The small GTPase Rab7 promotes fusion events between late endosomes and lysosomes. Rab7 activity is regulated by extrinsic signals, most likely via effects on its guanine nucleotide exchange factor (GEF) or GTPase activating protein (GAP). Based on their homology to the yeast proteins that regulate Ypt7 GTP-binding state, TBC1D15 and mammalian Vps39 (mVps39) have been suggested to function as the Rab7 GAP and GEF, respectively. We developed an effector pull-down assay to test this model. TBC1D15 functioned as a Rab7 GAP in cells, reducing Rab7 binding to its effector protein RILP, fragmenting the lysosome, and conferring resistance to growth factor withdrawal-induced cell death. In a cellular context, TBC1D15 GAP activity was selective for Rab7. TBC1D15 over-expression did not inhibit transferrin internalization or recycling, Rab7-independent processes that require Rab4, Rab5, and Rab11 activation. TBC1D15 was thus renamed Rab7-GAP. Contrary to expectations for a Rab7 GEF, mVps39 induced lysosomal clustering without increasing Rab7 GTP binding. Moreover, a dominant-negative mVps39 mutant fragmented the lysosome and promoted growth factor independence without decreasing Rab7-GTP levels. These findings suggest that a protein other than mVps39 serves as the Rab7 GEF. In summary, although only TBC1D15/Rab7-GAP altered Rab7-GTP levels, both Rab7-GAP and mVps39 regulate lysosomal morphology and play a role in maintaining growth factor dependence.
Consistent with a model where Vps39 is a Ypt7 effector rather than a GEF, Gyp7 addition causes the release of Vps39 from vacuolar membranes in vitro suggesting that GTP hydrolysis releases Vps39 from Ypt7 (21). No other candidate Ypt7 GEF has been identified. Regardless of whether Vps39 functions as a GEF, an effector, or fills another role, Vps39 clearly promotes vacuolar fusion. Yeast null for Vps39 exhibit severe vacuolar fragmentation (26-28). In addition, vacuoles lacking Vps39 or exposed to Vps39 antibodies are deficient for fusion in vitro (29). In vitro vacuole fusion is separated into four steps termed priming, tethering, docking, and fusion (reviewed in (30)). During the priming phase, cis-SNARE complexes are disassembled, rendering them competent to assemble into the trans-complexes that form during the docking stage and eventually drive membrane fusion. Both a large, hexameric Vps39-containing complex and Ypt7 are required for docking (29,31). The protein complex containing Vps39, Vps41, and the homotypic fusion and vacuole protein sorting (HOPS) complex (Vps11, 16, 18, and 33) may function as a Ypt7 effector in yeast, linking Rab7-GTP to the trans-SNARE pairing that completes the docking stage. After docking, membrane fusion can proceed in the absence of Ypt7 (21).

While lysosomal fusion has been studied in mammalian cells, much less is known about how Rab7 activity is regulated. Multiple Rab GAPs contain a TBC (Tre2/Bub2/Cdc16) domain (32-38). TBC1D15 has been identified as a putative Rab7 GAP based on its homology to Gyp7. Purified TBC1D15 accelerates GTP hydrolysis by Rab7 in vitro (39). However, GAPs are often promiscuous in vitro while exhibiting much more restricted activity in cells (40). The apparent homolog of yeast Vps39, mammalian Vps39 (mVps39), interacts with mammalian homologs of Vps41 and the HOPS complex (41). mVps39 clearly has an important role in lysosomal trafficking, but its mechanism of action is not completely understood. mVps39 over-expression promotes the centripetal movement of late endosomes/lysosomes in mammalian cells similar to what is seen in cells expressing constitutively-active Rab7 (42,43). mVps39 also promotes the exchange of Rab5 for Rab7 on endosomes (44). These results are consistent with mVps39 functioning as a Rab7 effector, a Rab7 GEF, or performing a function that is independent of Rab7 GTP binding state. Interestingly, the best studied Rab7 effector, RILP, has no yeast homolog. RILP binds selectively to Rab7-GTP and recruits the dynein-dynactin motor complex to facilitate vesicle movement toward the minus end of microtubules (13,15,45). The N-terminus of RILP is required to recruit dynein motors, but not to bind GTP Rab7, and thus an N-terminal truncation of RILP functions as a dominant-negative mutant (13,15). DN-RILP locks Rab7 in its GTP-bound state despite the fact that it inhibits Rab7-dependent fusion reactions (15). How mVps39 or TBC1D15 affect Rab7’s ability to bind its effector RILP has not been previously investigated.

