Membrane traffic operates by vesicles that bud from precursor organelles and are transported to their target compartment where they dock and fuse. Targeting requires tethering factors recruited by small GTPases and phosphoinositides whereas fusion is carried out by SNARE proteins. Here we report that vesicles containing the Q-SNAREs syntaxin 13 (Stx13) and syntaxin 6 (Stx6) together are targeted to a different endosomal compartment than vesicles containing only Stx6 using injection of artificial vesicles. Targeting by Stx6 requires Vps51, a component of the GARP/EARP tethering complexes. In contrast, targeting by both Stx6 and Stx13 is governed by Vps13B identified here as tethering factor functioning in transport from early endosomes to recycling endosomes. Vps13B specifically binds to Stx13/Stx6 as well as to Rab14, Rab6, and PtdIns(3)P. We conclude that SNAREs use a combinatorial code for recruiting tethering factors, revealing a key function in targeting that is independent of SNARE pairing during fusion.
In eukaryotic cells, communication between organelles is mostly mediated by trafficking vesicles that bud from precursor compartments and are transported along cytoskeletal tracks toward the destination membrane where they dock and fuse. To ensure that each trafficking vesicle is targeted to the correct compartment, unique identifiers (molecular “zip-codes”) are incorporated into vesicles as they bud off a donor organelle. Three types of macromolecules have emerged as major zip code candidates. They include (i) small GTPases mostly of the Rab and Arf/Arl families, (ii) phosphorylated variants of the membrane lipid phosphatidylinositol (PtdIns phosphates), and (iii) soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that execute membrane fusion in the secretory pathway. Together, they orchestrate a multilayered recognition system at the target site to ensure targeting specificity.

The first layer is represented by vesicle tethering that captures the trafficking vesicle and brings it close to the target membrane. It is mediated by tethering factors that are transiently recruited from the surrounding cytoplasm and released when the trafficking step is completed. Tethering factors are not only necessary for targeting but also appear to be sufficient as shown in elegant relocation assays where tethering factors were artificially mis-localized to mitochondria, resulting in mistargeting of the corresponding vesicles. So far, ~20 different tethering factors are known that fall into two major groups: multi-subunit tethering complexes (MTCs) and homodimeric coiled-coil proteins. Both class of tethering factors share certain characteristics including specific binding to zip code molecules (see below) or coat proteins, the ability to bridge membranes, and to regulate membrane
dynein.

The second layer of the recognition system is thought to be represented by the specificity of SNARE assembly between the tethered vesicle and the target membrane, which drives membrane fusion. Mammalian cells contain between 35 and 40 different SNAREs, and SNARE complexes involved in different intracellular fusion steps differ in their composition. It is still debated to what extent the combinatorial composition of the respective SNARE complex encodes specificity since some SNAREs can functionally substitute for each other as long as they belong to the same subfamily. However, there is general agreement that pairing specificity of SNAREs, despite some promiscuity, provides a final checkpoint for the accurate delivery of trafficking vesicles.

Thus, there appears to be a division of tasks between the first and second layer of recognition among the three classes of zip code molecules. Vesicle capture by tethering factors is thought to be mainly orchestrated by the small GTPases that can be switched on and off. In the GTP-bound “on” form, transport vesicles are specifically recognized by tethering factors. Moreover, in many cases, binding is enhanced by phosphorylated variants of the membrane lipid phosphatidylinositol (PtdIns phosphates). The seven known phosphoinositides as well as Rab GTPases are selectively associated with defined endomembrane compartments, thus providing identity signals that are recognized by tethering factors and other effectors. Simultaneous binding to Rabs and PtdIns phosphates thus stabilizes membrane association via tethering factors. Note that in some cases, the recognition may be indirect and involve additional proteins. For instance, the Dsl1 complex and GCC185 tethering factors recognize COPI and the AP-1 complex, respectively, which both are recruited via active Arf GTPases. Thus, small GTPases and PtdIns phosphates are considered as prime players in defining targeting specificity via recruitment of tethering factors, whereas the role of SNAREs is hitherto thought to be confined to the final fusion step.

Interestingly, certain tethering factors are also known to bind to SNARE proteins, and only subsets of SNAREs appear to be involved. As far as known, binding is mainly mediated by the N-terminal domains of SNAREs that are localized upstream of the SNARE motifs required for fusion. Some of these interactions are rather stable, allowing for crystallization of the complex such as the yeast Dsl1-complex or the complex between the N-terminal domain of syntaxin 6 (Stx6) and Vps51, a component of the multi-subunit GARP (golgi-associated retrograde protein)/EARP (endosome-associated recycling protein) tethering complexes. While such interactions may further stabilize the binding of tethering factors to trafficking vesicles, SNARE binding is primarily thought to promote the assembly of specific and functional SNARE complexes after tethering. SNAREs are generally not considered to play a role in targeting due to their broad distribution among intracellular membranes. As integral membrane proteins they can only be returned by membrane traffic to their site of action and are present on all vesicles of the respective recycling pathway. Thus, they lack the stage-specificity of the GTPases and PtdIns phosphates, which is needed for distinguishing each individual trafficking step, but a rigorous test of the involvement of SNAREs has so far not been feasible.

Recently, we have made the surprising observation that injection of artificial vesicles results in targeting and fusion of the liposomes with endogenous early endosomes if they contain the SNAREs mediating fusion of early endosomes (syntaxin 13 (Stx13), Stx6, vti1a, and VAMP4) as the only proteins. These observations show that an otherwise “naive” vesicle devoid of PtdIns phosphates and GTPases only requires SNARE proteins to be phased into the correct domain of the secretory pathway. In the present study, we have used the same approach to shed light on the underlying molecular mechanisms. Using the early endosomal SNAREs as example, we show that targeting specificity is mediated by cooperative capturing of SNARE-binding tethering factors in a combinatorial manner. These include the Vps51, a component of the GARP complex that is recruited by Stx6, and Vps13B, a large monomeric protein with unclear function that is recruited jointly by Stx6 and Stx13 13. We show here that Vps13B functions as a tethering factor in vesicle traffic between early and recycling endosomes. Our data suggest that at least some SNAREs can effectively recruit tethering proteins that alone or in combination define intracellular targeting.

