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Review

Functional homologies in vesicle tethering

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ABSTRACT

The HOPS multisubunit tethering factor (MTC) is a macromolecular protein complex composed of six different subunits. It is one of the key components in the perception and subsequent fusion of multivesicular bodies and vacuoles. Electron microscopy studies indicate structural flexibility of the purified HOPS complex. Inducing higher rigidity into HOPS by biochemically modifying the complex declines the potential to mediate SNARE-driven membrane fusion. Thus, we propose that integral flexibility seems to be not only a feature, but of essential need for the function of HOPS. This review focuses on the general features of membrane tethering and fusion. For this purpose, we compare the structure and mode of action of different tethering factors to highlight their common central features and mechanisms.

1. Introduction

Vesicles play a central role in eukaryotic cells, where they act as major vehicles for organic and inorganic compounds to be exchanged between the cellular compartments, the plasma membrane and the extracellular space. Thus, diverse transport vesicles connect organelles of the secretory and endocytic pathways, including vacuoles or lysosomes. A complex coordination of the cytoskeleton, motor proteins, vesicular coat proteins as well as Rab-GTPases, tethering factors, SNAREs and specific lipids is responsible for vesicle formation, stability, specific targeting and fusion. The most prominent organelle specific markers are Rab proteins. They are anchored to membranes and bind to various effectors, among them tethering factors [1–3]. These in turn seem to initially recognize incoming vesicles by specific Rabs. At the fusion site tethers then cooperate with membrane-bound SNAREs found on both vesicle and organelle membrane, and can promote their assembly. Subsequent tight folding of a ternary SNARE complex is then sufficient to promote bilayer mixing and delivery of the vesicle content to the target organelle [4].

Various families of tethering factors that localize on the membrane surface are essential for target-specific membrane fusion. Although they do not share any sequence homology and have considerably different structures, their function is similar. For example golgins act as homodimeric proteins and form long coiled-coils [5] whereas tethering factors of the CATCHR-family and the class C Vps (vacuolar sorting proteins) complexes HOPS (homotypic fusion and vacuole protein sorting) and CORVET (class C core vacuole/endosome tethering) form large multisubunit tethering complexes (MTCs) (reviewed in [6]). Our scientific focus is on the HOPS and CORVET complexes, which coordinate endosomal and lysosomal fusion processes. We therefore use the HOPS complex as prime example to discuss the need of conformational changes and protein flexibility during the process of membrane tethering and fusion. In addition, we will highlight and discuss the importance of structural flexibility in other known tethering events.

2. Architecture of HOPS

The HOPS complex consists of six different subunits, Vps11, Vps16, Vps18, Vps33, Vps39 and Vps41. Their sizes range from 79 to 123 kDa. Besides Vps33 (Fig. 1A), which belongs to the family of Sec1/Munc18 (SM) proteins, all other subunits share a similar domain architecture, each containing an N-terminal β-propeller and a C-terminal α-solenoid domain [7,8]. The other class C protein complex known, CORVET, shares four of the HOPS subunits, namely Vps11, Vps16, Vps18 and Vps33. Here Vps3 (in metazoans TRAP-1/Tgfbrap1) and Vps8 take the space of Vps39 and Vps41, respectively. We thus propose that the two complexes have a similar structure and common modes of action, but differ in the specificity

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for their binding partners, which define their function in endosomal trafficking.

HOPS and CORVET were initially identified in yeast [9–11], though the same complexes also exist in metazoan cells [12–14]. As almost all mechanistic studies were conducted with the yeast complexes, we will keep here to the yeast nomenclature.

Single particle negative stain EM data showed that the heterohexameric HOPS complex exists in different conformations resulting in elongated and more compact structures similar to an open and partly closed clamp ([34], Fig. 2A and E). There are no distinct intermediate states, indicating that the complex can take any conformational states between a 28 nm short and a 40 nm long extreme. The backbone of the complex formed by Vps11 and 18 is rather stiff whereas the head and tail comprising Vps16–Vps33–Vps41 and Vps39, respectively, appear to be flexible with possible hinge regions at the junction between these parts and the backbone. This results in a change of their inner distance between 7 and 14 nm and can be observed in the absence of any partner.

To get some more insight into the underlying structural mechanism different buffer conditions, HOPS variants and subcomplexes have been tested. Even though high salt and the introduction of different large tags on selected subunits increased HOPS integral stability, it did not reduce its flexibility significantly [34]. Vps16 and Vps33 can form a stable heterodimer localized in the head part of HOPS (Fig. 1B, 2D, H and R), and Vps11, Vps18 and Vps39 a stable heterotrimer (Fig. 2B and F). Both subcomplexes assemble to a heterohexamer of about 650 kDa only in the presence of Vps41 (Fig. 2A and E). Negative stain EM data showed that the Vps16–Vps33 dimer as well as the Vps11–Vps18–Vps39 trimer and the Vps18–Vps39 dimer are highly flexible (Fig. 2B–D and F–H). Corresponding to the complete HOPS complex, it was not possible to define fixed intermediate states. Interestingly, some of the hinges that are responsible for the smooth movement of the HOPS complex are undisturbed in the subcomplexes and localize within them (see also movies of [34]).

So far, there is very limited high-resolution structural information available from the single HOPS subunits. Computational analyses predict that their domain architecture (except Vps33) is similar to known vesicular coat proteins such as clathrin, the COP-II components, and coat nucleoporins [7,15–19]. However, only the structure of the β-propeller of Vps18 from *Saccharomyces cerevisiae* could be solved so far by X-ray crystallography [20].

