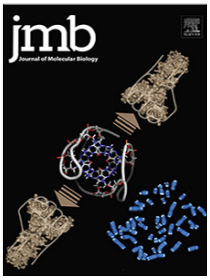

Identification of New Interactions between Endolysosomal Tethering Factors

Zsófia Simon-Vecsei [a]*, Ármin Sőth [a], Péter Lőrincz [a,b], András Rubics [a], András Tálas [c], Péter István Kulcsár [c] and Gábor Juhász [a,d]*

***a* - Department of Anatomy,** *Cell and Developmental Biology, Eötvös Loránd University, Budapest, Hungary*
***b* - Premium Postdoctoral Research Program,** *Eötvös Loránd Research Network, Budapest, Hungary*
***c* - Institute of Enzymology,** *Research Centre for Natural Sciences, Budapest, Hungary*
***d* - Institute of Genetics,** *Biological Research Centre, Szeged, Hungary*

Correspondence to Zsófia Simon-Vecsei and Gábor Juhász: Department of Anatomy, Cell and Developmental Biology, Eötvös Loránd University, Pázmány Péter sétány 1/C, Budapest H-1117, Hungary. *simon.vecsei.zsofia@ttk.elte.hu (Z. Simon-Vecsei), szmrt@elte.hu (G. Juhász)*
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Abstract

Proper functioning of the precisely controlled endolysosomal system is essential for maintaining the homeostasis of the entire cell. Tethering factors play pivotal roles in mediating the fusion of different transport vesicles, such as endosomes or autophagosomes with each other or with lysosomes. In this work, we uncover several new interactions between the endolysosomal tethering factors Rabenosyn-5 (Rbsn) and the HOPS and CORVET complexes. We find that Rbsn binds to the HOPS/CORVET complexes mainly via their shared subunit Vps18 and we mapped this interaction to the 773–854 region of Vps18. Based on genetic rescue experiments, the binding between Rbsn and Vps18 is required for endosomal transport and is dispensable for autophagy. Moreover, Vps18 seems to be important for β1 integrin recycling by binding to Rbsn and its known partner Vps45.

Introduction

Eukaryotic cells must maintain constant flux of proteins and lipids between different organelles, which is sustained by transport vesicles. The transport routes are precisely controlled by membrane fusion processes, which are supported by a conserved fusion machinery that consists of proteins of the small GTPase families (Rab (Ras-associated binding) and Arf (ADP-ribosylation factor) proteins), tethering factors, SNAREs (SNAP Receptor, SNAP: Soluble NSF attachment protein), SM (Sec1/Munc-18) proteins and molecular motors.[1] The maturation of endosomes is mainly regulated by Rab proteins, which in their GTP-bound active form can bind and recruit multiple effectors, such as motor and adaptor proteins and tethering factors. These later can bring the donor and acceptor membranes into contact and thus facilitate the homo- and heterotypic fusion of vesicles.[2] The fusion is executed by SNAREs that form a tight four-helical coiled coil complex and mediate the mixing of the lipid bilayers.[3]

Several tethering factors have been identified in the endosomal system: the early endosomal Rabenosyn-5 (Rbsn),[4] Early endosomal antigen 1 (EEA1),[5] the CORVET (Class-C core endosome vacuole tethering) complex[6]; the late endosomal HOPS (Homotypic vacuole fusion and protein sorting) complex[7] and in the recycling system the Golgi associated retrograde protein (GARP)[8] and endosome-associated recycling protein (EARP)[9]

complexes. Besides its role in the endosomal system, HOPS mediates the fusion of autophagosomes and secretory granules with the lysosome as well.[10–12]

The HOPS and CORVET complexes have been identified in yeast[6,7] and four of their subunits are shared: Vps11, Vps16, Vps18, Vps33 (aka. Class-C proteins). The additional subunits are CORVET-specific Vps3 and Vps8 and HOPS-specific Vps39 and Vps41.[6,13] Metazoan cells also express the above-mentioned shared subunits and the complex-specific Vps39, Vps41 and Vps8 as well.[14–17] The mammalian CORVET has the same subunit composition as the yeast one, except for the missing metazoan Vps3 subunit, which is replaced by one of the Vps39 homologs, Vps39-2 (also known as Tgfbrap1).[18,19] Interestingly, an alternative, tetramer form of CORVET (called mini-CORVET) was identified in Drosophila: Vps3 does not have a homologue in flies (and is not replaced by other homologous subunit) and Vps11 was not found as a complex subunit either.[20]

Recently the existence of other endosomal complexes has also been suggested: the Vps3 and Vps8 subunits are suspected to form a complex independently from the other subunits, playing a role in recycling pathways.[21] Accordingly, the two paralogs of Vps16 and Vps33, VIPAS39/SPE-39/VIPAR/Vps16B and Vps33B form the complex called CHEVI (class-C Homologs in Endosome-Vesicle Interaction) independently of HOPS or CORVET.[22] The exact function of this latter complex has not been clearly defined; it is proposed to act in recycling, biogenesis of lysosome-related organelles and bacterial clearance.[23,24] VIPAS39 is also a subunit of the FERARI (Factors for endosome recycling and retromer interaction) complex, also a heterohexamer containing the Sec1p/Munc18-like (SM) protein Vps45, two Rab-binding proteins: Rbsn (Rab5 binding) and Rab11-FIP5 (Rab11 binding), the dynamin-like EH domain containing protein 1 (EHD1) and the membrane-cytoskeleton linker Ankyrin-3 (ANK3).[25]

We have previously demonstrated that Drosophila miniCORVET colocalizes with Rabenosyn-5, and we could also detect a direct interaction of Rabenosyn-5 with the shared HOPS/CORVET subunit Dor/Vps18. Since the insect miniCORVET contains only one Rab binding subunit, this raised the possibility that miniCORVET functions as a tether by co-operating with other tethers, which may be also true for complete hexameric CORVET or HOPS complexes.[20] Rbsn is known as a Rab4/Rab5 and phosphatidylinositol-3-phosphate (PI3P) binding protein, which with its partner Vps45 is required for the homotypic fusion of early endosomes or the fusion of endosomes with clathrin-coated vesicles in both mammalian and fruit fly cells.[4,26,27]

Endosomal transport is under tight regulation and its disruptions lead to defective cargo delivery. Mutations in proteins controlling these pathways can cause pigmentation and liver defects, severe neurological and neurodegenerative diseases.[23,28] Of note, a point mutation of Rbsn causes intractable seizures, developmental delay and microcephaly, among others.[29] Mutations of Rbsn-5 or its partners lead to tumor formation in flies due to defective endosomal traffic resulting in the loss of epithelial polarity.[27]

Here we provide evidence that Rbsn binds to multiple HOPS/CORVET subunits mainly via Vps18. Vps18 binds Rbsn through amino acids (aa) 773–854, and the loss of this binding site has no effect on on autophagosome clearance. Moreover, we also observed a probably indirect interaction between Vps18 and Vps45, possibly via Rbsn, since the binding sites of Vps18 and Vps45 do not overlap on Rbsn. Our results highlight that endosomal tethering factors maintain a complex interaction network and several seemingly individual players such as Vps18 and Vps45 can cooperate to maintain proper endosomal trafficking.