Results

Liposomes containing endosomal SNAREs are correctly targeted. To assess targeting of vesicles injected into HeLa cells, we determined co-localization of the fluorescence-labeled vesicles with a set of marker proteins 5 min after injection (see also ref. 23). As depicted in Fig. 1a, these markers include APPL1 (adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper containing 1) for endocytic vesicles, EEA1 (early endosome antigen 1) for early endosomes, transferrin (Tfn) for early/recycling endosomes, M6PR (cation-independent mannose-6-phosphate receptor) for golgi/endosome transport vesicles, LBPA (lysobisphosphatidic acid) for late endosomes, LAMP1 (lysosomal-associated membrane protein 1) for lysosomes, GM130 for cis-Golgi, Golgin97 for the trans-Golgi network (TGN), and PDI (protein disulphide isomerase) for the endoplasmic reticulum. To quantify the degree of co-localization, the center of all spots in both channels was calculated and the distance of every injected vesicle to the closest particle was measured. As a control for accidental overlap, we carried out experiments in which cells were kept at 4 °C. As reference for our lipidosome experiments, we first injected enriched fractions of labeled early/recycling and late endosomes. To this end, HeLa cells were either preincubated with Alexa488-Tfn to label early/recycling endosomes, and transfected with
GFP-Rab7 to label late endosomes. Endosomes were then enriched using standard density gradient fractionation of post-nuclear supernatants (Supplementary Fig. 1a–d). Although the injected early/recycling endosomes are expected to contain at least some of these markers, the overlap between injected and endogenous EEA1/Tfn-positive endosomes, but not with other organelles, was much higher than in the 4 °C condition (Fig. 1b, c and Supplementary Fig. 2a), confirming our previous observation. Note that the injected early/recycling endosomes were functionally active: when endosomes derived from cells expressing a Tfn receptor (TfnR) containing a luminal GFP-tag were injected, surface exposure of the Green fluorescent protein (GFP) -tag was observable few minutes after injection (Supplementary Fig. 1e–h). Similarly, injection of late endosomes containing GFP-Rab7 resulted in specific and significant colocalization with LAMP1 that was reduced at 4 °C (Fig. 1d and
Supplementary Fig. 2b), showing that specific targeting occurs. Thus, isolated endosomes retain their targeting information during isolation.

Next, we injected artificial vesicles with a simple lipid composition (79.7% phosphatidycholine, 20% phosphatidylserine, and 0.3% Rhodamine–PE) containing either the four SNAREs mediating early endosome fusion including Stx13, vti1a, Stx6, and VAMP4 (referred to as EE-SNAREs) or the four SNAREs mediating late endosome fusion including syntaxin 7, vti1b, and M6PR. Early endosome vesicles showed major overlap with Tfn (93.5%) positive, whereas 14.8% of them were positive for M6RP. Overlap with M6PR was lower but still seen with the majority of this population (56.0%). In contrast, only an almost negligible proportion (2.8%) of the Stx6+/Stx13+ vesicles were Tfn positive, whereas 14.8% of them were positive for M6RP.

Targeting specificity is governed differentially by Stx13 and Stx6. In the following experiments, we focused on the early endosomal SNAREs in order to dissect which of the SNAREs are responsible for the targeting of the artificial vesicles. As shown in Fig. 2a, omission of the R-SNARE VAMP4 from the liposomes had no effect on the targeting specificity. Next, we analyzed liposomes containing two of the three Q-SNAREs (all three combinations, Fig. 2b), and finally only individual SNAREs (Fig. 2c). Here, an interesting dissociation was observable: vesicles containing Stx6 and Stx13 again behaved similar to liposomes containing protein-free liposomes injected as control (Fig. 1e and Supplementary Fig. 2f) that was comparable to that of injection of proteoliposomes (PLs) reconstituted with early endosomal SNAREs (4-EE-SNARE PL). White arrows indicate co-localization between injected endosomes and organelle markers. Scale bar, 2.5 µm.

In contrast, liposomes containing only Stx6 co-localized with vti1a. In contrast, liposomes containing Stx6 and Stx13 again behaved similar to liposomes containing early endosome SNAREs (4-EE-SNARE PL). White arrows indicate co-localization between injected liposomes and organelle markers. Scale bar, 2.5 µm.

In summary, these results demonstrate that Stx13- and Stx6-containing vesicles are targeted differentially to their endogenous counterparts.

To confirm that Stx13 is sorted away from CTxB-containing vesicles derived from recycling endosomes, we internalized Alexa Fluor 647-CTxB and Alexa Fluor 568-Tfn in GFP-Stx13 transfected cells and monitored vesicle budding using time-lapse imaging. Figure 3d shows an example of a CTxB-positive transport vesicle that originates from a Tfn- CTxB-, and Stx13-positive precursor but excludes Tfn and Stx13. We conclude that both Stx13 and Stx6 operate jointly in the recycling pathway of Tfn-positive endosomes, whereas vesicles containing Stx6 but devoid of Stx13 operate in the M6PR- and CTxB-positive retrograde pathway (Fig. 3e).
liposomes containing Stx6 with cytosol, separated the liposomes by flotation gradient centrifugation, and analyzed bound proteins by proteomics. Among other proteins, we identified Vps51 (also referred to as Ang 2 in humans), which is known to be part of the multi-subunit GARP/EARP tethering complexes. The association between Stx6 and Vps51 was confirmed by co-immunoprecipitation (Supplementary Fig. 3a) and by binding of Vps51 to liposomes containing full-length Stx6 but not ΔN-Stx6 (Fig. 4b). In addition, upon co-transfection of GFP-Vps51 and mRFP-Stx6, the proteins not only colocalized in the TGN as reported but also on small vesicles in cytoplasm (~54% of Stx6 vesicles; Fig. 4c, data not shown). These data agree with previous observations showing that Vps51 forms a specific complex with the N-terminal domain of Stx6 (ref. 22).

Is Vps51 both necessary and sufficient for the targeting of injected Stx6 liposomes? To this end, we established stable Vps51 knockdown cell lines (Fig. 4d), and measured targeting of injected Stx6 liposomes. Indeed, co-localization of these liposomes with M6PR-containing vesicles was significantly decreased (Fig. 4d). Next, we fused the C-terminal domain of the outer mitochondrial protein monoamine oxidase (MAO) to a construct containing Vps51 and GFP (Vps51-GFP-MAO; Supplementary Fig. 3b). This fusion protein was efficiently targeted to mitochondria (Supplementary Fig. 3c). Furthermore, Vps51-GFP-MAO co-precipitated with Vps52, suggesting that the fusion protein is still capable of forming GARP complexes (Supplementary Fig. 3d). Injection of Stx6 liposomes resulted in the gradual accumulation of the proteoliposomes (PLs) on the surface of Vps51-GFP-positive mitochondria (Fig. 4e and Supplementary Fig. 3e, 3f). No such accumulation was observed when protein-free liposomes were used or when Vps51 was omitted (Fig. 4e and Supplementary Fig. 3e, 3f). These data indicate that Vps51 is sufficient for orchestrating the capture of injected Stx6 liposomes. On the other hand, no intermixing between the GFP-labeled outer mitochondrial membrane and the Rh-PE-labeled lipid membrane was observed. Thus, Stx6, while being sufficient for targeting, is not able to mediate fusion with mitochondria. Furthermore, we observed that in transfected cells, endogenous Stx6- or M6PR-containing endosomes selectively accumulated around the labeled mitochondria, whereas no such accumulation was seen for Tfn- or Golgin97-positive vesicles (Fig. 4f). Moreover, no accumulation was observed in cells expressing GFP-MAO (Supplementary Fig. 3g) confirming that Vps51 is both necessary and sufficient for mitochondrial mistargeting of both injected Stx6 liposomes as well as endogenous Stx6/M6PR-containing trafficking vesicles.

