immunoblotted with anti-GFP antibody (upper panel). GST or GST-fusion proteins were loaded on SDS–PAGE gel and stained with Coomassie blue (lower panel). (B) GATE16 was co-immunoprecipitated with TRPML3 and TRPML3-loop from HEK293T cell extracts. Cells were co-transfected with the indicated constructs and immunoprecipitated with anti-GFP antibody. Samples were analyzed on Western blotting and immunoblotted with anti-FLAG antibody. The inputs, corresponding to 5% of the amount used for each immunoprecipitation, were blotted with anti-FLAG antibody (middle panel) and anti-GFP antibody (lower panel). (C) Same analysis as in (B) was done except that membrane fraction was used for the binding instead of whole cell lysate to better detect type-I and type-II GATE16. Note that only type-II GATE16 was found in co-IP analysis. (D) co-IP between TRPML3 and GATE16 increased in starvation condition. Same analysis as in (B) was done except that this time cells were kept in full media or serum-starved for 2 h.

TRPML3–GATE16 is dependent on the status of autophagy. Thus, we asked whether the interaction of TRPML3 with GATE16 can be increased upon induction of autophagy. Autophagy was induced by serum starvation for 2 h, and then, the level of co-immunoprecipitated GATE16 was compared between in fed and starved condition. As depicted in Fig. 2D, starvation of cells co-expressing TRPML3 and GATE16 resulted in the increased type-II level of GATE16 and more interaction with TRPML3. Taken together, these data indicate that the interaction of TRPML3 with GATE16 is modulated by autophagy and *vice versa* and that this interaction takes place exclusively at the organellar membrane.

3.4. TRPML3 facilitates GATE16 function in autophagy, leading to increased autophagosome formation

All ATG8 proteins are lipidated and recruited to autophagosomes on induction of autophagy and play a key role in autophagosome biogenesis [9]. To verify the role of GATE16 in autophagy, HeLa cells expressing GFP-GATE16 were observed by confocal microscopy in fed and starved condition. The images in Fig. 3A and the summary in Fig. 3D show that the number of GATE16-positive particles is initially low and most of GATE16 exists in soluble form. On the other hand, the number of particles or lipidated form of GATE16 is increased by double in starvation condition. Cells co-expressing TRPML3 also showed an increased number of the particles and starvation of the cells caused a marked increase of the particles (Fig. 3B and D). Since GATE16 plays an important role in autophagosome biogenesis, these results suggest that the interaction with TRPML3 facilitates the function of GATE16 in autophagy, leading to increased number of autophagosomes.

The overlap between TRPML3 and GATE16 was low in fed condition, but it was increased by starvation (Fig. 3B and E), confirming the binding results in Fig. 2. However, this result is quite different from that of LC3B, since TRPML3 is highly colocalized with LC3B in both fed and starved cells to a similar extent [4] (Fig. 3C and E). GATE16 was originally characterized as a factor essential for intra-Golgi protein transport and was shown to modulate this process by coupling NSF activity and the Golgi SNARE factor GOS-28 [10,16,17]. In contrast, no direct involvement of LC3 in membrane trafficking process has been reported [9]. Therefore, the difference shown in the summary of Fig. 3E may be due to the extra-autophagosomal localization and function of GATE16. This idea is supported by the experiment in Fig. 3B. When autophagy was induced in cell starvation condition (Fig. 3B, lower panel), the overlap of TRPML3 and GATE16 is markedly increased to the level of that of TRPML3 and LC3B, suggesting that GATE16 is recruited to