Results

Recently we identified miniCORVET as an early endosomal tether in Drosophila, which promotes the fusion of endosomes possibly by interacting with Rbsn-5 via the Class-C protein Vps18.[20] Identification of this interaction prompted us to examine whether human Rbsn also binds human Vps18. Using yeast two-hybrid (Y2H) experiments we could successfully detect an interaction between these human proteins (Figure 1(A)), in line with our previously published Drosophila data. Next, we confirmed this interaction in immunoprecipitation (IP) experiments: FLAG-Rbsn could precipitate HA-Vps18, and *vice versa*, FLAG-Vps18 could precipitate HA-Rbsn (Figure 1(B), upper panels). We got the same results using anti-HA immunoprecipitation (Figure 1(B), lower panels). We also successfully verified the interaction by detecting endogenous proteins in anti-FLAG IPs. Specific bands of endogenous Rbsn (Figure 1(C)) and Vps18 (Figure 1(D)) could be detected after expressing FLAG-Vps18 or FLAG-Rbsn, respectively, in HEK293 cells and precipitating the proteins with anti-FLAG resin. Since punctate, likely endosome-associated HA-Vps18 and FLAG-Rbsn also colocalize in Hela cells (Figure 1(E)) that is also seen in case of FLAG-Vps18 and HA-Rbsn, (Figure 1(F)), all of our results point to an *in vivo* interaction between these two proteins.

Vps18 is a shared subunit of two tethering complexes: the early endosomal CORVET and the late endosomal HOPS. To assess if Vps18 binds to Rbsn while being an integral part of these complexes or this interaction is independent of HOPS or CORVET subunits, we probed the

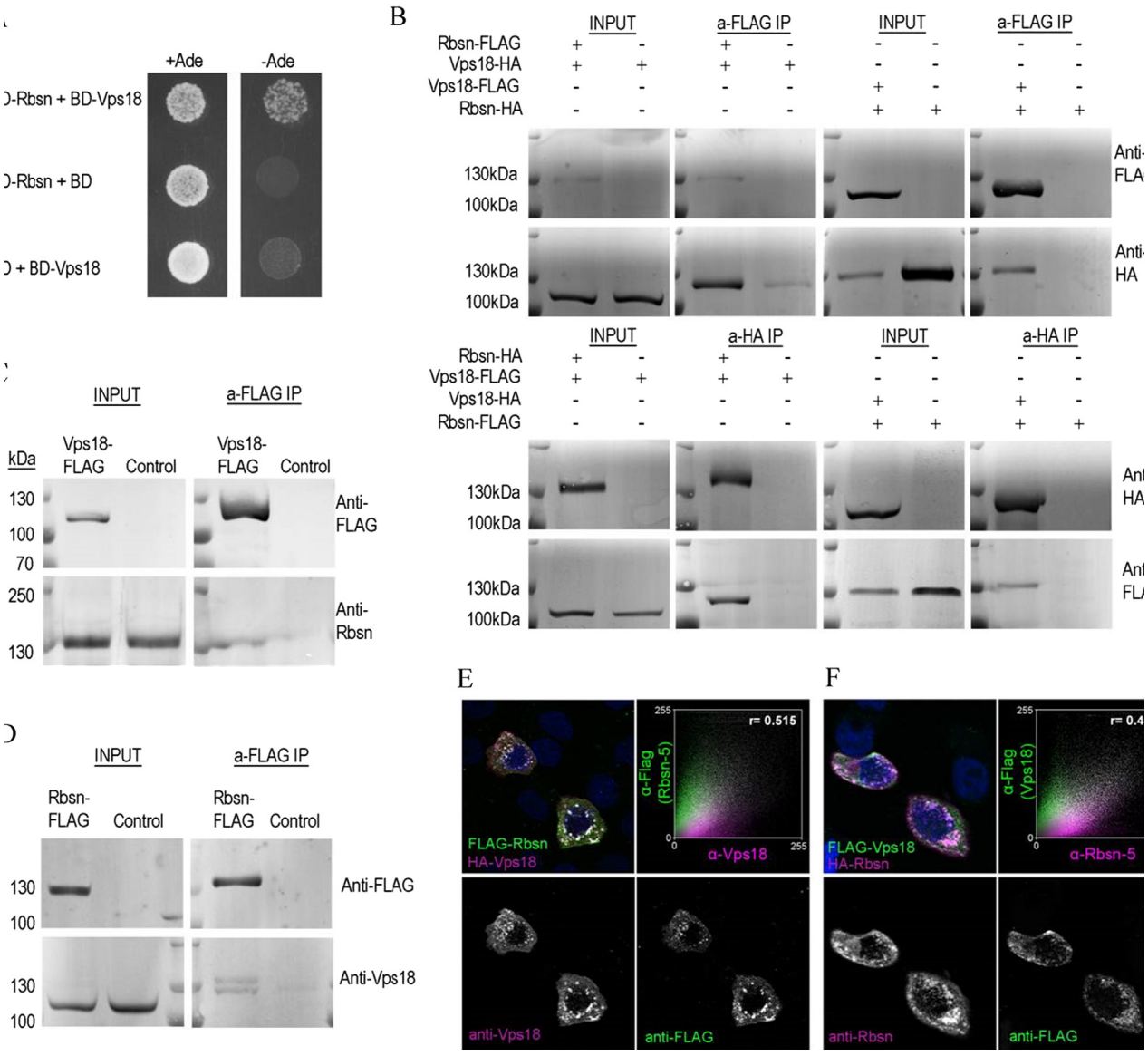

Figure 1. Rabenosyn-5 binds to the Class-C protein Vps18. (A) Y2H experiment using the full-length Rbsn (fused with activating domain, AD) and Vps18 (fused with binding domain, BD) proteins. Selection without adenine (-Ade) suggests direct binding of the two proteins. (B) Anti-FLAG (upper panel) and anti-HA (lower panel) immunoprecipitation experiments show the interaction of Rbsn with Vps18 in all combinations. (C) Endogenous Rbsn is detected by western blot in Vps18-FLAG immunoprecipitates. Control samples were cells transfected with empty vector. (D) Endogenous Vps18 binds to Rbsn-FLAG based on anti-FLAG immunoprecipitation (E) Vps18-HA and Rbsn-FLAG show clear colocalization in immunofluorescence analysis of transfected HeLa cells. (F) Rbsn-FLAG and Vps18-HA also show colocalization. Scatter plots and Pearson's r values are shown and both indicate colocalization (n = 15 in E; n = 6 in F). Representative images of at least two parallel experiments.

