RESEARCH PAPER

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The Vps39-like TRAP1 is an effector of Rab5 and likely the missing Vps3 subunit of human CORVET

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Membrane fusion in the endocytic pathway is mediated by a protein machinery consistent of Rab GTPases, tethering factors and SNAREs. In yeast, the endosomal CORVET and lysosomal HOPS tethering complexes share 4 of their 6 subunits. The 2 additional subunits in each complex – Vps3 and Vps8 for CORVET, and the homologous Vps39 and Vps41 for HOPS – bind directly to Rab5 and Rab7, respectively. In humans, all subunits for HOPS have been described. However, human CORVET remains poorly characterized and a homolog of Vps3 is still missing. Here we characterize 2 previously identified Vps39 isoforms, hVps39–1/hVam6/TLP and hVps39–2/TRAP1, in yeast and HEK293 cells. None of them can compensate the loss of the endogenous yeast Vps39, though the specific interaction of hVps39–1 with the virus-specific LT protein was reproduced. Both human Vps39 proteins show a cytosolic localization in yeast and mammalian cells. However, hVps39–2/TRAP1 strongly co-localizes with co-expressed Rab5 and interacts directly with Rab5-GTP in vitro. We conclude that hVps39–2/TRAP1 is an endosomal protein and an effector of Rab5, suggesting a role of the protein as a subunit of the putative human CORVET complex.

Introduction

In eukaryotic cells, endocytosis is a process that mediates both nutrient uptake and cellular quality control by promoting the formation of vesicles that remove cell surface receptors and other transmembrane proteins from the plasma membrane. The cargo is then either recycled back to the plasma membrane or fed into the degradation pathway toward the lysosomal compartment. For coordination of membrane traffic and finally fusion of the vesicle with the target membrane, a protein machinery consistent of Rab GTPases, tethering factors, and SNAREs is needed.[1]

Rab GTPases are molecular switches that cycle between membranes and the cytosol, and at the same time between a GTP-loaded and a GDP-bound form. Further factors are required to control the Rab cycle: Guanine exchange factors (GEFs) catalyze the exchange of GDP for GTP and thereby shift the Rab GTPase to its activated form. On the other site, GTPase activating proteins (GAPs) stimulate the GTPase activity manifold, and thus, inactivate the Rab.[2]

Rab GTPases recruit tethering factors that bridge the opposing membranes prior to fusion. These tethering factors come in 2 flavors, either as dimeric coiled-coiled proteins or as multisubunit tethering complexes (MTCs) that consist of 3 to 10 subunits and can form huge protein complexes.[3] MTCs might have functions beyond just tethering of membranes. Some of them bind also to SNAREs and fulfill a proofreading and chaperoning function in SNARE assembly, which corresponds to an active participation in the control of fusion events.[4-6]

Every fusion process in the endomembrane system requires a specific set of Rabs and tethering factors. In yeast, the Rab5-like Vps21 controls fusion events at the early endosome and binds to the coiled-coiled tether Vac1 (yeast EEA1), but also recruits the hexameric CORVET complex that has overlapping and unique functions.[7] The yeast Rab7-like Ypt7 protein localizes at the lysosome/vacuole and recruits the HOPS tethering complex that promotes fusion events.[8,9] HOPS is also a hexameric tether and shares 4 of its 6 subunits (Vps11, Vps16, Vps18, Vps33), the so-called class C core complex, with the endosomal CORVET. The HOPS complex has intensively been studied and is needed for vacuole fusion in vivo and in vitro.[10-13] Electron microscopy revealed a seahorse-like structure of about 30 nm.[8] Furthermore, its subunits Vps39 and Vps41 bind to the active form of yeast Rab7, and Vps33 – a Munc18/Sec1-like protein – interacts with the vacuolar SNAREs in yeast. Analogous to HOPS, the unique CORVET subunits Vps3 and Vps8 are effectors of the yeast Rab5 protein Vps21.

The HOPS and CORVET complex were initially found and studied in yeast.[9,14-16] In higher eukaryotes, HOPS subunits have been characterized, implicating a similar function as in yeast.[17-28] Due to the exogenous expression and the presence of multiple isoforms for some of the identified HOPS subunits it remains unclear if HOPS or CORVET is analyzed in some

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studies. The core subunits Vps18 and Vps11 have one clear homolog in mammalian cells and Vps16 and Vps33 are both expressed as an A and B isoform.[17-20,25-31] The CORVET subunit Vps8 and the HOPS Vps41 have one corresponding homolog in higher eukaryotes.[24,27] Vps39 has 2 homologs: Vps39–1/hVam6/TLP and Vps39–2/TRAP1.[21,32,33] Since the hVps39 proteins are also homologous to yeast Vps3, it was proposed that one of them is the missing metazoan Vps3 and part of the mammalian CORVET complex.[34,35]

Here, we characterized the human Vps39 homologs in yeast and in HEK293 cells and found that the 2 isoforms cannot compensate for a loss of yeast Vps39. In human cell lines, hVps39–2 was strongly recruited to endosomes, when Rab5 was co-expressed, whereas hVps39–1 remained cytosolic upon expression of Rab5 or Rab7. Furthermore, hVps39–2 bound to Rab5-GTP, suggesting a role of Vps3 in higher eukaryotes.