To test whether TBC1D15 and mVps39 function as the Rab7 GAP and GEF, respectively, we developed an effector pull-down assay using the Rab7 binding domain of RILP. As a biological correlate, the effects of these proteins on lysosomal morphology and growth factor dependence were monitored in the murine hematopoietic cell line, FL5.12. FL5.12 cells are immortalized, but not transformed and depend upon IL-3 for growth, proliferation, and survival (46-48). FL5.12 cells and other IL-3 dependent hematopoietic cell lines are often used to investigate the consequences of growth factor withdrawal because artifacts due to the withdrawal of nutrients, vitamins, and poorly defined factors present in serum are eliminated. These cells are also useful because of their distinctive lysosomal morphology. FL5.12 cells contain only 3-5 lysosomes per cell, more closely resembling the vacuolar architecture of yeast than the dispersed lysosomal staining pattern typical of many adherent mammalian cell lines. We report here that TBC1D15 was a selective Rab7 GAP in a cellular context while mVps39 did not affect Rab7-GTP binding state as would be expected for a Rab7 GEF. Both mVps39 and TBC1D15 modulated lysosomal morphology and played a previously unrecognized role as regulators of growth factor dependence.

**EXPERIMENTAL PROCEDURES**

**Antibodies, plasmids, and reagents.** Rab7 antibody (clone Rab7-117) and FLAG M2 antibody were obtained from Sigma. Human
Lamp1 antibody (clone eBioH4A3) was obtained from eBioscience. The myc antibody (clone 9B11) was obtained from Cell Signaling. The eGFP antibody (clone JL-8) was obtained from Clontech. Secondary antibodies were obtained from LI-COR Biosciences. Complete protease inhibitors were from Roche Diagnostics. BCA reagents used for protein assays were from Pierce Chemical Company. Murine TBC1D15 was amplified by PCR from pSport1 IMAGE clone 30054233 and an N-terminal myc tag added. The PCR product was TA sub-cloned into pEF6/V5-His (Invitrogen), sequence confirmed, and moved into the retroviral vector pBABEpuro. Human GFP-DN-RILP and GFP-RILP were generously provided by Cecilia Bucci (Università del Salento, Lecce, Italy). To generate an mVps39 expression construct, RNA was isolated from FL5.12 cells using a Qiagen RNAeasy kit. The mVps39 cDNA was generated using a Superscript III RT-PCR kit with Platinum Taq (Invitrogen) and TA cloned into the EF6/V5-His vector (Invitrogen). An N-terminal myc tag was added during RT-PCR. Sequencing of the entire insert identified four mutations in mVps39; this construct was renamed mVps39mut. Wildtype mVps39 was kindly provided by Dr. Robert Piper (University of Iowa) and cloned into pBABEpuro. GFP-Rab5-S34N was generously provided by Philip Stahl (Washington University School of Medicine). GFP-Rab11-S25N was obtained from Richard Pagano via Addgene (Plasmid 12678). All plasmids were sequence confirmed.

Cell culture and transfection. FL5.12 cells were maintained at low density in RPMI (Mediatech) supplemented with 10% FCS (Mediatech, Hyclone, or Sigma), 500 μM recombinant mouse IL-3 (BD Pharmingen or eBioscience), 10 mM HEPES (Mediatech), 55 μM β-mercaptoethanol (Sigma), antibiotics, and L-glutamine (Mediatech). Cell lines expressing transgenes were used in experiments within five days of thawing. Extracellular nutrient limitation was performed in RPMI made from chemical components that contained 10% dialyzed FCS (Invitrogen) in place of standard serum. Hela and 293T cells were maintained in DMEM (Mediatech) supplemented with 10% FCS. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