Class-C (Vps11, Vps16 and Vps33) and the complex-specific subunits (Vps39, Vps41, Vps8 and Tgfbrap) in FLAG-Rbsn immunoprecipitates as well. Importantly, all of these subunits could be detected after anti-FLAG IP (Figure 2(A) and (B)). Since the IP method detects both direct and indirect binding between proteins, we wanted to test whether these subunits could directly interact with Rbsn. Therefore, we turned to Y2H experiments using Rbsn and the HOPS and CORVET subunits. Although none of the subunits showed binding in yeast grown on Ade- medium (data not shown) that only identifies strong interaction,[30,31] the application of His selection revealed presumably weaker interactions between Vps16, Vps33, the HOPS-specific Vps39 and Rbsn (Figure 2(C)). These results altogether indicate that both HOPS and CORVET can potentially bind Rbsn

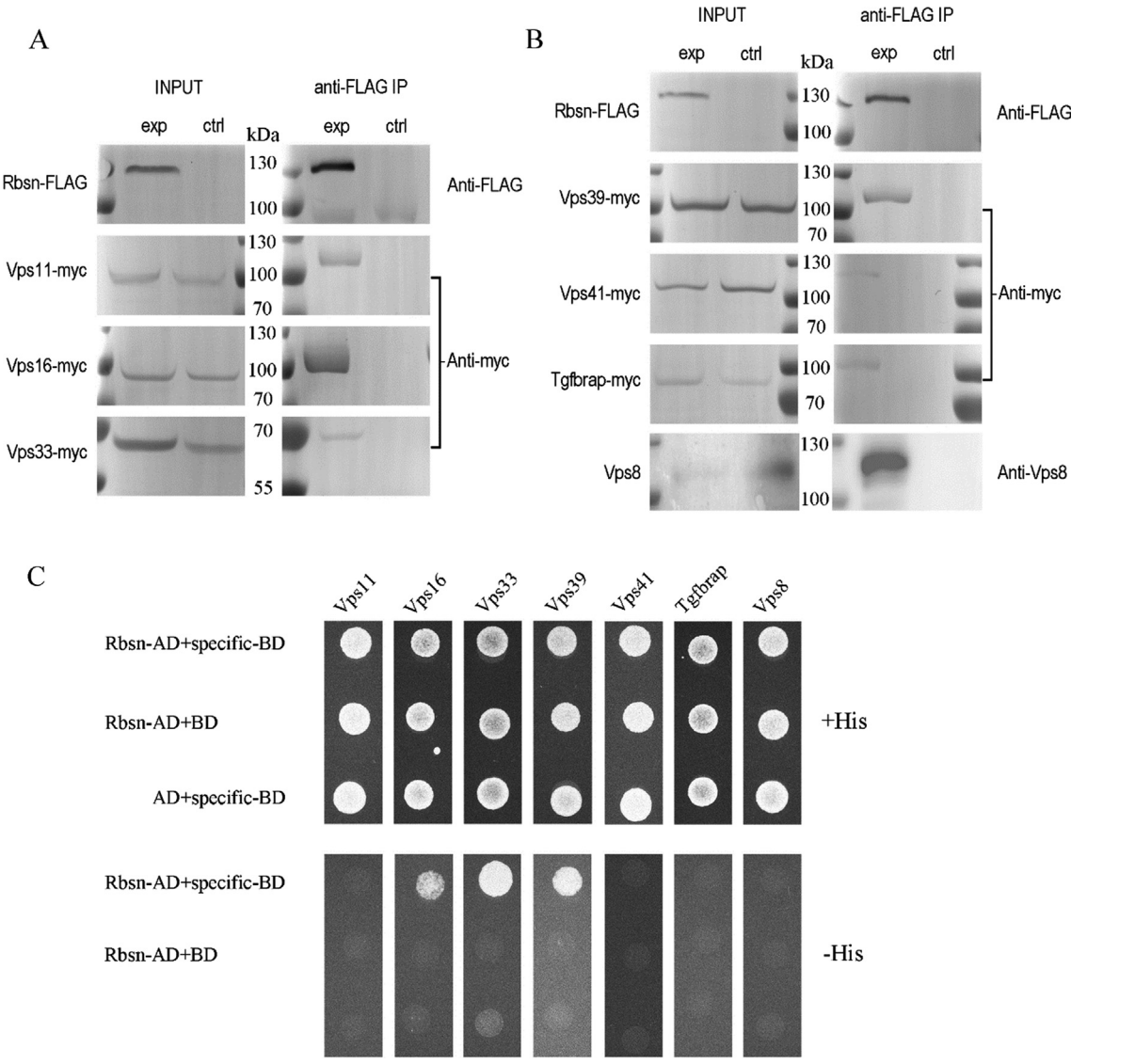

Figure 2. Rabenosyn-5 binds to multiple HOPS and CORVET subunits. Anti-FLAG immunoprecipitation in HEK293 cells transfected with Rbsn-FLAG and the indicated myc-tagged (A) Class-C subunits and (B) HOPS- or CORVET-specific subunits, shows interaction in all cases. (C) Y2H analyses of Rbsn (fused with AD) and Class-C or complex-specific subunits (fused with BD) selected on medium lacking histidine (-His) shows the probable binding of Rbsn with Vps16, Vps33, and Vps39. Representative images of at least two parallel experiments.

via multiple subunits, from which Vps18 seems to be the most relevant based on the positive result on stringent Ade selection.[30,31]

Next, we aimed to determine the specific site within Vps18 that is responsible for its binding to Rbsn. Based on earlier studies[32] and predictions (Figure S1(A); Phyre2),[33] we cut the protein to an N-terminal part (ND, amino acids (aa) 1–481, including the β-propeller domain), a middle-part (CLH, aa 482–854, including the clathrin heavy chain domain) and a C-terminal part (RING, aa 855–973, the RING domain of Vps18). We found that Rbsn binds to the CLH region of Vps18 (Figure 3(A)). We sought to narrow this binding site by the generation of shorter versions of CLH and we detected binding between Rbsn and amino acids 773–854 of Vps18 (Figure 3(B)). We created a Vps18 construct lacking this region to verify if the identified 773–854 is indeed responsible for Rbsn binding. Accordingly, Y2H experiments revealed that Vps18 lacking amino acids 773–854 can no longer interact with Rbsn (Figure 3(C)). Based on the predicted domain structure of Vps18 (Figure S1(A)), fragment 773–854 is located in an exposed position and indeed can serve as a site for protein interactions. To prove further the role of this part of Vps18 in Rbsn binding, we expressed and purified the identified fragment with N-terminal GST-tag in E. coli. Rbsn-FLAG could be detected from cell lysates after incubation with the purified