Results

Expression of human Vps39 homologs in yeast

The Saccharomyces cerevisiae vacuolar sorting protein Vps39 has 2 homologs in higher eukaryotes termed hVps39–1/hVam6/TLP and hVps39–2/TRAP1, which both have very similar domain structures (**Fig. 1A**). To clarify, which of the 2 subunits is the equivalent of yeast Vps39, we expressed both in *vps39*Δ cells, which have highly fragmented vacuoles. We decided to express the human isoforms under the control of the inducible strong *GAL1* promoter to investigate if they are able to rescue the fragmentation phenotype or are recruited to membranes by the Rab5-like Vps21 and yeast Rab7 Ypt7. Both isoforms were readily expressed in yeast upon induction with galactose (**Fig. 1B**), but both did not rescue the vacuole morphology and remained cytosolic (**Figs. 1C, 2A**). We thus conclude that neither isoform can complement yeast Vps39.

The human Vps39–1 homolog is transported to the nucleus by the Large T antigen

It was reported, that hVps39–1 interacts with the LT protein and is translocated to the nucleus by LT in human cell lines.[36] We thus asked if the human proteins that are expressed in yeast would change their localization upon co-expression of large T antigen (LT) from the Merkel cell polyomavirus (MCV). Indeed, we observed a complete shift of hVps39–1 to the nucleus (**Fig. 2A**). This interaction was specific, since hVps39–2, yeast Vps39 or yeast Vps3 were not translocated to the nuclear compartment (**Fig. 2B**). Furthermore, expression of the previously characterized LT W209A mutant, which cannot bind hVps39–1, blocked the nuclear translocation. We thus conclude that the missing complementation of the hVps39 isoforms is not due to misfolding, but likely reflects their specificity for the human complex.

The human Vps39–1 and Vps39–2/TRAP1 do not localize to defined vesicular structures

To unravel, which hVps39 isoform corresponds to yeast Vps3, we turned to metazoan cells. We therefore integrated a plasmid into the genome of HEK293 cells that codes for C-terminally HA- Strep-tagged Vps39–1 or Vps39–2 and generated inducible stable cell lines from this (**Fig. 3A**). We next followed the cellular localization via indirect immunofluorescence. As previously described, overexpressed hVps39–1 localized to the cytosol (**Fig. 3A–D**).[22,32] Even after removal of excess cytosolic staining by treatment of cells with methanol, we did not obtain improved staining. Likewise, a switch of the HA-Strep tag from the C- to the N-terminus of hVps39–1 did not have an effect. When we monitored the localization of hVps39–1 upon induction by a time course, we also found it immediately in the cytosol. Under certain conditions, TRAP1/hVps39–2 was reported to associate with inactive TGF-β receptors into patch-like structures, which required co-overexpression of both kinase deficient receptors.[37] In our HEK293 cells, overproduced TRAP1 localizes strictly to the cytosol, in agreement with the missing receptor overexpression (**Fig. 3A–C**).

The human Vps39–2/TRAP1 co-localizes with GFP-Rab5

The yeast HOPS complex is a well-studied effector of Ypt7/Rab7, and its mammalian counterpart binds to Rab7 via the RILP effector protein.[38] Hence, we asked if hVps39–2 would also be associated with Rab7 or would rather bind to Rab5. The latter would be expected, if it were the missing homolog of yeast Vps3 and a subunit of putative CORVET complex in humans. Strikingly, when we transfected the cells with GFP-Rab5 or GFP-Rab7, we found indeed hVps39–2 almost completely recruited from the cytosol to membranes of the enlarged Rab5 vesicles (**Fig. 4B**). We were, however, surprised that hVps39–1 did neither co-localize with Rab5 nor with Rab7 (**Fig. 4A**) as the interaction with hVps39 and Rab5 has been previously reported.[39] The possible reason for the discrepancy of these data with our observations could be due to different cell types, expression levels or a different splicing variant of the hVps39–1 in this particular study. We then wondered, if the missing recruitment of hVps39–1 to Rab-positive vesicles might be due to the activation state of Rabs and therefore tested the dominant active Rab mutants in our cells. Again, hVps39–1 localized in the cytosol pointing to the need of additional factors than Rab5 or Rab7 for its membrane recruitment (**Fig. 4C**).

The human Vps39–2/TRAP1 interacts with Rab5 and is part of a large complex

Next we investigated, whether hVps39–2 is indeed an effector of Rab5. For this, we conducted a pull-down experiment with recombinant purified GST-Rab5, GST-Rab7 or GST-Rab9 and cell lysate from our HEK293 cells. hVps39–2 was specifically retained from the lysate by Rab5-GTP, whereas hVps39–1 did not bind to any of the tested Rabs (**Fig. 5A–B**). These results confirmed our co-localization experiments and revealed hVps39–2 as an effector of Rab5 that localizes to early endosomes. In yeast, the Rab5-like Vps21 interacts with the CORVET complex. We thus asked if Vps39–2 as the putative homolog of CORVET Vps3 would also interact with other subunits. We therefore decorated our pull-down experiments with