GST-RILP pull-downs. Nucleotides 658 to 897 of Genbank accession number NM 001029938 (amino acids 220-299) of the murine RILP protein constituting the Rab7 binding domain of RILP were fused to the C-terminus of GST in the pGEX 4T-3 vector (Stratagene). GST-RILP was transformed into E. coli strain BL21. 250 ml of LB was inoculated with 1 ml of an overnight culture and grown at 37°C to an OD of 0.6 to 0.8. IPTG (EMD) was then added to a final concentration of 0.5 mM to induce protein production. The 250 ml culture was incubated for additional 3-4 hr at 30°C, after which the bacteria were spun down, washed with cold PBS, resuspended in 5 ml of cold lysis buffer (25 mM Tris-HCL pH 7.4, 1 M NaCl, 0.5 mM EDTA, 1 mM DTT, 0.1% TX-100, with Complete protease inhibitors), then sonicated. The bacterial lysates were cleared by centrifugation, and 5 ml of cold lysis buffer was added. Proteins were purified by adding 300 μl of a pre-equilibrated 50% slurry of glutathione sepharose 4B beads (GE Healthcare) to the lysate. Beads were incubated with lysates for 30 min. at room temperature then washed with lysis buffer, resuspended as a 50% slurry, and protein levels quantified using the BCA Assay. Mammalian cells to be analyzed in the pull-down were lysed in pull-down buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl2, 1% TX-100, protease inhibitors). Each pull-down was performed in 1 ml with 300 μg of cell lysate and 30 μg of beads pre-equilibrated in pull-down buffer. Beads were rocked overnight at 4°C, washed twice with cold pull-down buffer, and bound proteins eluted by adding 2X NuPAGE Sample buffer (Invitrogen) and incubating at 72°C for 10 min.

Flow cytometry and microscopy. Cells were analyzed on a Becton Dickinson LSR II flow cytometer. Viability was determined by vital dye exclusion (propidium iodide or DAPI, Invitrogen). To evaluate lysosomal morphology, cells were stained with 500 nM Lysotracker Red (Invitrogen) for 30 min. at 37°C and examined using a Nikon Eclipse TE2000 fluorescence microscope equipped with a Coolsnap CCD camera. Quantitation of the number of lysosomes was performed manually using the tagging function
available in ImagePro software. To evaluate Lamp1 clustering, transfected Hela cells growing on coverslips were fixed and permeabilized in IF block (PBS with 10% FCS, 2% paraformaldehyde, 0.3% saponin, and 0.05% sodium azide) for 15 min at room temperature. Cells were washed twice in IF wash (PBS with 0.03% saponin) before and after staining with antibodies.

**Transferrin internalization and recycling.** FL5.12 cells were washed with PBS and resuspended in serum-free medium with 1% bovine serum albumin (BSA). The cells were incubated at 37°C for 1 hr then washed with ice-cold PBS before labeling on ice with 100 μg/ml biotinylated transferrin (Invitrogen) in serum-free medium with 1% BSA. Cells were washed with ice-cold PBS to remove unbound transferrin and incubated at 37°C in complete medium with 20% FCS. At the indicated time points, cells were washed in ice-cold PBS with 2% FBS and 0.05% sodium azide, fixed with 1% paraformaldehyde (on ice for 30 min. followed by 10 min at RT), stained with streptavidin-APC (eBioscience), and analyzed on a BD LSRII flow cytometer.

**RESULTS**

**TBC1D15 is a Rab7 selective GAP.** TBC1D15 can function as a GAP for Rab7 in vitro (39). However, GAPs are often promiscuous in vitro while displaying selective activity in a cellular context. To test whether TBC1D15 serves as a Rab7 GAP in cells, we developed an effector pull-down assay in which the Rab7-binding domain of its effector protein RILP (49) was fused to GST and used to selectively isolate Rab7-GTP from cell lysates. Pull-down assays on cells expressing Rab7 mutants that are predominantly GDP- (Rab7-T22N) or GTP-bound (Rab7-Q67L) confirmed that Rab7-GTP was selectively isolated by GST-RILP (Fig. 1, A and B) (2,13,15). To further validate this assay, we evaluated Rab7-GTP binding status in cells expressing mutant or wild-type RILP. In keeping with published results (13,15), both DN-RILP and wild-type RILP increased Rab7-GTP levels (Fig. 1, C and D). The SifA protein from Salmonella enterica interferes with Rab7 recruitment to the Salmonella containing vacuole, possibly by serving as a competitive inhibitor (45). Unlike DN-RILP, SifA does not alter the Rab7 GTPase cycle (50). As expected, no change in Rab7-GTP levels was observed in cells expressing SifA (Fig. 1, C and D). These experiments demonstrate that the effector pull-down assay has the dynamic range to detect both reductions and elevations in Rab7-GTP binding.