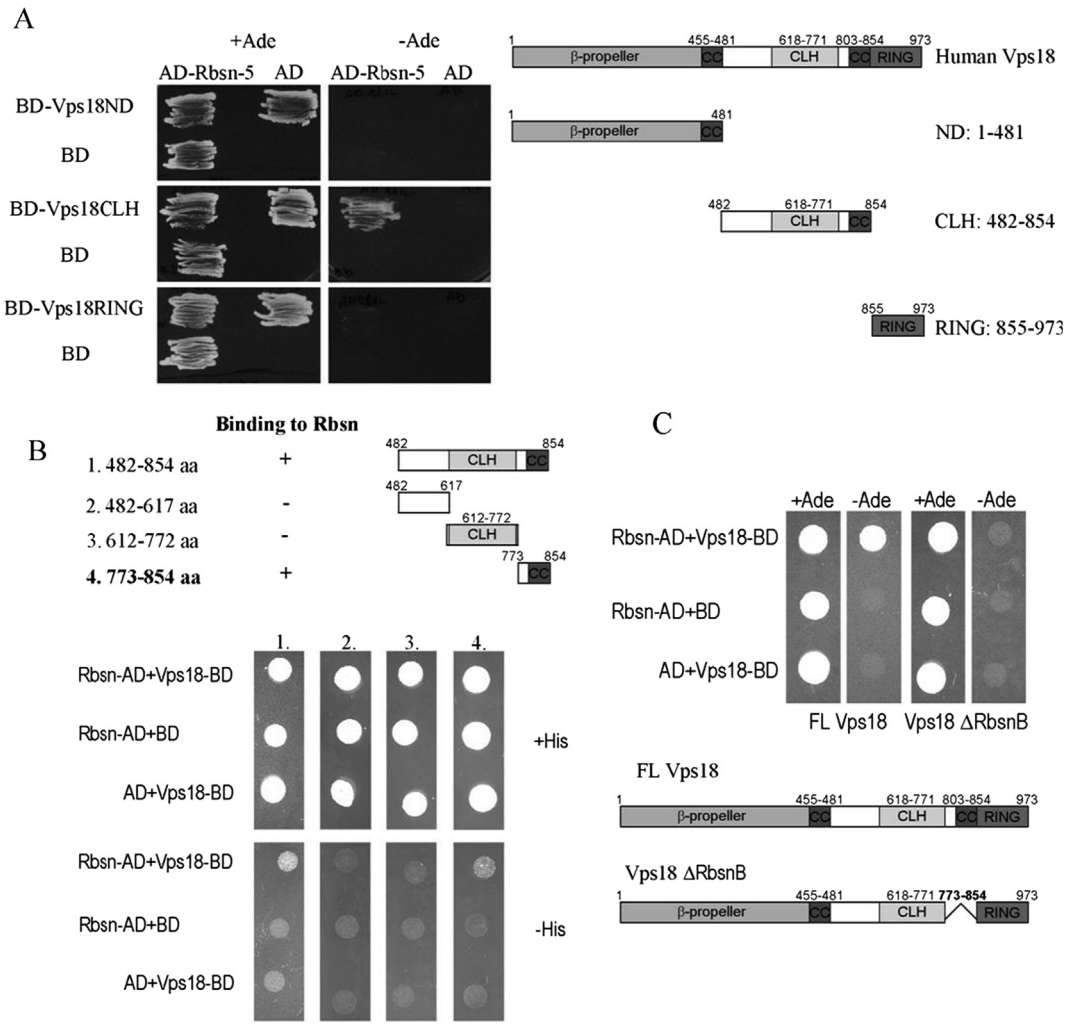

Figure 3. Identification of the Rbsn-binding site of Vps18. (A) N-terminal (ND: aa 1–481), middle (with clathrin-heavy chain domain, CLH: aa 482–854) or C-terminal parts (RING: aa 855–973) of Vps18 (fused with BD) were tested for binding to full length Rbsn (fused with AD), and interaction was detected between Rbsn and the CLH region (aa 482–854) of Vps18 on Ade selection medium. (B) Amino acids 482–854 were mapped further for narrowing the Rbsn binding site of Vps18. The indicated constructs fused to BD were probed for binding to full length Rbsn-AD. Amino acids 773–854 of Vps18 showed interaction with Rbsn on medium lacking histidine (-His). (C) Only full length Vps18 (FL Vps18) binds to Rbsn, Vps18 lacking the identified Rbsn-binding site (Vps18 ΔRbsnB) does not bind in Y2H experiments. Representative images of at least two parallel experiments.

fragment in GST pull down experiment (Figure S1 (B)). We also tested the interaction ability of the Rbsn binding site mutant Vps18 (Vps18 ΔRbsnB) with HOPS subunits using Y2H. Vps18 ΔRbsnB showed interaction with core subunits Vps11, Vps16 and Vps33, while it could not bind Vps41 (Figure S2). Taken together, the 773–854 fragment of Vps18 binds Rbsn and may have a role in the interaction with Vps41 as well.

To test the function of human Vps18, we generated Vps18 KO HEK293 cells with the self-cleaving plasmid method using CRISPR/Cas9.[34] WB and immunofluorescence confirmed the lack of Vps18 in our mutants (Figure 4(A), (B), (D)).

Being a subunit of HOPS, Vps18 is essential for autophagosome-lysosome fusion.[35] The key autophagy protein LC3 is conjugated to the forming autophagosome by lipidation and this process can be routinely followed by western blot, as the migration of the non-conjugated cytosolic form of LC3 (LC3I) is slower than the conjugated one (LC3II). Thus, if autophagosome clearance is inhibited, the accumulation of autophagosome associated LC3II can be detected. Since Vps18 is a HOPS-subunit, we wanted to know if the binding of Vps18 to Rbsn is required for autophagosome clearance. We observed increased LC3II levels in Vps18 KO cells compared to control ($p < 0,01$; 2,5-4x fold change compared to wild type HEK293 cells, Figure 4(C)