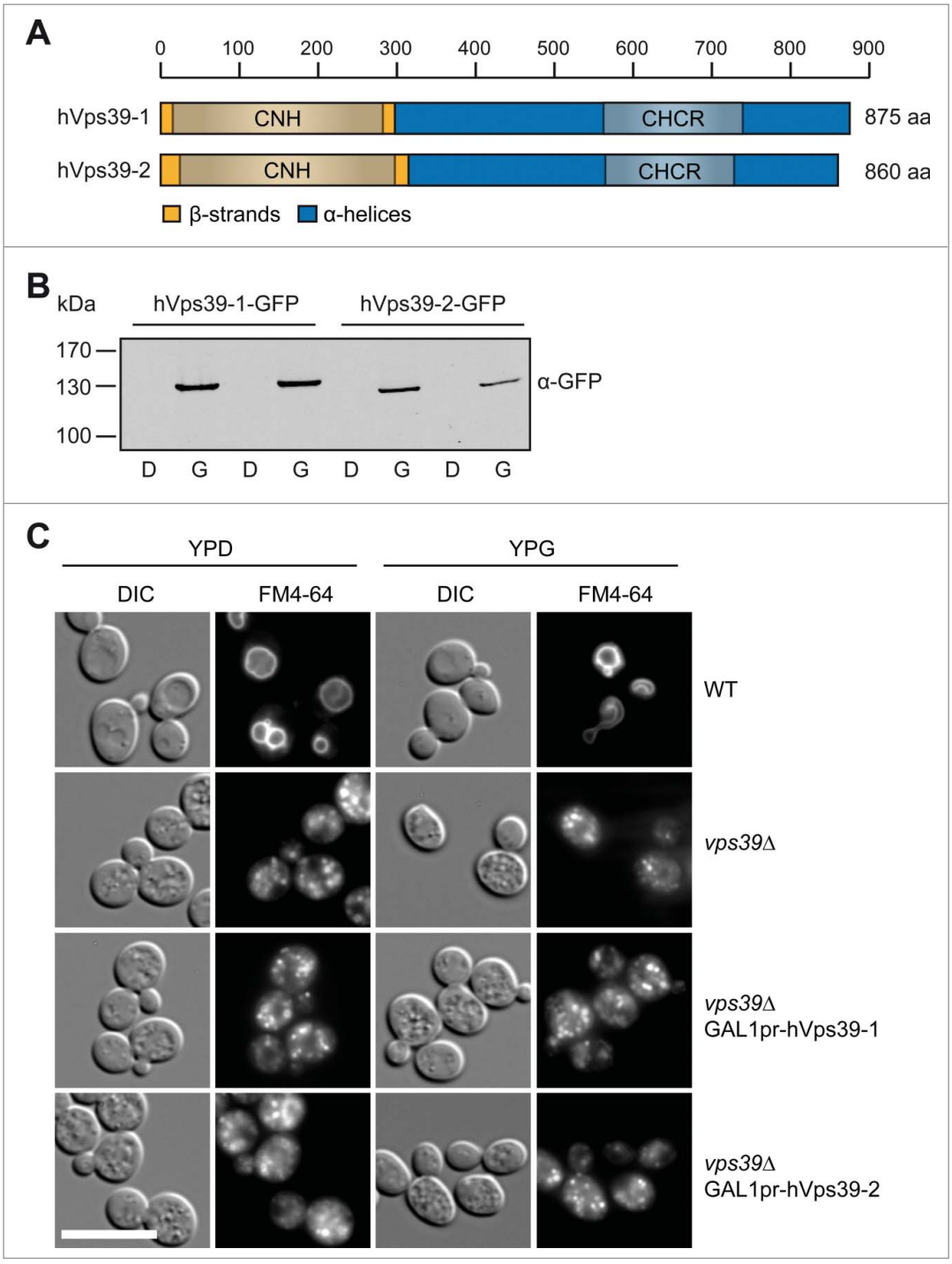

Figure 1. Expression of human Vps39 homologs in yeast. (**A**) Depicted is the predicted secondary structure and protein domains of hVps39–1 and hVPS39–2 using the predict protein algorithm (predictprotein.org, Technical University Munich, Germany) and the Prosite database (SIB ExPASy Bioformatics Resources Portal). CNH: Citron homology, CHCR: Clathrin heavy-chain repeat. (**B**) The human isoforms of Vps39 were integrated into the yeast genome and expressed under the control of the inducible *GAL1* promoter. Cells were grown over night in glucose (YPD) or galactose (YPG), lysed and analyzed on a blot membrane by decorating against the GFP-tag. (**C**) Cells expressing a genomically integrated form of human Vps39–1 or Vps39–2 in a strain lacking the yeast *VPS39*, were stained with the lipophilic dye FM4–64 and monitored by fluorescence microscopy. DIC, differential interference contrast.

an antibody against the core subunit hVps11 and indeed found the protein co-eluting with hVps39–1 from the GTP-form of GST-Rab5 (**Fig. 5A**). To determine the size of the formed complex, we loaded lysate from the hVps39–2/TRAP1 HEK293 cell line onto a Superose 6 column and followed the retention volumes on a western blot. We found hVps39–2/TRAP1 in 2 peaks, a smaller peak corresponding to about 650 kDa, a size equivalent to the yeast CORVET complex, a larger peak in the range of 200 kDa, which likely corresponds to the monomeric overexpressed protein (**Fig. 5C**). Importantly, hVps11 was recovered almost exclusively in the large complex, suggesting that it is part of the metazoan CORVET complex together with hVps39–2/TRAP1.