Consistent with the ability of TBC1D15 to function as a Rab7 GAP in vitro (39), the fraction of Rab7 bound to GTP was reduced in cells expressing TBC1D15 (Fig. 1, C and D). As a functional correlate, we tested whether TBC1D15 over-expression altered lysosomal morphology. TBC1D15 expression led to lysosomal fragmentation (Fig. 2, A and B). The degree of fragmentation was similar to that observed upon expression of the dominant-negative mutant Rab7-T22N. Fragmentation induced by TBC1D15 was not reversed by expression of Rab7-Q67L, possibly because high enough levels of the activated mutant were not achieved. In addition to fragmenting the lysosome, inactivating Rab7 decreases cellular sensitivity to growth factor withdrawal-induced apoptosis (4). Cells stably over-expressing TBC1D15 were also protected from growth factor withdrawal-induced death (Fig. 2C). Together, these results are consistent with a role for TBC1D15 as a Rab7 GAP.

**TBC1D15 can function as a Rab11 GAP in vitro** (39) and an association between TBC1D15 and Rab5 was detected in yeast two-hybrid assays (51). To determine whether TBC1D15 might also serve as a GAP for these Rabs, we measured transferrin endocytosis and recycling in cells over-expressing TBC1D15. Transferrin internalization depends on the function of both Rab4 and Rab5 while Rab4 and Rab11 promote transferrin recycling (3,52-58). Both transferrin internalization and recycling are, on the other hand, independent of Rab7 (3,59). To measure transferrin internalization, biotinylated transferrin was bound to FL5.12 cells on ice and its disappearance from the cell surface after warming cells to 37°C followed using flow cytometry.
Despite the fact that lysosomes were severely fragmented, transferrin was internalized at the same rate in TBC1D15 over-expressing and control cells (Fig. 3A). Transferrin internalization was delayed, however, in cells expressing dominant-negative Rab5-S34N. For recycling assays, FL5.12 cells were maintained in media containing biotinylated transferrin for 1 h at 37°C. The cells were then washed, placed in media containing excess unlabelled transferrin, and the clearance of biotinylated transferrin from cells as a result of recycling measured over time. While a GDP-locked mutant of Rab11 reduced the rate of transferrin recycling, cells over-expressing TBC1D15 recycled transferrin with kinetics that were indistinguishable from controls (Fig. 3B). These experiments indicate that in intact cells, TBC1D15 functions as a GAP for Rab7, but not Rab4, 5, or 11. We have therefore renamed TBC1D15 Rab7-GAP.

*mVps39 does not display the properties of a Rab7 GEF. *Yeast Vps39 mutants possess a fragmented vacuole (26,27,60,61). This may be because Vps39 functions as a GEF for the yeast ortholog of Rab7, Ypt7 (23). However, the GEF activity of Vps39 remains controversial. To test whether mVps39 can function as a Rab7 GEF, the effect of mVps39 over-expression on Rab7-GTP loading, lysosomal morphology, and growth factor withdrawal-induced apoptosis was evaluated. A mVps39 cDNA was derived from FL5.12 cells by RT-PCR. An N-terminal myc epitope tag was added to track the protein as other groups have established that this modification does not interfere with Vps39 function (28,42). Two splicing variants of mVps39 are expressed in cells. The cDNA we isolated from FL5.12 cells corresponded to the shorter isoform of Vps39 that lacks exon 3 resulting in the replacement of 12 amino acids in the longer isoform with a glycine residue. Based on the published sequence of mVps39, four point mutations had been introduced during the process of RT-PCR (Fig. 4A). Rather than repairing this construct or screening additional clones, we obtained the wild-type mVps39 cDNA from Dr. Robert Piper’s group (41,43). Because the mutations in our initial construct were in highly-conserved domains of the protein (Fig. 4, A and B), we included the mutant mVps39 (mVps39mut) in subsequent assays.