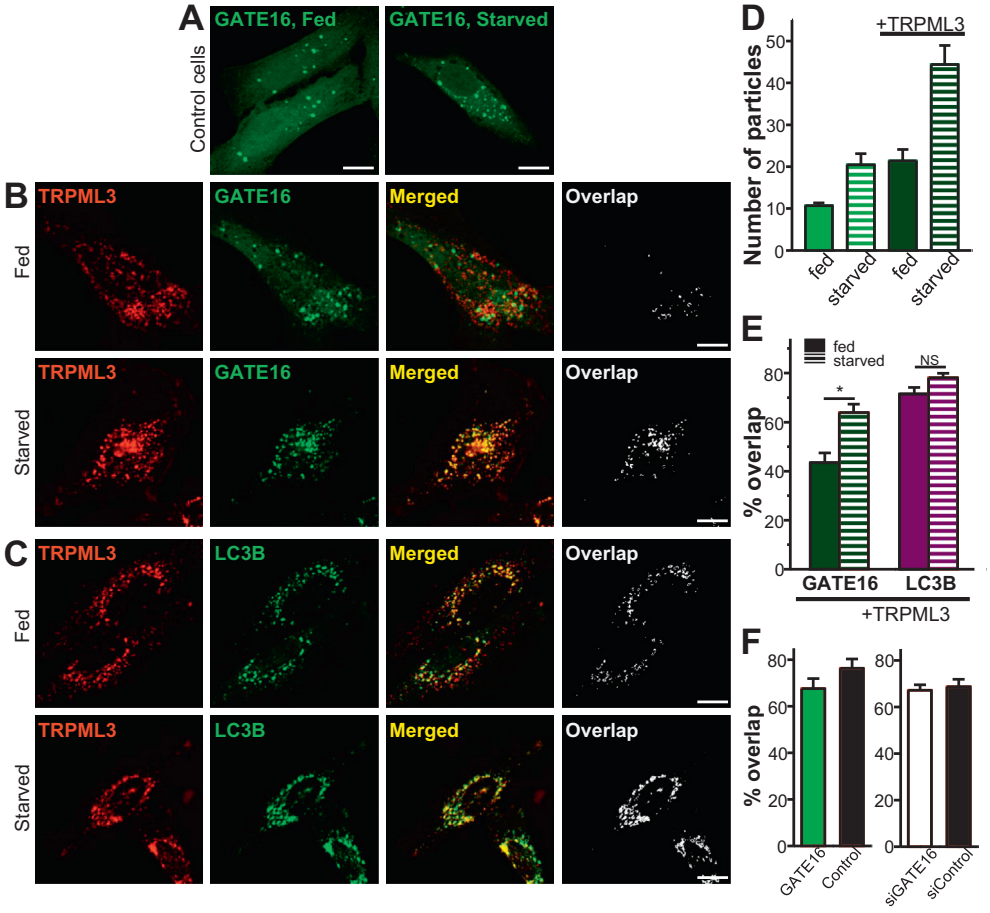

Fig. 3. Localization and function of TRPML3 and GATE16 in autophagy. (A) HeLa cells expressing GFP-GATE16 were kept in fed media or serum-starved for 2 h. HeLa cells expressing mCherry-TRPML3 and GFP-GATE16 (B) or mCherry-TRPML3 and GFP-LC3B (C) were treated as in (A). Bars indicate 10 μm. (D) The number of puncta of GFP-GATE16 in (A) and (B) was counted and given as mean ± SEM of 12–17 cells from 2–3 experiments. (E) The overlap between TRPML3 and GATE16 or TRPML3 and LC3B in fed or starved condition was determined with imageJ and given as mean ± SEM of 7–15 cells from 2–3 experiments(student *t* test, $^{*}p < 0.05$). (F) HeLa cells were transfected with mCherry-TRPML3, GFP-LC3B and FLAG-GATE16 or empty FLAG vector.(left panel). HeLa cells were treated with siRNA for GATE16 (siGATE16) or control non-targeting siRNA (siControl) for 2 d and then transfected with mCherrry-TRPML3 and GFP-LC3B (right panel). The overlap between TRPML3 and LC3B at indicated conditions was analyzed by ImageJ and plotted as mean ± SEM of 7–23 cells from 2–3 experiments.

autophagosomes from other cellular compartments upon induction of autophagy and further interacts with TRPML3, resulting in increased overlap.

3.5. GATE16 is not required for the recruitment of TRPML3 to autophagosomes

Since ATG8 proteins are implicated in selective cargo recruitment to the autophagosome [18], next we tested whether TRPML3 localization in autophagosomes is affected by GATE16. Recruitment of TRPML3 to autophagosome was monitored by GFP-LC3 co-transfection with mCherry-TRPML3 in HeLa cells. To increase the expression level of GATE16, FLAG-GATE16 or empty vector as a control was transiently transfected with the plasmid combination. By contrast, to see the effect of KD of GATE16, siRNA targeting GATE16 or non-targeting control siRNA that are shown in previous study [13] was treated in HeLa cells 2 d before the plasmids transfection. When we observed the overlap between TRPML3 and LC3B in the overexpression or KD of GATE16, we could not detect any difference (Fig. 3F). Therefore, it seems that TRPML3 is recruited to LC3B-positive autophagosomes independently of GATE16 or TRPML3–GATE16 interaction.