Discussion

In this study, we compared the human homologs of yeast Vps39 to identify the missing Vps3 homolog of metazoan CORVET. Even though both isoforms did not complement a yeast *vps39*Δ mutant, we were able to reproduce the specific interaction of hVps39–1 with the Merkel virus T-antigen. Upon

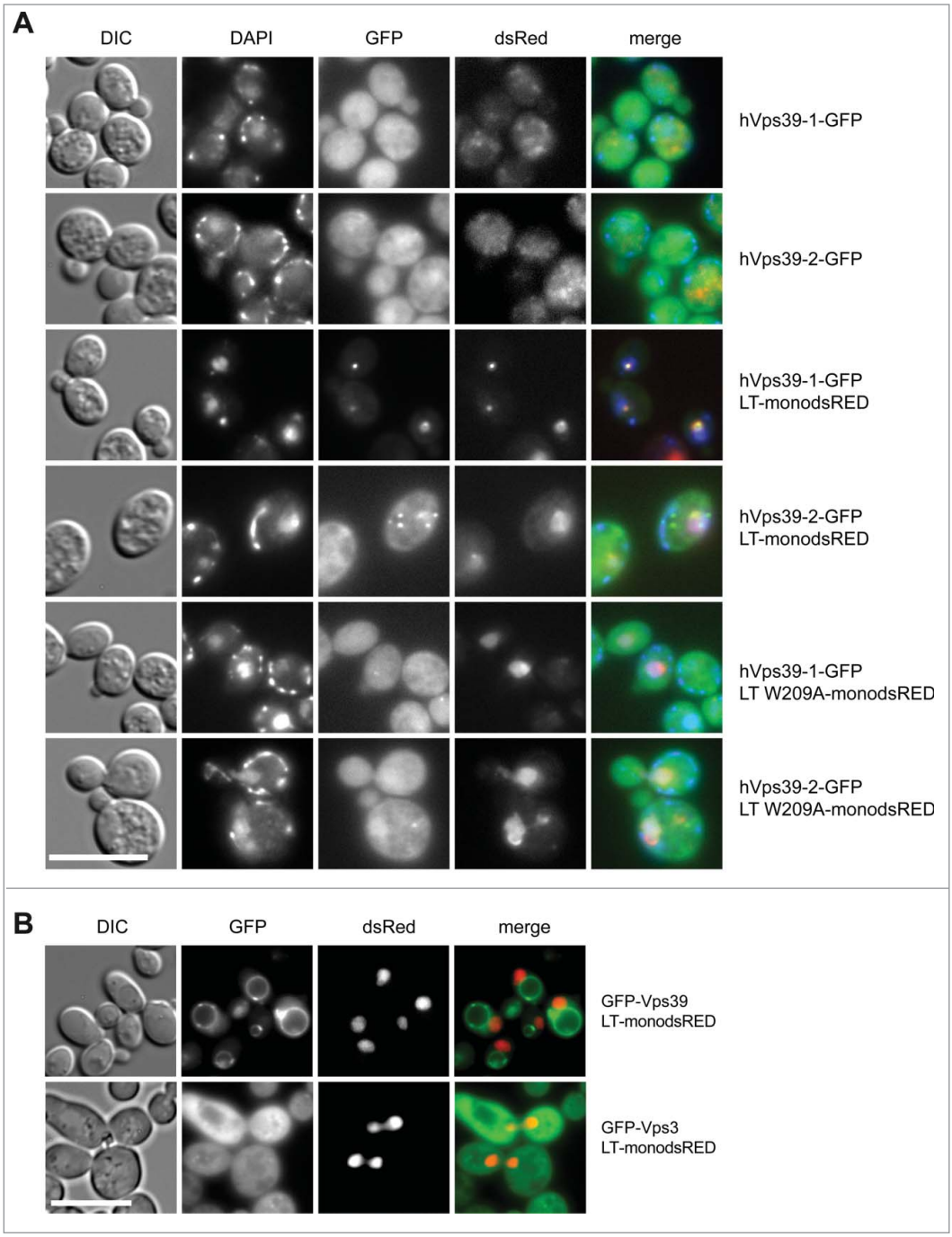

Figure 2. The human Vps39–1 homolog is transported to the nucleus by the Large T antigen. (**A**) The human isoforms of Vps39 were integrated into the yeast genome and expressed as C-terminally tagged GFP fusion proteins under the control of the inducible GAL1 promoter. The LT wt and mutant forms were ectopically expressed as C-terminal monodsRED fusions from a Cen plasmid. Cells were grown to early logarithmic phase, stained with DAPI, and monitored by fluorescence microscopy. (**B**) Yeast Vps39 and Vps3 were expressed as N-terminal GFP fusions under the control of the TEF1 promoter. The LT protein was expressed as described in (**A**). DIC, differential interference contrast. Scale bar, 10 μm.

expression in HEK293 cells, we observed a cytosolic localization, which is in good agreement with former studies, even though hVps39–1 could be localized to late endosomes and lysosomes by immuno electron microscopy.[24,22,32] For hVps39–2, we observed clear Rab5-GTP dependent interaction and co-localization. This indicates that hVps39–2/TRAP1 is the likely missing CORVET subunit in metazoan cells.

It was surprising to us that Vps39–1 did not bind to Rab7 in vivo or in vitro, analogous to the yeast homologs. It was shown that the Rab7 effector RILP is necessary to recruit the HOPS complex to membranes.[38] Up to now, no direct interaction could be observed between Rab7 and HOPS in metazoan cells, but it was reported that a not further specified form of hVps39 binds nucleotide-independently to Rab5 upon pull-down from cytosol.[39] Since CORVET had not been discovered, and hVps39–2/TRAP1 has a comparable size like hVps39–1, it is possible that the used antibody was also recognizing the hVps39–2 protein. At least in our hands, we only found an interaction of hVps39–2 with Rab5 in a strictly GTP-dependent manner.