If mVps39 functions as a Rab7 GEF, over-expressing mVps39 should increase the fraction of Rab7 that is GTP-bound. However, wild-type mVps39 did not alter Rab7-GTP levels (Fig. 1, C and D). To confirm that this mVps39 construct was fully functional, its ability to alter lysosomal morphology in HeLa cells was evaluated. Consistent with previous reports (42,43), transient expression of mVps39 in HeLa cells produced lysosomal clustering in 50% of the transfected cells, a 10-fold increase over background (Fig. 4, C and D). Lysosomal clustering is not readily detected in FL5.12 cells as they normally possess very few lysosomes (Fig. 2, A and B). Intriguingly, mVps39mut appeared to act as a dominant-negative protein. When introduced into HeLa cells, mVps39mut did not cluster Lamp1-positive structures (Fig. 4, C and D). Similarly, mVps39mut did not alter Rab7 GTP binding (Fig. 1, C and D). mVps39mut produced lysosomal fragmentation of a similar magnitude to that seen with the dominant-negative Rab7-T22N mutant when expressed in FL5.12 cells (Fig. 2, A and B). Taken together, this data strongly suggests that mVps39 does not promote lysosomal clustering or fusion by functioning as a Rab7 GEF.

In keeping with its effects on lysosomal morphology, mVps39mut dramatically increased growth factor-independent cell survival (Fig. 4E). Wild-type mVps39 neither fragmented the lysosome nor increased growth factor-independent cell survival (Fig. 2, A and B, Fig. 4F). Lysosomal fusion reactions are required for cells to derive bioenergetic benefits from autophagy (7,62). Cell lines over-expressing TBC1D15 or mVps39mut were not resistant to nutrient withdrawal (Fig. 4G). In contrast, cells expressing a Bcl-2 mutant, G145A, that does not interfere with autophagy (63) were resistant to nutrient deprivation-induced death. Thus, the growth factor-independent cell survival seen in cell lines expressing Rab7-GAP or mVps39mut did not result from a primary apoptotic defect.

How mVps39mut altered lysosomal morphology was not clear. mVps39mut did not interfere with Rab7 recruitment onto endosomal membranes; all Lysotracker Red-positive structures were GFP-Rab7-positive in control cells and in cells expressing mVps39mut (Fig. 4H). mVps39 facilitates the exchange of Rab5 for Rab7.
on endosomes—mVps39 RNAi induces swollen Rab5-positive endosomes (44). However, GFP-Rab5 localization was indistinguishable in control and mVps39mut-expressing FL5.12 cells (data not shown). Loss of acidification blocks vacuole fusion in yeast (64). Based on their strong staining with Lysotracker Red, the fragmented lysosomes in mVps39mut-expressing cells appeared to be acidified. Because mVps39mut fragmented lysosomes (Fig. 2, A and B) without affecting Rab7 GTP binding status (Fig. 1, C and D) reduced Rab7 GEF activity is also unlikely. If mVps39 functions as a Rab7 effector, mVps39mut might block the downstream effects of Rab7 activation. In this case, mVps39 would be expected to preferentially bind to the GTP-bound form of Rab7. Using a variety of detergents and buffers, we were unable to detect an association between mVps39 and Rab7 or Rab7-Q67L via co-immunoprecipitation. We were likewise unable to isolate mVps39 from cell lysates using recombinant GST-Rab7 or GST-Rab7-Q67L immobilized on glutathione beads. Taken together, these results suggest that mVps39mut alters lysosomal morphology through an indirect or even Rab7-independent action.

**DISCUSSION**

We developed an effector pull-down assay and used it to demonstrate that TBC1D15 functions as a Rab7-GAP in cells (Fig. 1 C and D and Fig. 2). Our observation that the activated mutant Rab7-Q67L did not reverse TBC1D15-induced fragmentation (Fig. 2, A and B) might be explained by a failure to attain sufficiently high levels of Rab7-Q67L or by the fact that expressing Rab7-Q67L to high levels is toxic (10). Alternatively, nucleotide cycling may be required for full Rab7 function. Dominant-active Rabs can produce results that are inconsistent with GAP over-expression (65). Although Rab7-Q67L is functional under at least some experimental conditions (59), “activated” mutants can inhibit a Rab-dependent process (57,58,66). By performing transferrin internalization and recycling assays (Fig. 3, A and B), we established that the GAP activity of TBC1D15 was selective for Rab7 in cells justifying the renaming of TBC1D15 as Rab7-GAP.