**The N-terminal domain of Stx6 mediates targeting by Vps51.** To clarify whether the N-terminal domain of Stx6 is required for targeting of the liposomes to the M6PR compartment, we injected liposomes containing an N-terminally truncated mutant of Stx6 (ΔN-Stx6, residues 169–255). No co-localization with any organelle markers was observable (Fig. 4a). Next, we incubated liposomes containing an N-terminally truncated mutant of Stx6 targeting of the liposomes to the M6PR compartment, we injected

**Vps13B is recruited to Stx6- and Stx13-containing vesicles.** As shown above, liposomes containing both Stx6 and Stx13 differ from Stx6 liposomes by being targeted to Tfn-positive endosomes as well as to M6PR-positive vesicles. This is surprising since
liposomes containing only Stx13 are not specifically targeted (Fig. 2c). Moreover, almost all of the endogenous endosomes involved in Tfn recycling contain both SNAREs. These data imply that recycling endosomes recruit tethering proteins that recognize both Stx6 and Stx13. The only known protein suggested to bind to both SNAREs on early endosomes is EEA1. Since the presence of additional hitherto unknown tethering factors cannot be excluded, we used SNARE-containing liposomes to search for cytoplasmic proteins specifically binding to both SNAREs. Among others, we detected Vps13B, one of four Vps13 paralogs in mammals that is also referred to as COH1 due to its association with Cohen syndrome, a severe inheritable disease (see Supplementary Table 1). Analysis by immunoblotting confirmed the presence of Vps13B on liposomes containing both SNAREs.
Fig. 3 SNAREs are selected during budding of transport vesicles. a Association of Stx6 and Stx13 with transport vesicles containing either Tfn or M6PR. Endosomal compartments in cells expressing mRFP-Stx6 and GFP-Stx13 were labeled either by internalization of Alexa Fluor 633–Tfn or by immunostaining for anti-M6PR antibody. White arrows indicate Stx6(+)/Stx13(−) vesicles. Most of them are negative for Tfn (arrows, top), but several of them are positive for M6PR (arrows, bottom). Scale bar, 5 μm. Right: intensity plots of the line scans shown by yellow lines in the left images. Stx6(−)/Stx13(+) vesicles (shadowed by light yellow) co-localize with both Tfn (top) and M6PR (bottom), but Stx6(−)/Stx13(−) vesicles are negative for Tfn and positive for M6PR. b, c Snapshots of tubular structures (T) extending from vesicle endosomes (V) from a movie. Both domains are triple positive for Stx13, Stx6, and Alexa633–Tfn. Intensity plots of the line scans are shown on the right side. c A tubule is shown that contains the retrograde marker Alexa647–cholera toxin β (CTxB) and that is positive for Stx6 but negative for Stx13. Right panels show line scans shown by lines in the left panels. d Snapshots from a movie showing budding of a CTxβ-positive vesicle (white circles in the last frame) from a globular endosome that is positive for CTxβ but negative for Stx13 and Tfn. Scale bar, 1 μm. e Summary showing sorting of cargos and SNARE proteins in early endosomes.

whereas no or only reduced binding was observable to liposomes containing either of the SNAREs (Fig. 5a). Note that using this assay, we were unable to detect an interaction with EEA1 (Fig. 5a).

Human Vps13B is a large protein of 3997 amino acids (NP_689777). Vps13B was originally identified in yeast but is now known to be conserved in eukaryotes. In yeast, Vps13p is widely distributed in the endosomal system and has been invoked both in membrane traffic and in the formation of membrane contact sites. The human Vps13 gene family contains four members (Vps13A-D) that appear to have functionally diversified. Recently, Vps13B was shown to be primarily localized to the Golgi apparatus and to be an effector of Rab6, suggesting a function in the organization of Golgi membranes.

To confirm the interaction between Stx6 and Stx13 and Vps13B, we immunoprecipitated endogenous Vps13B from detergent extracts of HeLa cells. Co-precipitation with both Stx6 and Stx13 but not with VAMP4, Stx4 or Stx16 was observed (Fig. 5b). Conversely, Vps13B co-precipitated with Stx6 and Stx13 but not with the other tested SNAREs (Fig. 5c). To test whether the N-terminal domain of Stx13 is required for Vps13B-binding, we transfected cells, together with FLAG-tagged Vps13B with (i) GFP-tagged Stx13, (ii) Stx13 with the N-terminal domain being truncated (GFP–ΔN-Stx13), and (iii) a chimera in which the N-terminal domain of Stx13 was replaced with that of Stx6 (GFP–Stx13–6). FLAG–Vps13B was then immunoprecipitated using an anti-FLAG antibody. Full-length GFP–Stx13 and GFP–Stx13–6 but not ΔN–Stx13 co-precipitated, suggesting that the both N-terminal domains are required whereas the nature of the SNARE motif is probably not relevant (Fig. 5d).

In HeLa cells, Vps13B was previously shown by immunocytochemistry to be concentrated as a peripheral membrane protein of the Golgi apparatus, but the authors also noted that the protein has a more widespread distribution that was not confined to the Golgi. Immunolabeling for endogenous Vps13B confirmed these findings (Fig. 5e), with very similar patterns being observable when a Vps13B–GFP and FLAG–Vps13B construct was expressed (see Fig. 6c and Supplementary Fig. 5b, data not shown). To examine whether Vps13B is associated with Stx13 and Stx6 containing transport vesicles in the endosomal region, we focused on the peripheral region of the cell, again using expression of tagged SNAREs to facilitate localization. As shown in Fig. 5e, f, endogenous Vps13B displayed co-localization with vesicles positive for both mRFP–Stx6 and GFP–Stx13 (34%) whereas co-localization with vesicles containing either mRFP–Stx6 or GFP–Stx13 was low (7% and 10%, respectively). Furthermore, partial (~40%) colocalization was also found with TfnR both in the cell periphery (Fig. 5g). In contrast, overlap of Vps13B with endogenous EEA1 and M6PR, respectively, was lower than TfnR (Fig. 5g), and no overlap was observable with the late endosomal markers CD63 and GFP–Rab7, with the mitochondrial marker mitofilin, or with the autophagosomal marker LC3, regardless of whether autophagocytosis was induced by starvation (Supplementary Fig. 4a). Together, these findings suggest that in the cell periphery, Vps13B selectively associates with recycling endosomes carrying Stx6 and Stx13, in addition to its abundant presence in the “perinuclear cloud” including recycling and late endosomes, lysosomes, and the vesicles of the TGN.