hVps39–2 was described to cycle between patches at the plasma membrane and the cytosol.[37] If this protein is the missing homolog of yeast Vps3, is an important issue, since it provided direct evidence for the existence of a CORVET complex in metazoa. We found that hVps39–2 co-localizes with Rab5 at vesicular structures, which represent enlarged endosomes. Furthermore, it binds to Rab5-GTP, analogous to the CORVET subunits Vps3 and Vps8 in yeast and co-elutes with hVps11, a core subunit of HOPS and CORVET (**Fig. 5**). Finally, we confirmed that both proteins reside in a large complex. This analysis also revealed that overexpression generated a large monomeric pool of hVps39–2, which likely

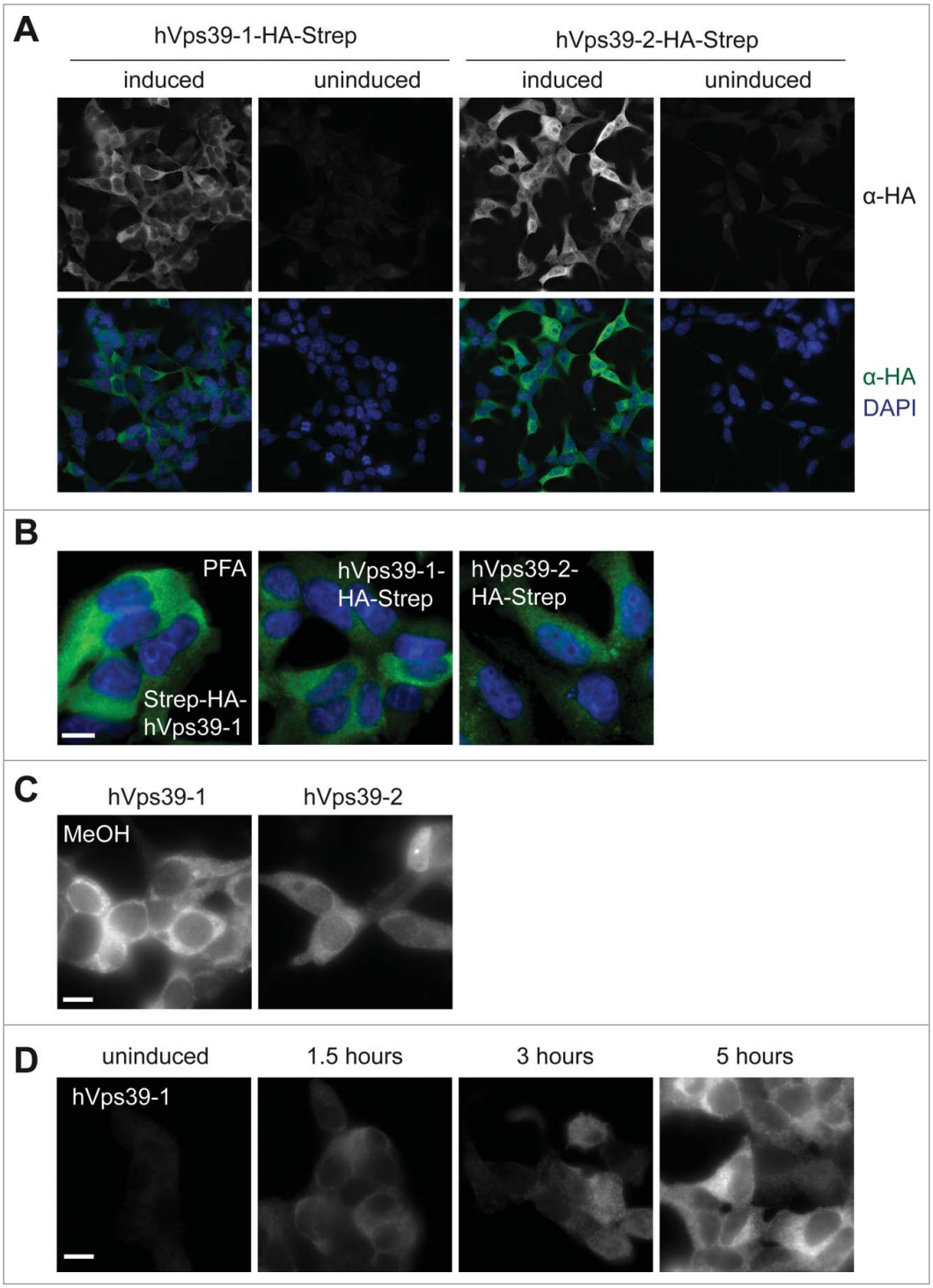

Figure 3. The human Vps39–1 and Vps39–2/TRAP1 do not localize to defined structures. (**A**) HEK293 cells were generated that genomically express inducible hVps39–1-HA-Strep or hVps39–2/TRAP1-HA-Strep. The cells grew on a coverslip and were induced with 1 μg/ml tetracycline. After 24 hours, cells were fixed and subjected to immunostaining with a primary antibody against the HA epitope, and a secondary antibody conjugated to a cyanine dye (Cy2). Afterwards, the specimen was briefly stained with 4,6-diamidino-2-phenylindole (DAPI). (**B**) The position of the HA-Strep tag does not affect the localization of the protein. HEK293 cells with stably expressed N-terminal Strep-HA-tagged version of hVps39–1 were analyzed as before. The shown specimens were processed for staining as in (**A**). (**C**) Alternative fixation to wash out the cytosolic signal. Cells were subjected to methanol fixation prior to staining as in (**A**). (**D**) Time course of hVps39–1 localization after induction of protein expression with 1 μg/ml tetracycline. At the depicted time points, cells were subjected to fixation and staining as in (**A**). Scale bar, 10 μm.