Multiple studies indicate that mVps39 plays a key function in lysosomal fusion but its mechanism of action remains unclear. It is often assumed that mVps39 is a Rab7 GEF. However, the function of the homologous protein in yeast is subject of debate, as Vps39 possesses the properties of both a Rab7 GEF and an effector in different assays performed in different laboratories (21,23-25). Although it is impossible to rule out that under some circumstances mVps39 can function as a Rab7 GEF, our finding that mVps39 promotes lysosomal clustering (Fig. 4C) without altering Rab7-GTP levels (Fig. 1, C and D) strongly suggests that increasing the exchange of GDP for GTP is not the principal mechanism by which mVps39 promotes lysosomal fusion reactions. The effector pull-down assay has the dynamic range to detect either an increase or a decrease in Rab7-GTP levels (Fig. 1, C and D) supporting our proposal that Rab7 GTP binding is not altered by mVps39. The fact that a dominant-interfering mutant of mVps39, mVps39mut, produced lysosomal fragmentation (Fig. 2, A and B) without decreasing Rab7 GTP binding (Fig. 1, C and D) is also consistent with the proposal that mVps39 does not function as a Rab7 GEF. As mVps39mut does not block Rab7 recruitment (Fig. 4H) or grossly affect Rab5 localization (data not shown), mVps39mut may interfere with lysosomal fusion down-stream from Rab7. This model would be consistent with yeast studies suggesting that Vps39 functions as a Ypt7 effector protein (21,24,25). It is also possible that mVps39 promotes lysosomal fusion through indirect effects on Rab7. Intriguingly, yeast Vps39 has recently been shown to function as a GEF for Gtr1 and Gtr2, GTPases that regulate the activity of the TOR kinase in response to nutrient signals (67). Mammalian TOR translocates to a Rab7-positive compartment in response to signaling through the Rag GTPases that are homologous to the Gtr proteins (68,69). Thus, mVps39 may impact Rab7 indirectly by influencing mTOR signaling via the Rag GTPases. Consistent with this model, Rab7 activity is modulated by growth factors that regulate mTOR activity (10). Defining the precise mechanism by which mVps39 promotes lysosomal fusion reactions will require additional studies that may be facilitated by the dominant-negative mVps39 mutant described here.
The GST-RILP effector pull-down assay we have developed should help to clarify how alterations in Rab7 activity affect human disease processes. Many microorganisms directly target Rab7-dependent fusion reactions to block degradation of the pathogen-containing phagosome in the lysosome (5). Evaluating Rab7 activation state using GST-RILP pull-down assays may provide key mechanistic insights into how intracellular pathogens avoid lysosomal degradation. Activating mutations in Rab7 have been described in Charcot-Marie-Tooth disease type 2B (70-72). It would be interesting to determine whether Rab7-GTP binding is altered in other forms of this peripheral neuropathy. Reductions in Rab7 activity and or lysosomal fusion may also contribute to tumor initiation or progression. We demonstrate that Rab7-GAP (Fig. 2C) and mVps39 (Fig. 4E) regulate cellular growth factor dependence. Altering Rab7-GAP or mVps39 activity might contribute to oncogenesis not only by increasing growth factor-independent cell survival, but also by blocking autophagy, thereby increasing genomic instability (73-76). Microarrays have detected increased TBC1D15/Rab7-GAP mRNA in cervical cancer, decreased mVps39 mRNA levels in B cell lymphomas and prostate cancer, and reduced Rab7 mRNA levels in some B and T cell leukemias (Oncomine database, [77]). Microarray studies are likely to tell only part of the story as the activity of these proteins is likely to be regulated post-translationally. A more complete understanding of how signal transduction pathways regulate and are regulated by Rab7-GAP and mVps39 will facilitate future investigations of whether or these proteins play a role in cancer and other diseases.

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FIGURE LEGENDS

Fig. 1. Measurement of Rab7-GTP levels using an effector pull-down assay. A) HEK 293T cells were transfected with plasmids encoding GFP or GFP-tagged Rab7, Rab7-Q67L, or Rab7-T22N. The Rab7 binding domain of RILP coupled to GST (GST-RILP) or GST alone was used to precipitate the GTP-bound form of Rab7 from cell lysates. Western blots were scanned using a LI-COR infrared imaging system. No signal was detected in any experiment when eluates from beads coupled to GST alone were evaluated (not shown). (B) The amount of Rab7 isolated by GST-RILP (GTP-bound Rab7) was expressed relative to the total amount of Rab7 in the sample (INPUT) and then normalized to the amount of wild-type Rab7-GTP detected. Means from triplicate gels are presented. Similar results were obtained in four independent experiments. C) GST-RILP pull-downs were performed as in (A) using cells transiently expressing the indicated constructs. D) The results in (C) were quantified and normalized as in (B). Similar results were obtained in FL5.12 cells. Results are representative of three independent experiments. Error bars, SD.