Vps13B binds to specific Rab proteins and phosphoinositides. Most of the established tethering factors operate by coincidence detection, i.e. they simultaneously bind to zip code molecules such as specific small GTPases including Rabs, Arfs, and phosphoinositides to restrict their distribution and regulate the function. When analyzing Vps13B immunoprecipitates by proteomics, both Rab6 and Rab14, which regulate Tfn recycling pathway prior to Rab11 and after Rab5 and Rab4 (ref. 39), were detected. For further exploration, we expressed tagged variants of the endosomal GTPases Rab4, Rab5, Rab6, Rab11, and Rab14 in HeLa cells, followed by immunoprecipitation of the Rabs and immunoblotting for endogenous Vps13B. A specific interaction was observed for Rab6 (confirming ref. 36) and for Rab14. None of the other Rabs were detected (Fig. 6a).

In contrast, GFP–Rab6 was almost exclusively localized to structures resembling the TGN, overlapping with endogenous Vps13B (36%). These structures, however, displayed a more punctate pattern associated with Rab6-positive TGN structures (Fig. 6d insert).

Since yeast Vps13 has recently been shown to specifically bind to PtdIns(3)P, we asked whether mammalian Vps13B displays a similar phosphoinositide binding preference. First, using a cell-free extract of cells expressing Vps13B–GFP, a PIP strip assay was carried out, and bound Vps13B was detected with a GFP antibody. As shown in Fig. 6c, specific binding to PtdIns(3)P but not to any other phosphoinositide was observed. Second, we used beads coated with anti-GFP antibody to isolate Vps13B–GFP, followed by washing and incubation with liposomes. Binding was only observed when the liposomes contained PtdIns(3)P (Fig. 6f).

These data suggest that Vps13B specifically binds to PtdIns(3)P-containing membranes although a contribution of other endogenous proteins binding to Vps13B cannot be completely ruled out (Fig. 6d).

Vps13B functions as a tethering protein. The data above show that Vps13B specifically interacts with Stx6, Stx13, and with...
PtdIns(3)P, and they confirm and extend the notion that Vps13B specifically interact with select Rab GTPases—all features typical for bona fide tethering factors. In the final set of experiments, we therefore asked whether Vps13B does indeed function as tethering factor that is responsible for the targeting of Stx6+/Stx13+ liposomes to TfnR-containing compartments. To this end, we targeted Vps13B to mitochondria by expressing a Vps13B-GFP-MAO construct (Supplementary Fig. 5a), thus using the same strategy as for Vps51 above, resulting in mitochondrial localization of the fusion protein (Supplementary Fig. 5b). Due to low
expression levels and low efficiency of transfection, experiments involving the injection of liposomes were not feasible. Instead, we tested whether endogenous liposomes were re-distributed. Intriguingly, TfnR-positive endosomes became scattered (Fig. 7a), associated with a dramatic reduction of the perinuclear index (Fig. 7a) that was not seen for organelles containing M6PR (Fig. 7a) or Golgin97 (Supplementary Fig. 5c). At higher magnification, some TfnR-positive endosomes seemed to distribute along mitochondria (Fig. 7a). Next, we generated stable cell lines expressing Vps13B shRNA in HeLa cells, resulting in significant downregulation of the protein (Fig. 7b and Supplementary Fig. 5d, e). We then injected Stx6/Stx13 liposomes. Targeting of these liposomes to Tfn-positive endosomes was significantly decreased, whereas targeting to the M6PR-positive compartment was not affected (Fig. 7c). These results suggest that Vps13B operates as a tethering factor in the Tfn recycling pathway, which is governed by a combination of the Q-SNARE proteins Stx6 and Stx13.

The Tfn/TfnR complex is known to be recycled to the plasma membrane by two different pathways: (i) a Rab4 and Rab35-dependent recycling pathway (fast recycling) in which vesicles formed at the early endosomes in the cell periphery are directly targeted to the plasma membrane, and (ii) a Rab11-dependent pathway (slow recycling) in which tubulo-vascular transport organelles are first delivered to the perinuclear recycling endosome from where they are returned to the plasma membrane (Fig. 7g). To examine at which step Vps13B operates, we monitored the fate of endocytosed Alexa Fluor 568-Tfn over time in wt and Vps13B KD cells using time-lapse microscopy. While neither the rate of Tfn clearance (Fig. 7d) nor the surface levels of M6PR and TfnR (Supplementary Fig. 6a) were changed significantly, we noted a conspicuous loss of perinuclear accumulation in Vps13B KD cells, particularly evident 15–30 min after the pulse (Fig. 7d). A similar re-distribution of the cell periphery was seen for Rab11 and Rab14, both specific for the late recycling pathway, whereas markers for early endosomes (EEA1), late endosomes (CD63), retrograde pathway (M6PR), and mitochondria (Mitofusin2) remained unchanged (Supplementary Fig. 6b). The TGN (Golgin97) was weakly fragmented, as previously reported.

These data suggest that Vps13B may act as a tethering factor in the trafficking step between early and recycling endosomes. To confirm this interpretation, we immunosolated TfnR containing vesicles from cell extracts with anti-TfnR antibody and analyzed them by immunoblotting for the presence of endosomal and lysosomal markers. As shown in Fig. 7e, Vps13B knockdown resulted in a reduction of co-distribution of TfnR with Rab11, an increase in Rab35 and Rab4, and also an increased presence of LAMP1. Moreover, the overall protein levels of TfnR were reduced in Vps13B KD cells, which was prevented by addition of chloroquine, a lysosomal inhibitor (Fig. 7f).

**Discussion**

Using artificial vesicles with a minimalistic composition as tools, we have shown that SNAREs alone, in the absence of Rab proteins or phosphoinositides, are capable of directing trafficking vesicles to the correct target compartment in the endosomal pathway. Targeting is surprisingly accurate even though it does not appear to be as precise as that of endogenous trafficking organelles. Moreover, our data suggest that targeting information is encoded in the N-terminal domains of SNAREs instead of in the SNARE motifs. The N-terminal domains are known to be critical for the recruitment of additional proteins. Most prominently, such proteins include Sec1/Munc18 (SM) and CATCHR (complex associated with tethering containing helical rods) proteins that are required for regulating SNARE assembly during fusion. Similarly, complexes between individual tethering factors and individual SNAREs were previously reported (see Introduction). However, these interactions are so far mainly thought to ensure that SNAREs or SNARE acceptor complexes are present and ready for fusion at the site where tethering occurs.