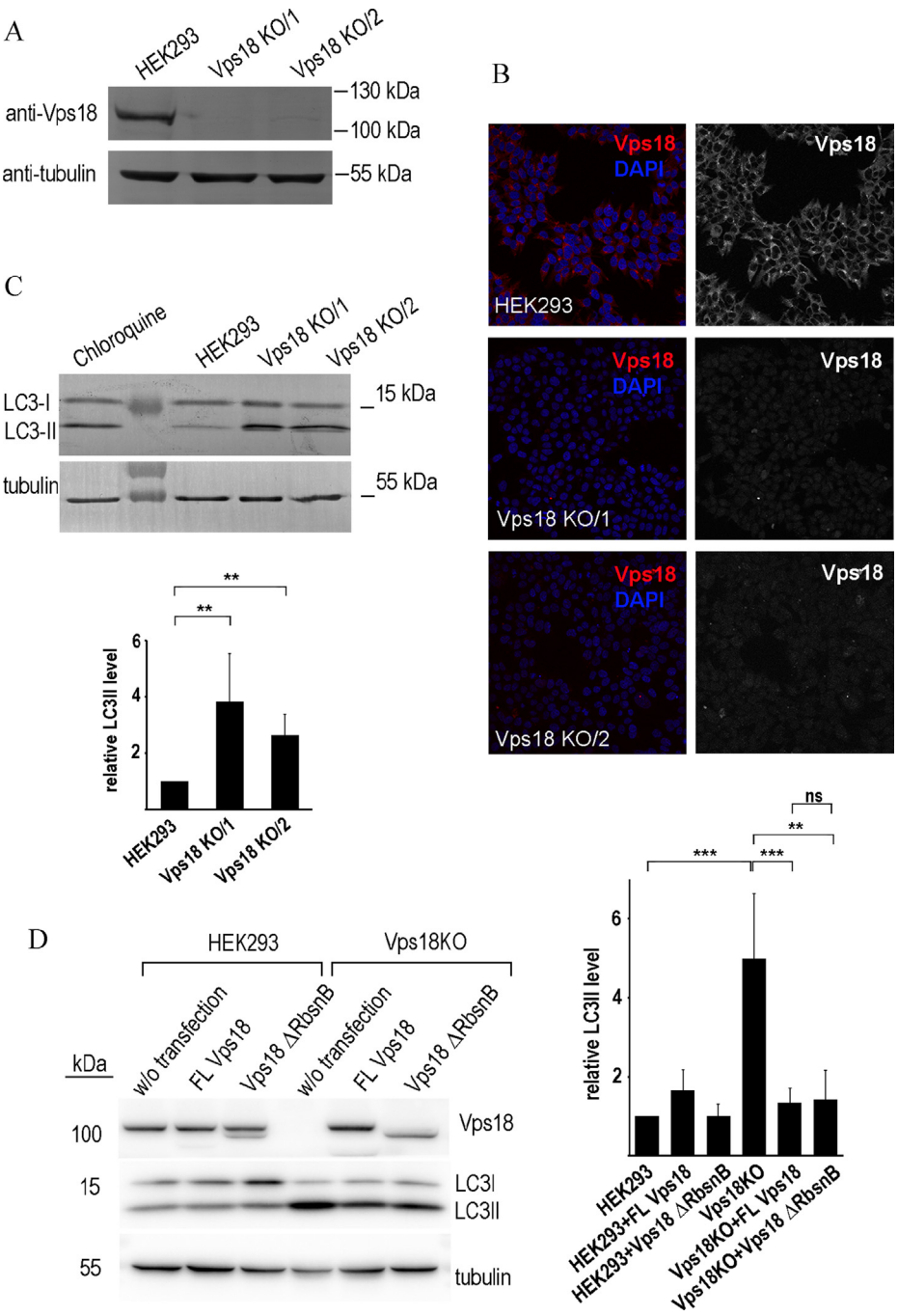

Figure 4. LC3 lipidation is independent from the Rbsn binding site of Vps18. (A, B) Loss of Vps18 was verified in Vps18 KO HEK293 cell lines by (A) Western blot and by (B) immunocytochemistry. (C) The level of the lipidated LC3 (LC3II) increased in wild type HEK293 cells treated with the autophagic degradation inhibitor chloroquine similar to Vps18 KO cells (compared to control HEK293). (D) The Vps18-Rbsn interaction is not required for the lipidation of LC3. Wild type and Vps18 KO HEK293 cells expressfull length Vps18 (FL Vps18) or Vps18 lacking the Rbsn-binding site (Vps18 ΔRbsnB) were analyzed. Band densities on C and D were quantified by ImageJ software, normalized to tubulin and compared to HEK293 cells. t-test was used for statistical analysis based on five experiments. Error bars mark SDs. **, $p < 0,01$; ***, $p = 0,001$; ns = not significant.

and (D)), similar to when cells were treated with chloroquine, which is a known inhibitor of autophagic degradation.

Next, we generated Vps18 KO and HEK293 cell lines stably expressing full length (FL Vps18) or Rbsn binding site mutant Vps18 (Vps18 ΔRbsnB).

Both proteins could rescue the Vps18 loss-induced increased LC3II phenotype to the same extent, while they had no obvious effect on the LC3II level in wild type cells (Figure 4(D)). Immunofluorescence demonstrated that both the autophagy cargo p62 and Lamp1 positive lysosomes accumulate in KO cells, indicating a failure of lysosomal degradation, which is a hallmark of loss of HOPS function (Figure S3). Both of these phenotypes could be rescued by FL Vps18 (Figure S3(C) and (H)). The accumulation of p62 could be reversed by Vps18 ΔRbsnB ($p = 0{,}018$; Figure S3(I) and (J)), but the distribution of lysosomal Lamp1 was not properly restored based on statistical analysis ($p = 0{,}063$; Figure S3(D) and (E)). These results were also confirmed by ultrastructural analyses: the accumulation of multivesicular bodies (MVB) and small lysosomes was evident in Vps18 mutant cells compared to controls (Figure S4(B)), and this could be rescued by both FL Vps18 (Figure S4(C) and (E)) and Vps18 ΔRbsnB (Figure S4(D) and (E)). However the rescue effect of the latter, while statistically significant, was not as complete as in the case of the full length protein (Figure S4(D) and (E)). These results indicate that the Rbsn-Vps18 binding is not required for autophagy, but it is important for endosome to lysosome progression.

The SM protein Vps45 was identified as an interacting partner of Rbsn[4] and both proteins are part of the recently identified FERARI complex as well.[25] Additionally, both Vps45 and Rbsn are indispensable for endocytosis and Rab5-mediated endosomal fusion in Drosophila and C. elegans.[36,27]

Since multimeric tethering complexes can share subunits and individual proteins can interact with multiple partners (such as the shared HOPS/CORVET Class-C subunits), it is conceivable that Vps45-Rbsn proteins bind Vps18 as well as FERARI subunits. To test this possibility, we performed anti-FLAG IP using Rbsn-FLAG or Vps18-FLAG and Vps45-myc proteins, finding that both FLAG-tagged proteins successfully precipitated Vps45-myc (Figure 5(A)). Next, we examined the type of binding between Vps18 and Vps45 by Y2H experiments to find a probably direct interaction only between Rbsn and Vps45 (Figure 5(B)). Based on these, we propose that Rbsn bridges the other two proteins. Next we tested if Rbsn could bind Vps18 and Vps45 simultaneously. We mapped that it is the N-terminal part of Rbsn (aa 1–421) that binds Vps18 (Figure 5(C)). Since the binding site of Vps45 has been mapped to the same region (aa 100, 101 and aa 105, 109),[37] it seemed possible that the two binding sites overlap. To test this, we mutated the amino acids required for Vps45 binding to alanine and investigated if the binding of Vps18 is also abolished. We found that the Vps45 binding site mutant Rbsn versions could still interact with Vps18 (Figure 5(D)). Beside this, we could identify a specific region of Rbsn ND (aa 285–420), which only interacts with Vps18 but not with Vps45 (Figure 5(E)). Altogether these data indicates that the two binding sites do not overlap. Additionally, we found that the N-terminal part of Rbsn (aa 1–421) may contain a binding site for other HOPS subunits: Vps16, Vps33 and Vps39 as well (Figure 5(C)). Our IP and binding site-mapping results raise the possibility that Rbsn, Vps45 and Vps18, can form a – perhaps transient - complex.

Based on earlier findings, the Rbsn-Vps45 interaction is required for proper β1 integrin recycling in mammalian cells.[37] Accordingly, Rbsn-5 and Vps45 mutant fruit fly epithelial cells lose epithelial polarity[27] and Rbsn-5 is required for the correct localization of Fasciclin III and β-integrin in developing wing epithelial cells.[38] We observed that the cortical actin network of Vps18 KO cells was underdeveloped (Figure S5(A)). Based on phalloidin stainings we found that Vps18 KO cells have shorter projections compared to wild type cells, which can be fully rescued by FL Vps18 and partially also by Vps18 ΔRbsnB (Figure S5(B)). Additionally, we investigated integrin recycling in Vps18 KO cells using an antibody-based assay.[21] We labelled β1 integrins after serum deprivation, then allowed their internalization. Integrin recycling was stimulated by FBS and was followed for the given time points. We observed the accumulation of β1 integrin-containing intracellular vesicles in the absence of serum (0 time point, arrows, Figure S5(C) and (D)). β1 integrin signal became dispersed 5 min after serum addition in wild type cells (5 min time point, Figure S5(C)). In contrast, the intracellular accumulation of β1 integrin did not change in Vps18 KO cells after 5 min, suggesting impaired integrin recycling (arrows, Figure S5(C)). FL Vps18 could rescue integrin-recycling significantly ($p = 0{,}01$), while Vps18 ΔRbsnB did not reverse the β1 integrin accumulation in a statistically significant fashion (Figure S5(D)).