accumulated in the cytosol and thus masked the endosomal localization observed upon co-expression with Rab5 (**Fig. 4B**). Taken together, we show that hVps39–2 is an endosomal protein that is recruited by Rab5 and resides in a complex of about 650 kDa. These findings provide evidence for the existence of a human CORVET complex, which may have also isoforms of other class C core subunits, such as Vps16A or Vps16B. For example, Vps33B and Vps16B were assigned to fulfill a function in phagocytosis and early endocytic pathway, whereas Vps33A and Vps16A are involved in endolysosomal fusion.[17,18,26,28,29,40]

Future studies will need to address whether hVps39–2 indeed associates with other subunits of the class C core, foremost the CORVET-specific subunit hVps8, and if trafficking routes of the endocytic pathway are affected upon the knock-down of the protein. Furthermore, ultrastructural analyses of hVps39–2 localization will be important to assign its specific function in metazoan cells.

Methods

Yeast expression constructs and yeast strains

Saccharomyces cerevisiae strains used in this study are summarized in Supplemental Table S1. Deletion of genes, promotor

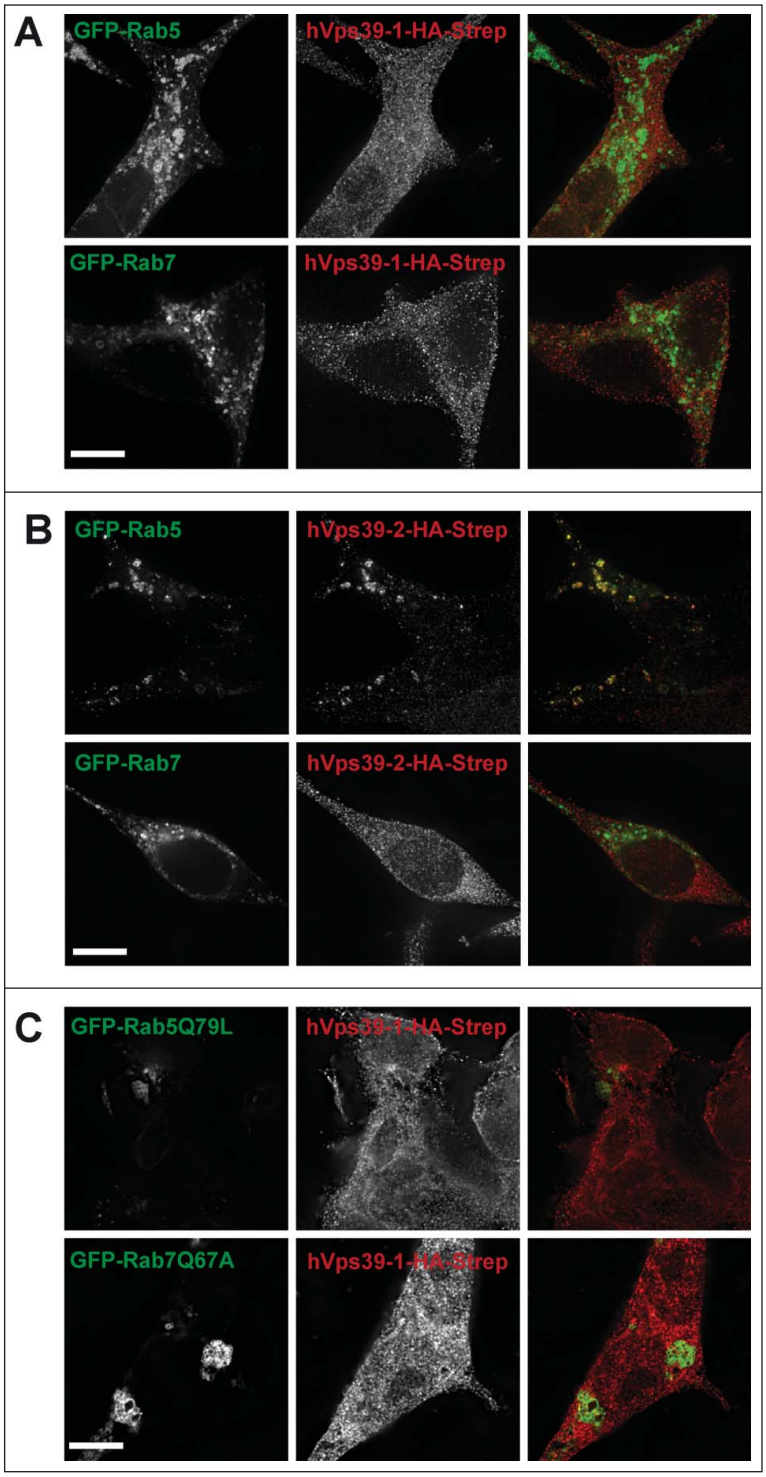

Figure 4. The human Vps39–2/TRAP1 co-localizes with GFP-Rab5. (**A-C**) The stable cell lines were transiently transfected with GFP-Rab5, GFP-Rab7, GFP-Rab5Q79L or GFP-Rab7Q67A and protein expression was induced 8 hours later with 1 μg/ml tetracycline. After a 16 hours incubation period, the cells were fixed, subjected to immunochemistry with a primary antibody against the HA epitope, and secondary antibody conjugated to a cyanine dye (Cy3). Depicted images were subjected to a 3D deconvolution and subsequently presented as a sum projection of 4 to 8 slices of a z-stack. Scale bar, 10 μm.