Our findings document that SNARE proteins may play a much more important role in vesicle targeting than previously appreciated. They highlight a second function of some of the SNAREs that becomes relevant immediately after vesicle budding and that is independent of (although connected with) their established role in membrane fusion. The emerging picture shows further that it is not only the individual SNARE but rather the specific combination of SNAREs that, at least in the examples studied here, decides about the recruitment of tethering factors and thus the destination of the vesicles. Such combinatorial coding may explain how specificity can be achieved despite the broad distribution of individual SNAREs, particularly in the endocytic limb of the secretory pathway. Indeed, only a fraction of the dozen or more SNAREs operating in the endocytic pathway would be needed for creating a specific SNARE combination for each trafficking step, even if constraints apply for meeting the “QabcR-rule” for functional SNARE complexes. Differential sorting of SNAREs during the budding of a trafficking compartment is thus a critical determinant of vesicle targeting whereas less specificity may be involved in SNARE-pairing required for the final fusion step. This also agrees with previous findings showing that in vitro, tethering/docking of early endosomes, in contrast to fusion, is
Fig. 5 Vps13B binds to Stx6- and Stx13-positive vesicles. a Recruitment of Vps13B and EEA1 to liposomes containing Stx6, Stx13, Stx6, and Stx13 together. For details, see legend to Fig. 4b. α-Tubulin was used as loading control, because the protein binds to liposomes dependent on the lipid composition (ref. 23). b, c Co-immunoprecipitation of Vps13B and SNARE proteins; b shows co-precipitation of Stx6 and 13 but not of any other SNARE with Vps13B. c Conversely, Vps13B co-precipitates with Stx6 and Stx13 but not with Stx16 and Stx4. The bottom panel shows that all SNAREs were efficiently immunoprecipitated. d The N-terminal domain of Stx13 is necessary for binding to Vps13B. Lysates from cells expressing Vps13B-FLAG and GFP-Stx13, GFP-ΔN-Stx13, or GFP-Stx13-Stx6 chimera were immunoprecipitated with anti-FLAG antibody and probed by immunoblotting for GFP. e Immuno-fluorescence for Vps13B of cells expressing mRFP-Stx6 and GFP-Stx13. Arrows in the insert indicate triple-positive vesicles. Scale bar, 10 µm. f Co-localization between Stx6, Stx13, and Vps13B in the peripheral region of the imaged HeLa cells. Vesicles were counted from nine images, obtained in three independent experiments, with the degree of overlap shown in the bar graph on the right (numbers denote total number of vesicles analyzed). g Co-localization of Vps13B with TfnR, EEA1, M6PR, and Golgin97. White arrows show co-localized vesicles. Scale bar, 10 µm. Intensity plots of exemplary line scans are shown on the right. A bar graph shows the percentage of TfnR-, EEA1-, M6PR-positive vesicles, which are also positive for Vps13B.
independent of SNARE pairing\textsuperscript{43}. In a way it makes sense to select the correct SNAREs during budding rather than having the vesicle shipped to the right place and then, in the end, a decision needs to be made whether fusion is allowed to proceed or aborted due to lack of pairing specificity. Sorting of SNAREs appears to be mediated by specific interactions with coat proteins such as clathrin adaptors\textsuperscript{44–46}. For instance, Stx13 is known to interact with the BLOC-1 complex, which controls the formation of recycling...
endosomal tubules\textsuperscript{47}. However, more work is required for understanding how exactly individual SNAREs are selected or excluded during the formation of a trafficking vesicle from a common precursor membrane, and to establish whether SNARE-mediated targeting by this mechanism also occurs in other domains of the secretory pathway.

While the injection of SNARE-containing, but otherwise “naïve”, vesicles revealed sufficiency of SNAREs for targeting and fusion, the SNARE-encoded targeting signals are embedded in complex regulatory networks in which Rab switching and PtdIns phosphates play major roles\textsuperscript{15}. Functional tethering factors usually interact with all three of these signal classes, either directly...
or via intermediate coat proteins, and thus it is probably the combination of all signals that fine-tunes the destination of a given vesicle. However, with few exceptions, it is not known how exactly these three signal types interact with each other and how the hierarchy between them (if any) is structured. We hope that the approach used here will allow for individual testing of each of the components, thus providing an experimental approach to addressing these unresolved issues.

Vps51 is part of the GARP complex and the more recently identified and structurally related EARC complex (refs. 30,31). The GARP complex is required for the return of M6PR from endosomes to the TGN. In yeast, GARP is known to be an effector of the RabGTPase Rab6 and Arl1 (ref. 33). Fusion at the TGN is thought to involve the SNAREs Stx6, vti1a, and VAMP4, but, intriguingly, syntaxin 16 instead of Stx13 (ref. 34). In this context, our data revealing that in the absence of Stx13, Stx6 liposomes, regardless of whether vti1a is present or not, are targeted to M6PR-positive compartments can be easily interpreted as Stx6, via binding to the GARP complex by interacting with Vps51, being decisive for targeting. Vps51 is both necessary and sufficient for governing targeting since not only injected Stx6 liposomes but also endogenous M6PR vesicles are mis-directed to mitochondria upon expression of a Vps51-MAO construct. Intriguingly, the EARC complex that also contains Vps51 regulates endosomal recycling. It remains to be established whether both complexes allow for Vps51 binding to Stx6, and if this is the case, how differential targeting of Stx6-containing vesicles is regulated.

Vps13 is originally found in yeast during classical screens for defects in vacuolar sorting. Apparently, Vps13p is required for trafficking from the yeast endosome to the Golgi complex, but it is also involved in endosomal recycling in yeast. Vps13p is involved in processes such as sporation and autophagy, but its mechanism of action is unclear (reviewed in ref. 34). Surprisingly, in an elegant study, it has recently been shown that Vps13 proteins operate as lipid transfer proteins, capable of transferring phosphatidyserine and phosphatidylethanolamine between liposomal membranes. The activity is mediated by the N-terminal domain whose crystal structure revealed a large hydrophobic cavity capable of binding ~10 glycerolipid molecules. In yeast, Vps13p has been identified at membrane contact sites connecting endoplasmic reticulum, mitochondria, endosomes, and vacuoles, suggesting that Vps13p mediates lipid transfer at such sites. Interestingly, in mammals, different Vps13 isoforms localize to distinct contacts: Vps13A is enriched at contact sites between the ER and mitochondria (Vps13A), Vps13C at contacts between the ER and late endosomes/lysosomes, and both isoforms were also found at ER contacts to lipid droplets. Our data now reveal that Vps13p is required for the targeting of TfnR-containing vesicles from early to recycling endosomes. Vps13p shares key properties with other tethering factors such as the ability to bind to Rab proteins, PtdIns phosphates, and SNAREs. Furthermore, EM imaging showed that yeast Vps13p has an elongated structure that is intriguingly similar to the HOPS tethering complex in shape and size. However, HOPS is a multiprotein complex with no significant sequence homology. Thus, Vps13p does not belong to one of the established classes of tethering proteins including long coiled-coil long proteins and multi-subunit complexes and may be the first representative of a novel and third class of tethering proteins.