Discussion

In this present work, we revealed novel interactions between different tethering factors acting in the mammalian endolysosomal system. We showed that the early endosomal tether Rbsn interacts with other tethering complexes: the early endosomal tether CORVET and the late endosomal tethering complex HOPS as well. Among the HOPS/CORVET subunits, Rbsn seems to bind Vps18, and perhaps also to Vps16, Vps33 and Vps39 directly, of which the binding to Vps18 is likely the strongest based on yeast two hybrid experiments, as we observed growth on stringent selection medium lacking adenine only in the case of Vps18-Rbsn interaction. Yeast cells transformed with Vps16, Vps33 and Vps39 could

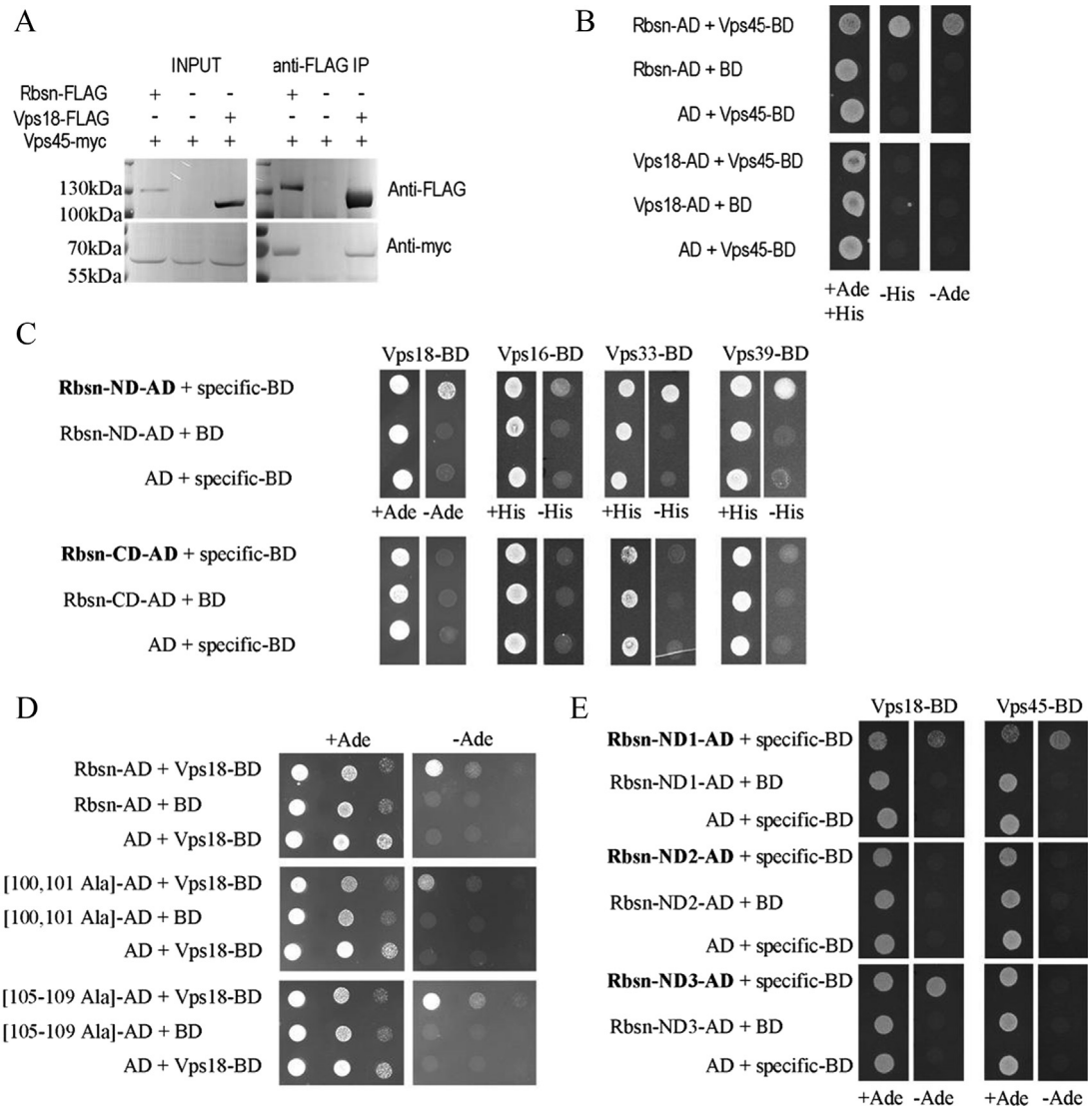

Figure 5. Vps18 indirectly interacts with Vps45, possibly via Rbsn. (A) Anti-FLAG IP showed interaction between Vps45-myc - Vps18-FLAG and Vps45-myc – Rbsn-FLAG proteins. (B) Vps45 (fused with BD) does not bind directly to Vps18 (fused with AD) in Y2H. Interaction was selected on medium without histidine (-His) or adenine (-Ade). (C) The N-terminal part of Rbsn contains binding sites for the HOPS subunits: Vps18, Vps16, Vps33 and Vps39. Rbsn-ND: aa 1–421; Rbsn-CD: aa 422–784. Y2H selection for protein interaction was done on medium lacking histidine (-His). (D) Vps45 binding site mutant Rbsn versions still interact with Vps18 in Y2H. The Rbsn constructs contain the following aa changes: Rbsn [100, 101 Ala]: Arg100Ala, Ser101Ala and Rbsn [105–109 Ala]: Asp105Ala, Phe106Ala, Lys107Ala, Lys108Ala, His109Ala. (E) Amino acids 285–420 (ND1) of Rbsn binds Vps18, and do not bind Vps45. Rbsn-ND1: aa 1–140; Rbsn-ND2: aa 141–284; Rbsn-ND3: aa 285–420. Y2H selection for protein interaction was done on medium lacking adenine (-Ade). Representative images of at least two parallel experiments.

only grow on histidine selection medium in which interactions with lower affinities can be also detected.[30,31] The Rbsn binding of Vps18 occurs through aa 773–854 of Vps18. Previous works identified the RING domain of Vps18 (aa 854–973) as required for proper HOPS assembly that binds other subunits of the complex: Vps16, Vps33 and Vps41.[32] However, our results indicate that aa 773–854 of Vps18 has a role in the binding of HOPS-specific subunit Vps41 as well (while it is dispensable for the binding of core subunits Vps11, Vps16 and Vps33). Beside Vps18, Vps41 also binds to Vps16 and Vps33, and hence complete HOPS complexes can be formed in this way. This can explain why in our rescue experiments the Rbsn binding site mutant Vps18 (Vps18 ΔRbsnB) can rescue LC3 lipidation and p62 accumulation of Vps18 KO cells.