Fig. 2. Over-expression of TBC1D15 disrupts lysosomal morphology and blocks growth factor withdrawal-induced cell death. A) FL5.12 cells stably expressing the indicated constructs or empty vector (VEC) were stained with Lysotracker Red in the presence of IL-3 and live cells analyzed by fluorescence microscopy. Scale bar, 5 μm. B) Lysosomal fragmentation was quantified by counting the number of Lysotracker Red-positive structures present per cell. In all cases where the number of lysosomes increased, there was a concomitant decrease in the size of Lysotracker Red-positive structures. From 40 to 70 cells were evaluated for each sample. Error bars, SEM. C) Viability following IL-3 withdrawal was measured in cells expressing empty vector (VEC) or myc-tagged TBC1D15 by vital dye exclusion and flow cytometry. A representative experiment of three independent experiments is shown. Error bars, SD.

Fig. 3. Over-expression of TBC1D15 does not interfere with transferrin internalization or recycling. A) FL5.12 cells expressing empty vector, Rab5-S34N, or TBC1D15 were surface labeled with transferrin as described in the Experimental Procedures and transferrin internalization followed using flow cytometry. B) FL5.12 cells expressing empty vector, Rab11-S25N, or TBC1D15 were labeled with biotinylated transferrin for 1 hr at 37°C and recycling measured as described in the Experimental Procedures section. Mean values from four independent experiments are shown. Error bars, SEM.

Fig. 4. Characterization of mVps39mut. A) Location of the four point mutations present in mVps39mut. mVps39mut is derived from the shorter isoform (isoform 2) of mVps39. Exon 3, replaced by a glycine in
the short form of mVps39 due to alternative splicing, is shown in orange. The citron homology domain (citron) is required for mVps39 localization to lysosomes and to induce lysosomal clustering (42,43). The clathrin homology domain (CLH) may mediate protein-protein interactions with Vps41, the HOPS complex, and mVps39 itself (41,42). The K775R and V843I mutations occur in a region homologous to “domain II” of the yeast protein. In yeast, the deletion of domain II blocks Vps39 function by eliminating its association with membranes, Vps11, and Vps18 (23).

B) Cross-species alignment of the four regions containing mutations in dominant negative Vps39. The mutated residue is shown in red. Residues absolutely conserved in invertebrates are shown in blue. Hs, Homo sapiens; Mm, Mus musculus; Gg, Gallus gallus; Xi, Xenopus laevis; Tr, Takifugu rubripes; Dm, Drosophila melanogaster; Sc, Saccharomyces cerevisiae.

C) HeLa cells were transfected with empty vector (CONT) or plasmids encoding myc-tagged mVps39 or mVps39mut, fixed, permeabilized, and stained with anti-myc and anti-Lamp1 antibodies. Scale bar, 10μm.

D) Quantification of the results shown in (C). For each condition, 130 to 180 cells were examined in three independent experiments. (E,F) Cells expressing myc-tagged mVps39mut (E) or myc-tagged mVps39 (F) were evaluated for growth factor dependence as in Fig. 2C. A representative experiment of at least three independent experiments is shown. Error bars, SD.

G) FL5.12 cells stably expressing the indicated transgenes were withdrawn from amino acids and glucose for the indicated intervals. The Bcl-2 G145A mutant was utilized because it does not interfere with autophagy. Viability was determined by vital dye exclusion and flow cytometry. A representative experiment is shown; similar results were obtained in three independent experiments. Error bars, SD.

H) GFP-Rab7 was introduced into FL5.12 cell lines stably expressing empty vector (CONT) or mVps39mut. Cells were stained with Lysotracker Red and live cells examined by fluorescence microscopy. Cells expressing relatively low levels of GFP-Rab7 were selected for evaluation. Scale bar, 5 μm.
A

VEC Rab7-T22N TBC1D15 TBC1D15 + Q67L mVps39 mVps39mut

LysoTracker

DIC

B

![Graph showing lysosomes per cell](image)

C

![Graph showing percent viable over time](image)