In summary, Vps13 proteins appear to be ‘moonlighting’ as they are involved both in lipid transfer and tethering. This is fascinating since both functions require a connection between two different membranes before the second step—membrane fusion or lipid transfer—is carried out. It is possible that yeast Vps13p represents an ancient form that embodies both functions, whereas in mammals these functions have diversified in the four related isoforms (Vps13 A–D). Indeed, unlike Vps13A and C, Vps13B is not associated with the ER (Supplementary Fig. 4a) as it lacks the FFAT motif needed for ER association. Instead, Vps13B was previously found to be mainly associated with the Golgi where it was reported to regulate the integrity of Golgi stacks due to its function as an effector of Rab6 (ref. 36). Moreover, the homology of the N-terminal domain of Vps13B with those of Vps13A and Vps13C is lower, but it needs to be tested whether Vps13B can function as lipid transfer protein or not. Functional diversification of the mammalian Vps13 isoforms is also supported by the fact that nonfunctional variants of individual isoforms cause different diseases. Moreover, the protein ATG2A may be an additional, albeit more distantly related member of the mammalian Vps13 family: it has high homologies with Vps13 in the N- and C-terminal regions, exhibits a similar elongated seahorse-like shape, and it mediates ER-phagophore association and/or tethering.

In conclusion, we show that distinct sets of SNAREs that are selected during budding of a trafficking vesicle determine targeting by selectively recruiting different tethering factors. This is a new function of SNAREs that is independent of their role in catalyzing fusion and also appears to be independent of the rules
governing SNARE pairing between two membranes in preparation for fusion. Combinatorial coding overcomes the “disadvantage” of the broad distribution of SNAREs. Vps13B is the first example of a tethering factor that requires the simultaneous presence of two SNAREs for recruitment, and it is conceivable that additional tethering factors exist recognizing different SNARE combinations. Our injection of artificial vesicles with a defined composition provides a powerful tool for the identification of tethering mechanisms and for determining the specificity in the vesicle targeting of vesicles.

Methods

Materials. All phospholipids were obtained from Avanti Polar Lipids. Primary antibodies were used obtained from the following companies: anti-APl1 (3185), anti-IC3B (4599), and anti-Rab7 (9367) from Cell Signaling; anti-EEA1 (61206) and anti-GM130 (560257) from BD Biosciences; anti-M6P (ab2733), anti-LAMP1 (ab24170), anti-mito-talinin (ab110329), anti-mito-tusion2 (ab101055), and anti-cathepsin D (ab3613) from Abcam; anti-Tf receptor (sc-65882) from Santa Cruz Biotechnology; anti-LRP (Z-FPLP) from Echelon; anti-BFP (R10367), anti-Golg97 (A-21270), anti-(150 mM KCl, 20 mM Hepes (pH 7.5)) containing 1 mM DTT. After dispersal by ammonium salt) (molar ratios) and the solvents were evaporated. The dried

SNARE combinations. Our injection of artificial vesicles with a defined composition provides a powerful tool for the identification of tethering mechanisms and for determining the specificity in the vesicle targeting of vesicles.

Protein recruitment to liposomes. The preparation of cytosol fractions was carried out as described21. Briefly, HeLa cells were homogenized in homogenization buffer at 230 mM sucrose containing 1:200, 1:1,000, 1:1200, and 1:15,400, respectively. For PLs containing only two SNAREs, a protein:phospholipid ratio of 1:2000 was used for each SNARE protein, and for PLs containing only one SNARE, the ratio was 1:1000. Finally, 4 LE-315h, a ratio of 1:2000 was used for each of the four SNARE proteins.

Immunostaining. Injected HeLa cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 10 min and washed with PBS three times. The cells were incubated overnight at 4 °C with primary antibody diluted 1:300–1000 in PBS containing 0.5% saponin and 1% goat serum. The coverslips were then washed three times with PBS and incubated with Cy3- or Cy5-labeled secondary antibodies, diluted at 1:600 in PBS containing 0.5% saponin and 1% goat serum for 90 min at room temperature. After washing with PBS, the coverslips were mounted in VECTASHIELD (Vector Laboratories) for immunostaining of cell surface proteins, cells were incubated with primary antibody at 4 °C for 60 min and cells were washed with PBS and then fixed with 4% paraformaldehyde in PBS for 10 min. After washing with PBS, the cells were incubated with Cy2- or Cy3-labeled secondary antibodies in PBS containing 0.5% saponin and 1% goat serum for 60 min at room temperature.

Internalization of Tfn and CtxB. For measuring internalization and recycling of Tfn, cells were starved for 3 h in Dulbecco’s modified Eagle medium (DMEM) containing 0.2% bovine serum albumin and then incubated with 5 μg/ml Alexa Fluor 568– or Alexa Fluor 634-conjugated Tfn. At 4 °C for 60 min. After cells were washed with ice-cold PBS, they were incubated in pre-warmed serum containing DMEM for 1 h. For CtxB internalization, Vero cells were incubated with 10 μg/ml Alexa Fluor 647-conjugated CtxB at 4 °C for 60 min. After being washed, the cells were incubated in pre-warmed injection medium (F12 medium (Invitrogen), supplemented with 10% fetal calf serum (FCS), 10 mM HEPES (pH 7.5), and 100 units/ml of penicillin and streptomycin) and observed by confocal microscopy.

Cell culture and preparation of early/late endosomes. HeLa cells and Vero cells were grown in DMEM (Lenza GmbH) with the following additions: 10% FCS (PAA laboratories GmbH), 4 mM glutamine (Lenza GmbH), and 10 units/ml each of penicillin and streptomycin (Lenza GmbH). Plasmid DNAs were transfected into HeLa cells using Lipofectamine LTX reagents according to the manufacturer’s instructions (Thermo Scientific).

For the generation of knockdown cell lines for Vps51 and Vps13B, five different target shRNA-containing vectors (GE Dharmacon) were used for transfection, followed by the selection of stable cell lines using 1 μg/ml puromycin. Screening was performed by Western blotting. For the generation of a cell line stably expressing GFP-Rab7, the plasmid was transfected with Lipofectamine 2000 (Invitrogen), and stably expressing cells were selected using 600 μg/ml G418-containing medium. Among several clones, a line was selected in which the expression levels and their distribution of GFP-Rab7 in the cells were comparable to those of endogenous Rab7.