As subunits other than Vps18 can also bind Rbsn, there is a possibility that Rbsn also acts as a scaffold during HOPS/CORVET assembly as it does in the case of FERARI.[25] There are no unam-

biguous findings regarding the assembly mechanism of endosomal tethering factors, which can potentially occur on a given membrane or protein surface that can accelerate their assembly by the enrichment of the subunits. It is also possible that HOPS and CORVET may transform into each other during endosome maturation on a given platform because intermediate complexes were found in yeast.[6,39] In our earlier Drosophila work, we proposed that the assembly of HOPS and CORVET takes place independent of each other, probably in the cytosol, since either complex is functional in the absence of the other complex, and depleting any Class-C subunits causes the loss of the early and late endosomal localization of CORVET- and HOPS-specific subunits, respectively. This indicates that only a fully assembled miniCORVET or HOPS is able to associate to endosomal membranes.[20,40] CORVET functions as an early endosomal tether on Rab5-positive endosomes,[6,13,18] and being a Rab5 effector, there is a possibility that Rbsn promotes CORVET assembly. Whereas we cannot fully rule out the model of a step-by-step assembly on a target membrane facilitated by Rbsn binding to Rab5 and PI3P in human cells,[4] in Drosophila this seems not to be the case as depletion of Rbsn-5 did not cause miniCORVET complex to lose its early endosomal localization.[20] HOPS was suggested to be transformed from CORVET through intermediate complexes in yeast,[6,39] however no such intermediates were found in Drosophila lysates, and their assembly appears to be independent from each other.[20,40] To our knowledge, the existence of these intermediate complexes and complex assembly have not been investigated in human cells yet.

Another possibility is that the identified Rbsn-HOPS interaction has a role in the mediation of the heterotypic fusion of Rab5- and Rab7-positive endosomes (Rab5 binding by Rbsn and Rab7 binding by Vps39 and Vps41). Since the miniCORVET of insects contains only one Rab binding subunit, miniCORVET needs a partner to function properly,[20] and FERARI complex also needs partners during Rab5 positive (via Rbsn) and Rab11 positive (via Rab11FIP5) endosomal fusions.[25] Based on these findings we find it very likely that Rbsn promotes CORVET/HOPS mediated vesicular transport, and as the Rbsn-Vps18 interaction turned out to be dispensable for autophagosome-lysosome fusion, we think that the interaction is specific for endosomal trafficking. This is in line with our rescue experiments, as Vps18 ΔRbsnB could fully rescue the elevated LC3 lipidation and p62 accumulation phenotypes of Vps18 KO cells, while it could not restore the increased number of MVBs and lysosomes as effectively as FL Vps18 did.

Vps45 is a SNARE-binding SM protein,[41] and it is the main interacting partner of Rbsn in several organisms.[4,27,36] Thus, we also tested the binding between Vps45 and CORVET/HOPS. To our surprise, Vps45 appeared to indirectly associate with Vps18, suggesting that their interaction is achieved via Rbsn binding, as different regions of Rbsn are needed for Vps45 and Vps18 binding, respectively.

The Rbsn-Vps45 binding was shown to be required for proper β1 integrin recycling in mammalian cells,[37] and Rbsn-5 is required for the correct localization of β-integrin and epithelial polarity in insects.[27,38] We found that HEK293 cells lacking Vps18 have altered cortical actin morphology and impaired β1 integrin recycling. Our results and earlier findings raise the possibility that this binding has an important cell physiological relevance as without either Rbsn, Vps45 or Vps18 the formation of cell adhesion structures is impaired, without which the viability of a cell dramatically decreases: they may undergo cell death or in some cases malignant transformation can occur.

Taken together, our results indicate that Rbsn can bind to CORVET/HOPS mainly through Vps18. This interaction is dispensable for autophagy, but it appears to be important for endocytic progression.

Materials and Methods

Cloning

For yeast two-hybrid experiments the coding sequences of Rbsn, Vps45, HOPS and CORVET subunits were amplified from HeLa cDNA template, then cloned into pGADT7 AD and pGBKT7 BD vectors with NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). For the identification of the binding sites of Vps18 and Rbsn, the appropriate part of the coding region (see details on Figure 3(A) and (B) and Figure 5 (C)) were amplified and cloned to pGBKT7 vector with the same technique. For the generation of Rbsn-binding site mutant of Vps18, 1–2316 and 2563–2922 nucleotides of Vps18 were amplified and assembled into GBKT7 vector with NEBuilder HiFi DNA Assembly Cloning Kit. The Vps45 binding-site mutants of Rbsn were generated by site directed mutagenesis. For co-immunoprecipitation experiments, full-length proteins were cloned into mammalian expression vectors containing CMV promoter and N-terminal HA- or FLAG tags.[42] Myc-tagged proteins were generated by amplification of the N-terminal myc-tag and the coding region of HOPS and CORVET subunits in pGBKT7 vector with the following primers:

5′-CCGCCACCAAGCTTGGTACCACTATAGGGC GAGCCGCC-3′ and
5′-CCCGAATTCGGCGGCCGCGGCCC CAAGGGGTTATGC-3′.

Then they were cloned into Acc-Not sites of pCMV3 vector. Vps18 and Rbsn binding-site mutant of Vps18 (without any tags) were cloned

into the same vector and used in western blot experiments. The sequences were checked with Sanger sequencing (Microsynth AG, Switzerland).

Yeast two-hybrid

Yeast strain AH109 was transformed with 300 ng pGADT7 and the same amount of pGBKT7 constructs for each reaction (Frozen EZ Yeast Transformation II, ZYMO Research). The cotransformants were selected on SD (synthetic defined) media, which lacks triptophan and leucine (SD-WL) and the protein interactions were identified on SD-WL medium without adenine or histidine. The yeasts were incubated for 48 hours in 30 °C in every case.

Cell culture and transfection

HEK293 (human embryonic kidney) and HeLa cells were grown at 37 °C in a humidified atmosphere with 5% CO2, in the following medium: high glucose DMEM (Dulbecco's Modified Eagle Medium, Lonza) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FBS, Lonza), 2 mM L-glutamine (Lonza), 100 U/ml penicillin and 100 mg/ml streptomycin. For western blot and immunoprecipitation experiments, 1.5 million HEK293 cells were placed in T25 flasks and transfected after 24 hours. Transfection was performed with TransIT-LT-1 (Mirus) transfection reagent according to the manufacturer's instructions. 2500 ng plasmid-constructs were added for each flask.

For immunohistochemistry HEK293 and HeLa cells were placed on poly-L-lysine (Sigma) coated glass discs (VWR) in 24 well plates. One hundred thousand cells were placed on each disc and transfection was performed after 24 hours, with the same procedure as mentioned above. 500 ng plasmid-constructs were added for each disc.

Immunoprecipitation

24 hours after transfection cells were scraped on ice in cold phosphate buffer saline (PBS), then centrifuged with 6000G for 5 min at 4 °C. Cells were resuspended in 500 μl lysis buffer (50 mM TRIS-HCl, pH: 7,5, 150 mM NaCl, 0,1% Triton X-100, 5 mM EDTA, 1 mM PMSF), then incubated on ice for 20 min. Samples were sonicated twice with 10% amplitude for 8 seconds, then centrifuged with 20000G for 5 min at 4 °C. INPUT samples were made from the supernatant, 40 μl each. 20 μl anti-FLAG or 20 μl anti-HA beads (Sigma) were used for each sample. Sample were rotated for 1.5 hours at 4 °C-on, then sedimentated with 5000G for 5 min at 4 °C and rotated with wash buffer (same as the lysis buffer without protease inhibitor) for 3 × 10 min at 4 °C. 20 μl wash buffer and 8 μl 6x Laemmli buffer was added after the last wash and the samples were boiled for 5 min at 100 °C. Samples were analyzed by western blot.