3.6. GATE16 is distinctive from LC3B in expression and function

To better dissect the difference between GATE16 and LC3B shown in Fig. 3, we used TRPML3(D458K), a DN form of TRPML3 that does not possess channel function. As channel function of TRPML3 is not required for the interaction (Fig. 2), it is possible that DN TRPML3(D458K) interacts with GATE16. Our previous study revealed that expression of the DNTRPML3(D458K) inhibits autophagy and consequently reduces formation of autophagosomes in response to various cell stressors [4]. Consistent with the previous report, co-expression of TRPML3(D458K) with LC3B decreased the number of autophagosomes and completely blocked the overlap between TRPML3 and LC3B (Fig. 4B). Surprisingly, however, GATE16 is robustly expressed in the vesicular compartments and substantially colocalized with TRPML3 in the same set of experiment (Fig. 4A and summary in Fig. 4C and D), although autophagy is inhibited by DN TRPML3(D458K). These results, combined with the data in Fig. 3B and E, clearly show that a considerable amount of GATE16 is localized in extra-autophagosomal compartments regardless of the status of autophagy. Accordingly, the GATE16 particles shown in Figs. 3 and 4 may not be all autophagosomes, but they include some other organelles. This is important because both TRPML3 and GATE16 have been implicated not only in autophagy but also in intracellular protein trafficking processes. Taken together, TRPML3 and GATE16 interact at the autophagosome and extra-autophagosomal organellar membrane to regulate autophagy and perhaps membrane trafficking events as well.

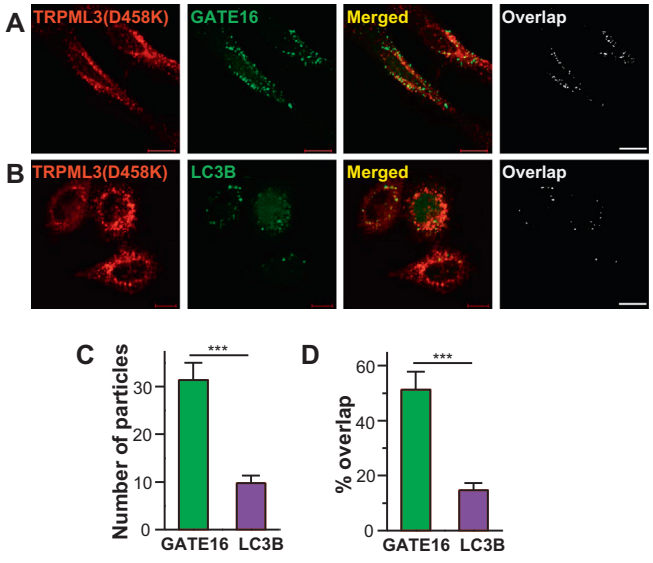

Fig. 4. Effect of inhibition of autophagy by DN TRPML3(D458K) on the behavior of GATE16 and LC3B. HeLa cells expressing GFP-GATE16 (A) or GFP-LC3B (B) with mCherry-TRPML3(D458K) were analyzed by confocal microscopy. Bars indicate 10 μm. (C) The number of puncta of GFP-GATE16 and GFP-LC3Bin (A) and (B) was counted and given as mean ± SEM of 11–18 cells from 1–2 experiments (***$p < 0.005$). (D) The overlap between TRPML3 and GATE16 or TRPML3 and LC3B was determined with imageJ and given as mean ± SEM of 12–28 cells from 1–2 experiments (***$p < 0.005$).

Another important distinctive feature between LC3B and GATE16 is that they act at different stages of autophagosome formation: LC3B is involved in elongation of the phagophore membrane whereas GATE16 is essential for a later stage in autophagosome maturation, possibly in sealing of autophagosomes [14]. It is suggested that the difference of their function might be due to their ability to recruit essential factors to the phagophore [9]. Since TRPML3 exclusively binds to GATE16, not to LC3B (Fig. 2A), the binding with TRPML3 may be a factor that makes a difference between them. How might TRPML3 affect autophagosome biogenesis? TRPML3 is a Ca^{2+} permeable channel and thereby can generate Ca^{2+} efflux from organellar lumen to cytosolic side. Although it is well known that Ca^{2+} is required for the membrane fusion events during autophagy, how Ca^{2+} is supplied in the process has not been understood. Here we suggest that the specific binding of TRPML3 to GATE16 can provide Ca^{2+}, only in the proximity of GATE16 to regulate autophagosome maturation and sealing process that requires Ca^{2+}.

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