Early endosomes were prepared as in Koike and Jahn25. For the isolation of late endosomes, GFP-Rab7 stably expressing HeLa cells were used. The cells were harvested by trypsin/EDTA treatment (Lonza GmbH), followed by washing once with fresh culture medium and once with internalization medium (OptiMEM, containing 10 mM glucose; Invitrogen). The cellular pellets were resuspended in homogenization buffer with protease inhibitor (Complete EDTA-free (Roche)) and cracked using a ball homogenizer with a clearance of 0.02 mm. The homogenate was centrifuged at 2000 × g for 15 min and the PNS (post nuclear supernatant) fraction was layered on top of a Nycodenz gradient consisting of 3 ml each of ice-cold Nycodenz solutions of 28%, 19%, 7.3%, respectively, followed by centrifugation at 100,000 × g for 60 min at 4 °C in a Beckman SW41 rotor. The 19%/28% boundary (late endosome-rich fraction) was concentrated while changing the buffer HB150 using a VIVASPIN 2 (30,000 MWCO; Sartorius).

Microinjection. About 2 μl (mole)plasmoliposomes and 10 μg/ml DAPI (4,6-diamino-2-phenylindole; Sigma) (injection marker) in HB150 were filled in Femtotips (Eppendorf); 1 x 104 HeLa cells were plated on poly-l-lysine (Sigma-Aldrich). After 24 h of incubation, the plasmid was injected into a 35-mm petri dish (Becton Dickinson) filled with pre-warmed injection medium (F12 medium (Invitrogen), supplemented with 10% FCS, 10 mM HEPES
2. Stenmark, H. Rab GTPases as coordinators of vesicle trafficking. Nat. Rev. Mol. Cell Biol. 12, 362–375 (2011).

3. Donaldson, J. G. & Jackson, C. L. ARF family G proteins and their regulators: roles in membrane transport, development and disease. Nat. Rev. Mol. Cell Biol. 4, 631–657 (2003).

4. Di Paolo, G. & De Camilli, P. Phosphoinositides in cell regulation and membrane dynamics. Nature 443, 651–657 (2006).

5. Jahn, R. & Scheller, R. H. SNARE-engines for membrane fusion. Nat. Rev. Mol. Cell Biol. 7, 631–643 (2006).

6. Yu, I. M. &Hughson, F. M. Tethering factors as organizers of intracellular vesicle traffic. Annu. Rev. Cell Dev. Biol. 26, 137–156 (2010).

7. Chia, P. Z. C. & Gleeson, P. A. Membrane tethering. F1000Prime Rep. 6, 74 (2014).

8. Spang, A. Membrane tethering complexes in the endosomal system. Front. Cell Dev. Biol. 4, 35 (2016).

9. Willett, R. et al. COG complexes form spatial landmarks for distinct SNARE complexes. Nat. Commun. 4, 1553 (2013).

10. Wong, M. & Munro, S. The specificity of vesicle traffic to the Golgi is encoded in the golgin coiled-coil proteins. Science 346, 1256898 (2014).

11. Yang, B. et al. SNARE interactions are not selective. Implications for membrane fusion specificity. J. Biol. Chem. 274, 5649–5653 (1999).

12. Liu, Y. & Barlowe, C. Analysis of Sec23p in endoplasmic reticulum/Golgi transport reveals cellular redundancy in SNARE protein function. Mol. Biol. Cell 13, 3314–3324 (2002).

13. Rothman, J. E. Mechanisms of intracellular protein transport. Nature 372, 55–63 (1994).

14. Faushauer, D., Sutton, R. B., Brunger, A. T. & Jahn, R. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc. Natl Acad. Sci. USA 95, 15781–15786 (1998).

15. Behnia, R. & Munro, S. Organelle identity and the signposts for membrane traffic. Nature 438, 597–604 (2005).

16. Stroop, C., Collins, K. M., Fratti, R. A. & Wickner, W. Purification of active HOPS complex reveals its affinities for phosphoinositides and the SNARE Vam7p. EMBO J. 15, 1579–1589 (1996).

17. Simonsen, A. et al. EEA1 links PI(3,5)K function to Rab5 regulation of endosome fusion. Nature 394, 494–498 (1998).

18. Zink, S., Wenzel, D., Wurm, C. A. & Schmitt, H. D. A link between ER tethering and COP-I vesicle uncoating. Dev. Cell 17, 403–416 (2009).

19. Brown, F. C., Schindelhauer, C. H. & Pfeffer, S. R. GCC185 plays independent roles in Golgi structure maintenance and AP-1-mediated vesicle tethering. J. Cell Biol. 194, 779–787 (2011).

20. Hong, W. & Lev, S. Tethering the assembly of SNARE complexes. Trends Cell Biol. 24, 35–43 (2014).

21. Ren, Y. et al. A structure-based mechanism for vesicle capture by the multisubunit tethering complex Dbl1. Cell 139, 1119–1129 (2009).

22. Abascal-Palacios, G. et al. Structural basis for the interaction of the golgi-associated retrograde protein complex with the t-SNARE syntaxin 6. Structure 21, 1696–1706 (2013).

23. Kosik, S. & Jahn, R. Probing and manipulating intracellular membrane traffic by microinjection of artificial vesicles. Proc. Natl Acad. Sci. USA 114, E9883–E9892 (2017).

24. Brandhorst, D. et al. Homotypic fusion of early endosomes: SNAREs do not determine fusion specificity. Proc. Natl Acad. Sci. USA 103, 2701–2706 (2006).

25. Zwingl, D. et al. Early endosomal SNAREs form a structurally conserved SNARE complex and fuse liposomes with multiple topologies. EMBO J. 26, 9–18 (2007).

26. Ohiya, T. et al. Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes. Nature 459, 1091–1097 (2009).

27. Antonin, W. et al. A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. EMBO J. 19, 6453–6464 (2000).

28. Popoff, V. et al. The retromer complex and clathrin define an early endosomal retrograde exit site. J. Cell Sci. 120, 2022–2031 (2007).

29. Bonifacio, J. S. & Hierro, A. Transport according to GARP: receiving retrograde cargo at the trans-Golgi network. Trends Cell Biol. 21, 159–167 (2011).

30. Schindler, C., Chen, Y., Pu, J., Guo, X. & Bonifacio, J. S. EARPs is a multisubunit tethering complex involved in endocytic recycling. Nat. Cell Biol. 17, 639–650 (2015).

31. Simonsen, A., Gauliier, J. M., D’Arrigo, A. & Stenmark, H. The Rab5 effector EEA1 interacts directly with syntaxin-6. J. Biol. Chem. 274, 28857–28860 (2000).

32. McBride, H. M. et al. Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. Cell 198, 377–386 (1999).

33. Balkova, I. et al. Deletions in the VPS13B (COH1) gene as a cause of Cohen syndrome. Hum. Mutat. 30, E845–E854 (2009).