Western blot

Protein samples were made from HEK293 cells 24 hours after transfection. Cells were washed with ice-cold PBS and scraped on ice in lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM KCl, 10 mM EDTA, 1 mM PMSF, 1% Triton X-100). They were incubated for 16 min on ice and centrifuged with 20.000g for 8 min at 4 °C. The protein amounts of the supernatants were measured by Bradford reagent (Thermo Scientific) and the concentrations of the samples were calculated from the absorbance values obtained with bovine serum albumin (BSA) calibration line. Samples were boiled with 6X Laemmli buffer at 100 °C for 5 min. 20μg protein per sample were applied on the 10% SDS polyacrylamide gel. Electrophoresis was performed in standard circumstances, then proteins were transferred to PVDF membranes. The following primary antibodies were used: anti-FLAG (Sigma, M2, mouse, 1:2000), anti-tubulin (DSHB, mouse, 1:1000), anti-HA (Roche, rat, 1:1000), anti-Vps18 (Abcam, rabbit, 1:3000), anti-myc (Sigma, rabbit, 1:500), anti-Rabenosyn-5 (Proteintech, rabbit, 1:2000), anti-Vps8 (Atlas, rabbit, 1:500), anti-LC3 (Nanotools, mouse, 1:200). For development of specific protein bands, alkaline phosphatase (AP) secondary antibodies were used in 1:5000 dilution. Protein detection were performed with NBT-BCIP (Sigma). Band intensities were analyzed by ImageJ software. For chloroquine treatment cells were incubated in cell culture medium containing 100 μM chloroquine (Sigma) for 1 hour.

Immunocytochemistry

Immunocytochemistry was performed 24 hours after transfection. Incubations were performed at room temperature, if not mentioned otherwise. First, the cells were washed twice with PBS, then fixed with 4% paraformaldehyde (PFA) for 20 min, washed with PBS for 15 min, permeabilized with PBTX (PBS supplemented with 0.1% Triton), then blocked for 30 min. The antibodies were diluted in the blocking solution consists of PBTX, 5% fetal calf serum (FCS) and 0,01% sodium azide. Samples were incubated with the primary antibodies at 4 °C for 16 hours. Anti-FLAG (Sigma, M2), anti-Rbsn-5 (Proteintech) and anti-Vps18 (Abcam) primary antibodies were used in 1:200 dilution. After that samples were washed with PBTX, then with PBTX supplemented with 4% NaCl and with PBTX again. Blocking solution was used for 30 min, then secondary antibodies were applied for 1 hour in 1:600 dilution. DyLight594 conjugated anti-mouse (Vector Laboratories) and fluorescein isothiocyanate

(FITC) conjugated anti-rabbit (Sigma) secondary antibodies were used. Samples were washed three times with PBTX, then incubated with PBTX, 4%NaCl, Hoechst nucleus dye (1:200 dilution, sigma) for 15 min, then washed with PBTX and rinsed with PBS. Samples were mounted with 4 μl Vectashield mounting medium (Vectar Laboratories).

Microscopy and image analysis

Samples were examined with Zeiss AxioImager M2 microscope with ApoTome 2 confocal unit, photos were taken using Zeiss Efficient Navigation (ZEN) software.. Colocalization was measured with ImageJ software (National Institutes of Health) by calculating the Pearson correlation coefficient (Pearson's r value) using the *coloc* plugin. The Mander's coefficient plugin was used to generate scatter plots.

Generation of Vps18 mutant cell line

Vps18 KO cells were established with the self-cleaving plasmid method by CRISPR/ Cas9.[34] The genomic target site of the gRNAs are at the border of the first exon–intron site of the Vps18 genomic sequence. The target coding gRNA was cloned into BpiI sites of the pmCherry-gRNA plasmid[43] with oligonucleotides below: 5′-caccGGG CATCCCCCACTCGGGTA-3′ and 5′-aaacTACCC GAGTGGGGGATGCCC-3′. HEK293T cells cultured on 12-well plates were seeded a day before transfection at a density of 1×10^5 cells/well respectively. Cells were co-transfected with plasmids containing the following sequences: I. specific gRNAs and SpCas9 enzyme; II. Targetless (TL) gRNA; III. a GFP cassette and puromycin resistance expressing plasmid containing the target site of the TL gRNA. The following amounts of plasmid DNA (760 ng of Cas9 and specific gRNA, 470 ng of TL gRNA and 760 ng of the GFP coding plasmid) was mixed with 200 ul DMEM and 4 ul Turbofect reagent and was incubated 30 min prior adding to the cells. Two days after transfection cells were selected for puromycin for 15 days and then single cell clones were prepared by serial dilution of the cells. GFP positive single cell clones were identified by High Content Screening (HCS) and were cultured in the presence of conditioned medium of untransfected HEK293 cells. Vps18 KO cells were identified by PCR (data not shown) and Western blot. From 10 possible KO cell lines 3 proved to be Vps18 null-mutant. One of them was used in these experiments.

Generation of cell lines stably expressing Vps18

Wild type or Vps18 KO HEK293 cells were transfected with CMV vector containing the coding sequence of the full length (FL) or the Rbsn binding site mutant version (ΔRbsnB) of Vps18. Cells were selected with hygromycin for two weeks and checked for Vps18 expression by WB.

Statistics

The datasets were analyzed by SPSS (IBM). The appropriate statistical tests were used, according to the distribution of the data. In the case of normal distribution t-tests were implemented. In the case of non-normal distribution Mann-Whitney U-tests were performed.

CRediT authorship contribution statement

Zsófia Simon-Vecsei: Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Ármin Sőth:** Investigation, Methodology, Validation, Visualization, Writing - original draft. **Péter Lőrincz:** Conceptualization, Investigation, Methodology, Writing - original draft. **András Rubics:** Investigation, Methodology. **András Tálas:** Investigation, Methodology. **Péter István Kulcsár:** Investigation, Methodology. **Gábor Juhász:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - original draft.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2021.166965.

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Abbreviations used:
Rbsn, Rabenosyn-5; Vps, Vacuolar Protein Sorting; Rab, Ras-Associated Binding; Arf, ADP-Ribosylation Factor; SNARE, SNAP Receptor, (SNAP: Soluble NSF Attachment Protein); SM protein, Sec1/Munc-18 protein; EEA1, Early Endosomal Antigen 1; CORVET, Class-C Core Vacuole/Endosome Tethering; HOPS, Homotypic Fusion and Vacuole Protein Sorting; GARP, Golgi-Associated Retrograde Protein; EARP, Endosome-Associated Recycling Protein; CHEVI, Class-C Homologs in Endosome–Vesicle Interaction; FERARI, Factors for Endosome Recycling and Retromer Interaction; EHD1, EH Domain Containing Protein 1; ANK3, Ankyrin-3; PI3P, Phosphatidylinositol-3-Phosphate; LC3, Microtubule-Associated Proteins 1A/1B Light Chain 3B; HEK293, Human Embryonic Kidney 293; Y2H, yeast two-hybrid method

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