exchange, and tagging were done by homologous recombination with PCR-amplified fragments.[41] hVps39–1/hVam6/TLP (AN: BC068559) and hVps39–2/TRAP1 (AN: BC020548) were cloned into a pRS406 plasmid and integrated in the genome of a *vps39*Δ yeast strain. The constructs were expressed under the control of the inducible *GAL1* promoter and tagged with GFP at the C-terminus, where indicated. The large T antigen, wt and mutant form, were amplified from pDsRed.cLT206+57KT and pDsRed.cLT206+57KT.W209A plasmids and cloned into a pRS413 Cen yeast expression vector harboring a *TEF1* promoter or in a pRS426 2μ vector with a *GPD* promoter.[36,42,43]

Microscopy of yeast cells

Yeast cells were grown to mid logarithmic phase in yeast extract/peptone medium (YP) containing 2% glucose (YPD) or galactose (YPG). To maintain plasmids, cells were grown in synthetic complete medium lacking selected amino acids (SDC). They were harvested by centrifugation, washed once with synthetic complete medium supplemented with all amino acids, and visualized at room temperature. For FM4–64 (Invitrogen) labeling of the vacuoles, cells were treated as described before.[44] Images were acquired using a Leica DM5500 B microscope (Leica, Mannheim, Germany) with a SPOT Pursuit-XS camera (Diagnostic Instruments, Sterling Heights, MI) using filters for GFP, dsRED, mCherry, FM4–64, and YFP.

Mammalian expression constructs and generation of inducible cell lines

To express the hVps39–1/hVam6/TLP and hVps39–2/TRAP1 with a C-terminal HA-Strep tag, we used the Flp-In HEK293 cell system (Invitrogen). The manufacturer's plasmid pcDNA5/FRT/TO (Invitrogen) was digested with NotI and XhoI and was subsequently ligated with an annealed oligo, coding for the HA-(NMHTG-linker)-Strep peptide. The coding sequences of Vps39–1/hVam6/TLP (AN: BC068559), purchased from Open Biosystems (cat. no.: MHS1010–97228208) and hVps39–2/TRAP1 (AN: BC020548), requested from the RZPD Berlin, Germany (ID IRATp970B1121D6) were amplified and cloned into the resulting plasmid via BamHI and NotI restriction sites. Flp-In HEK293 cells (Invitrogen) containing a FRT recombination site and expressing the tet repressor from a genomically integrated plasmid, were co-transfected with the resulting plasmids and the pOG44 vector (Invitrogen) for co-expression of the FRT recombinase, according to the manufacturer's protocol. HEK293 cells were cultured in DMEM high glucose, L-glutamine (PAA) substituted with 10 % FCS, 100 U/ml Penicillin-Streptomycin, 15 μg/ml blasticidine and 100 μg/ml hygromycine B. The isogenic surviving

colonies were pooled and tested for protein expression by immunostaining and protein gel blotting with an HA antibody (Covance).

Transient transfection, immunostaining and microscopy of inducible HEK293 cells

For transfection, HEK293 cells were seeded on glass cover slips in 6-well plates (Greiner Bio One) and transfected with GFP-Rab5, GFP-Rab7, GFP-Rab5Q79L, or GFP-Rab7Q67A (kindly provided by Dr. Francis Barr, Oxford, UK) using 0.4 μg of DNA and Effectene (Qiagen) according to the manufacturer's protocol. The transfected or untransfected cells were induced for protein expression with 1μg/ml tetracycline for 16 hours and fixed with 3% PFA/PBS for 20 minutes at RT. Fixed cells were washed once with PBS and fixation was then quenched, using 50 mM NH_4Cl/PBS for 10 minutes. Where indicated, cover slides with attached cells were immerged in methanol for 10 minutes at −20°C. After washing with PBS, the cells were subjected to permeabilization buffer (PBS buffer containing 1 mg/ml saponin and 2 mg/ml BSA) for 30 minutes. Cells were incubated with a 1:1000 dilution of HA antibody (Covance) and, after washing 3 times with permeabilization buffer, with a 1:400 dilution of cyanide dye-conjugated anti-HA antibody Cy2 or Cy3 (Jackson). The cells were then washed 2 times with permeabilization buffer, once with ddH_2O and mounted in ProLong Gold Antifade Reagent (Invitrogen). Images were acquired on an imaging system (Deltavision Elite; GE Healthcare) based on an inverted microscope (model IX-71; Olympus), equipped with an UAPON 100× (1.49 NA) oil immersion objective, an InsightSSI light source (Applied Precision), excitation and emission filters for DAPI, FITC, TRITC, mCherry and a CoolSNAP HQ2 CCD camera (Photometrics). Stacks of 8 or 15 images with 0.3-μm spacing were subjected to 3D deconvolution using SoftWoRx 5.5 software (Applied Precision). Processing of the images was performed with ImageJ 1.49a (National Institutes of Health).