34. Reznikowska, W. et al. Yeast and other eukaryotic organisms for studies of VPS13 proteins in health and disease. Traffic 18, 711–719 (2017).
35. Velayos-Baeza, A., Vettori, A., Copley, R. R., Dobson-Stone, C. & Monaco, A. P. Analysis of the human VPS13 gene family. Genomics 84, 536–549 (2004).

36. Seifert, W. et al. Cohen syndrome-associated protein, COH1, is a novel, giant golgi matrix protein required for golgi integrity. J. Biol. Chem. 286, 3765-37675 (2011).

37. Dellibovi-Ragheb, T. & Altan-Bonnet, N. Cloud storage for endosomes. Nature 500, 682 (2012).

38. Dellibovi-Ragheb, T. & Altan-Bonnet, N. Cloud storage for endosomes. Nat. Rev. Mol. Cell Biol. 10, 597–608 (2009).

39. Linford, A. et al. Rab14 and its exchange factor FAM116 link endocytic recycling and adherens junction function in migrating cells. Dev. Cell 22, 952–966 (2012).

40. Vamand, M., et al. Functional cross-talk between Rab14 and Rab4 through a dual effector, RUFY1/Rabip4. Mol. Biol. Cell 21, 2746-2755 (2010).

41. Rzepnikowska, W. et al. Amino acid substitution equivalent to human chorea-associated protein, COH1, is a novel, giant Golgi matrix protein required for golgi integrity. J. Biol. Chem. 286, 3765-37675 (2011).

42. Christoforidis, S., McBride, H. M., Burgoyne, R. D. & Zerial, M. The Rab5 effector EEA1 is a core component of endosome docking. Mol. Biol. Cell 19, 5327–5337 (2008).

43. Miller, S. E., Collins, B. M., McCoy, A. J., Robinson, M. S. & Owen, D. J. A SNARE-adaptor interaction is a new mode of cargo recognition in clathrin-coated vesicles. Nature 450, 570–574 (2007).

44. Pryor, P. R. et al. Molecular basis for the sorting of the SNARE VAMP7 into endocytic clathrin-coated vesicles by the ArfGAP Hrb. Proc. Natl Acad. Sci. USA 109, 827–832 (2012).

45. Reales, E., Sharma, N., Low, S. H., Fölsch, H. & Weimbs, T. Basolateral sorting of syntaxin 4 is dependent on its N-terminal domain and the API1B clathrin adaptor. J. Biol. Chem. 283, 1997–2006 (2008).

46. Huang, L., Kuo, Y.-M. & Gitschier, J. The pillid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. Nat. Genet. 23, 329–332 (1999).

47. Cai, H., Reinsch, K. & Ferro-Novick, S. Coats, Tethers, Rabs, and SNAREs work together to mediate the intracellular distribution of a transport vesicle. Dev. Cell 12, 671–682 (2007).

48. Christoforidis, S., McBride, H. M., Burgoyne, R. D. & Zerial, M. The Rab5 effector EEAI is a core component of endosome docking. Nature 397, 621–625 (1999).

49. Mizuno-Yamasaki, E., Rivera-Molina, F. & Novick, P. GTPase networks in membrane traffic. Annu. Rev. Biochem. 81, 637–659 (2012).

50. Gillingham, A. K., Sinha, R., Torres, I. L., Lilley, K. S. & Munro, S. Toward a comprehensive map of the effectors of Rab GTPases. J. Cell Biol. 193, 8901–8912 (2012).

51. Pérez-Victoria, F. J., Mardones, G. A. & Bonifacino, J. S. Requirement of the human GARP complex for mannose 6-phosphate-receptor-dependent sorting of cathepsin D to lysosomes. Proc. Natl Acad. Sci. USA 109, E9792–E9801 (2018).

52. Boyken, J. et al. Molecular architecture of the multisubunit homotypic fusion and vacuole protein sorting (HOPS) tethering complex. Proc. Natl Acad. Sci. USA 109, 1991–1996 (2012).

53. Bröcker, C. et al. Molecular architecture of the multisubunit homotypic fusion and vacuole protein sorting (HOPS) tethering complex. Proc. Natl Acad. Sci. USA 109, E9792–E9801 (2018).

54. Mallard, F. et al. Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. J. Cell Biol. 156, 653–664 (2002).

55. Bankaitis, V. A., Johnson, L. M. & Emr, S. D. Isolation of yeast mutants defective in protein targeting to the vacuole. Proc. Natl Acad. Sci. USA 83, 9075–9079 (1986).

56. Brickner, J. H. & Fuller, R. S. SOI1 encodes a novel, conserved protein that functionally interacts with the small GTPase RAB6 at the golgi complex and modulates the function of two TGN localization signals. J. Cell Biol. 139, 23–36 (1997).

57. Luo, W. & Chang, A. Novel genes involved in endosomal traffic in yeast revealed by suppression of a targeting-defective plasma membrane ATPase mutant. J. Cell Biol. 138, 731–746 (1997).

58. Dalton, L. E., Bean, B. D. M., Davey, M. & Conibear, E. Quantitative high-content imaging identifies novel regulators of Neol trafficking at endosomes. Mol. Biol. Cell 28, 1539–1550 (2017).

59. Kumar, N. et al. VPS13A and VPS13C are lipid transport proteins differentially localized at ER contact sites. J. Cell Biol. 217, 3625–3639 (2018).

60. Lang, A. R., Peter, A. T. J., Walter, P. & Kornmann, B. ER–mitochondrial functions can be bypassed by dominant mutations in the endosomal protein Vps13. J. Cell Biol. 210, 883–890 (2015).

61. Park, J. S. et al. Yeast Vps13 promotes mitochondrial function and is localized at membrane contact sites. Mol. Biol. Cell 27, 2435–2449 (2016).

62. De, M. et al. The Vps13p–Cdc31p complex is directly required for TGN late endosome transport and TGN homotypic fusion. J. Cell Biol. 216, 425–439 (2017).

Acknowledgements
We are grateful to D. Czernik for assistance with mass spectrometry analysis, U. Ries for technical support, S. Rizzoli for kindly providing the Matlab algorithm for examining the co-localization (all Göttingen), and K. Nakayama (Kyoto) for providing mRFP-Rab14 constructs. We also thank A. Stein and H.D. Schmitt (Göttingen) for comments on the manuscript; S.K. was supported by the Uehara memorial foundation.

Author contributions
S.K. carried out all experiments. S.K. and R.J. designed and discussed the experiments and wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-09617-9.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Journal peer review information: Nature Communications thanks Josef Rizo and the other anonymous reviewer for their contribution to the peer review of this work. Peer reviewer reports are available.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019