GST-Rab pull-down

The bacterial pFAT2 expression vectors (T7 polymerase hexahistidine–GST) containing Rab5 or Rab7 were kindly provided by Dr. Francis Barr (Oxford, UK). Bacterial expression and purification was performed as described before.[45] HEK293 cells were cultured in 3 times 15 cm dishes per construct as described above and were harvested with a cell scraper after washing with PBS. The cells were centrifuged for 5 minutes at 100 × g, 2°C and the pellet was resuspended in lysis buffer (50 mM Hepes, pH 7.4, 150 mM KCl, 5mM $MgCl_2$, 2 mM β-mercaptoethanol, 5 % glycerol) with protease inhibitor cocktail. Lysis was performed by passing the cells 30x through a cell cracker using a 8.004 ball (EMBL, Heidelberg). Cell debris was removed by centrifugation for 30 minutes at 2°C and 100.000 g in a TLA-55 rotor (Beckmann). The supernatant was added to nucleotide preloaded Rab GTPases, as described in the following. A 1-μmol amount of recombinant GST-Rab5 or GST-Rab7 was incubated in 500 μl of 20 mM HEPES, pH 7.4, 20 mM EDTA, 100 mM KCl, 2 mM β-mercaptoethanol, and 1 mM GTPγS or GDP. After an incubation for 20 min at 30°C, $MgCl_2$ was added to a final concentration of 30 mM. The samples were then loaded onto 50 μl of prewashed GSH Sepharose (GE Healthcare) and incubated for 2 h at 4°C. After incubation, the loaded beads were pelleted by centrifugation and washed with nucleotide buffer lacking EDTA and containing 5 mM $MgCl_2$. The beads were then incubated with HEK293 cell lysate in a total volume of 500 μl in the presence of 1 mM GTPγS or GDP. After 1 h at 4°C, the beads were harvested by centrifugation and washed 2 times with buffer containing 5 mM $MgCl_2$ and once without. Proteins were then eluted with 20 mM EDTA for 20 minutes at 30°C. The eluates were TCA-precepitated, resuspended in SDS loading buffer and subjected to a SDS-PAGE, followed by western blotting and decoration with HA antibody (Covance).

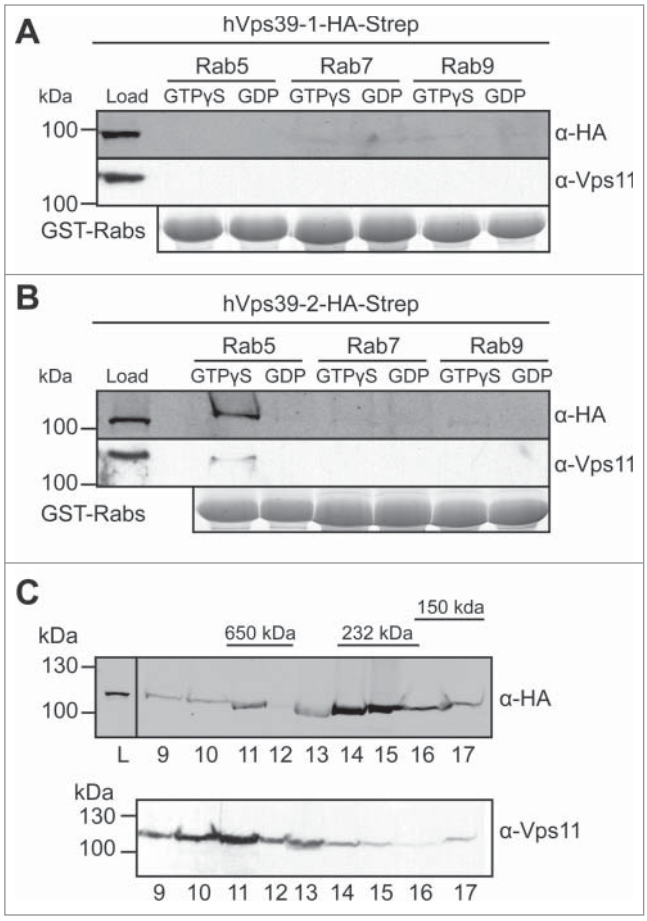

Figure 5. The human Vps39–2/TRAP1 interacts with Rab5 and forms a complex. (**A–B**) Purified GST-Rab5, GST-Rab7 and GST-Rab9 were immobilized at GSH-sepharose, preloaded with GTP or GDP, and incubated with HEK293 cell lysate. Bound proteins were eluted with EDTA, resolved on SDS–PAGE, and detected with antibodies to the HA tag on a western blot membrane. A Coomassie-stained loading control of GST-Rabs is shown. (**C**) Western blot analysis of gel-filtrated HEK293 cell lysate. Cleared HEK293 cell lysate was loaded onto a Superose 6 column and collected in fractions of 1 ml volume. The fractions were TCA-precipitated, resolved on SDS-PAGE and probed with antibodies to hVps39–2/TRAP1-HA-Strep and hVps11. Sizes of the respective markers are shown on top.

Size exclusion chromatography

HEK293 cells induced for expression of hVps39–2/TRAP1-HA-Strep were cultured in a 15 cm dish as described above. After washing with PBS, cells were harvested with a cell scraper in detergent lysis buffer (50 mM HEPES/KOH, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 5% glycerol, 0.5% NP-40) with protease inhibitor cocktail. The cells were incubated on ice for 15 minutes and then centrifuged at 20,000 g at 4°C for 20 minutes. The supernatant was subjected to a Superose 6 10/300 GL column (GE Healthcare), pre-equilibrated with lysis buffer. Fractions of 1 ml were collected, and proteins were precipitated by 13% TCA. The samples were resolubilized in SDS loading buffer, separated on a 7.5% SDS-PAGE gel, and transferred on a nitrocellulose membrane. Blots were decorated with an antibody against the HA tag from mouse (Covance) and against hVps11 from rabbit (abcam).

Note

A recent study of Zerial and coworkers (Perini et al.) confirmed that hVps39-2, here called Tgfbrap1, interacts with hVps8 in the human CORVET complex and thus nicely complements our study.[46]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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