Crystal structures of human Vps33 and its homologue from *Chaetomium thermophilum* (pdb ID: 4JC8 and pdb ID: 4BX8, respectively) clearly show that it differs from the other subunits and folds like a SM protein. SM proteins are implicated in binding to SNAREs [21–24] and were shown to exist in open and closed conformations with a hinge region between the N-terminal domain 1 and the C-terminal domains 2 and 3 ([25], Fig. 1A and D). When compared to other structures of SM proteins, the Vps33 structures are similar to the open conformations ([25,26], Fig. 1D). A model for the closed conformation of Vps33 from *C. thermophilum* was built based on the Munc18-1 syntaxin-1 complex, pointing to the possibility that the Vps33 domain 3 could exist in an open and closed conformation [27].

In addition, both proteins were co-crystallized with a fragment of Vps16 (pdb ID: 4BX9 and pdb ID: 4KMO, respectively) giving first insights into the interaction between HOPS subunits [27,28]. The Vps16 fragment comprises amino acids 642–736 for the human protein, and 505–834 for its homologue from *C. thermophilum*, which corresponds to the second half of their α-solenoid domain. The structure shows a relatively small interface between both subunits, which is mainly dependent on the contact made by the α4-helix of Vps16. Already the mutation of two distinct residues of Vps33 (A411D/H451D or A411D/L454E in Vps33 from *C. thermophilum*; K429D, Y438D/P or I441K in human Vps33) or Vps16 (A669/R725 in human Vps16) is sufficient to disrupt this interaction (Fig. 1A, [29]). It has so far not been possible to obtain high-resolution structural data of any of the other subunits or subcomplexes [28]. One explanation for that could be a possible intrinsic flexibility of subunits and a reduced stability of the single subunits in solution. The question now is whether biologically relevant binding partners might be able to stabilize the complex.

3. HOPS and interactors

3.1. HOPS recognizes small GTPases

Eukaryotic cells express various small, membrane-associated GTPases, so called Rab proteins that are involved in intercellular vesicular transport. They are rate limiting for vesicular fusion

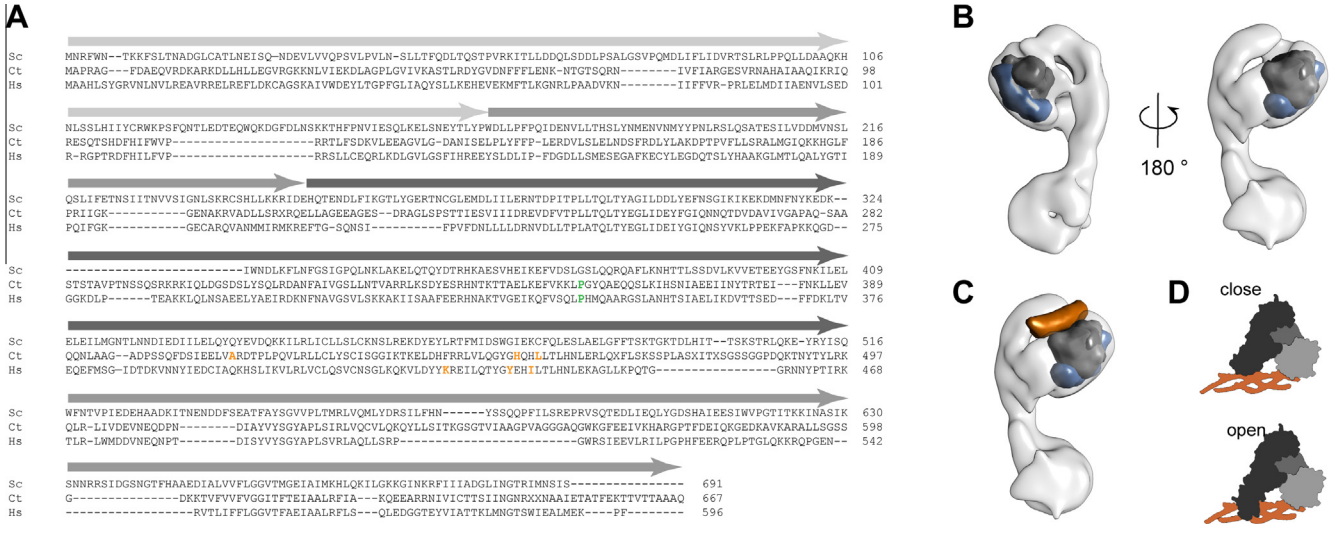

Fig. 1. Structural information on the HOPS complex. In (A) The domain structure of Vps33 is shown with an alignment of protein sequences from *S. cerevisiae* (Sc), *C. thermophilum* (Ct) and *H. sapiens* (Hs) as presented in [27] with domain 1 (light grey), domain 2 (grey), domain 3 (dark grey). Critical amino acids for Vps16 interaction are highlighted in orange, the hinge-proline [27] is labeled in green. (B) For Vps33 (grey) and Vps16 (blue) densities calculated from crystal structures (pdb-ID: 4KMO) were fit into the head part of the whole HOPS complex (Chimera, [156]). (C) The predicted position of the SNARE complex (brown, density calculated from pdb-ID: 1FSC) bound to Vps33 then adapts to the groove in the HOPS-head. (D) The open and closed conformation of Vps33 with the ternary SNARE complex is presented as modelled by [27]. The colour code for the domains refers to (A).

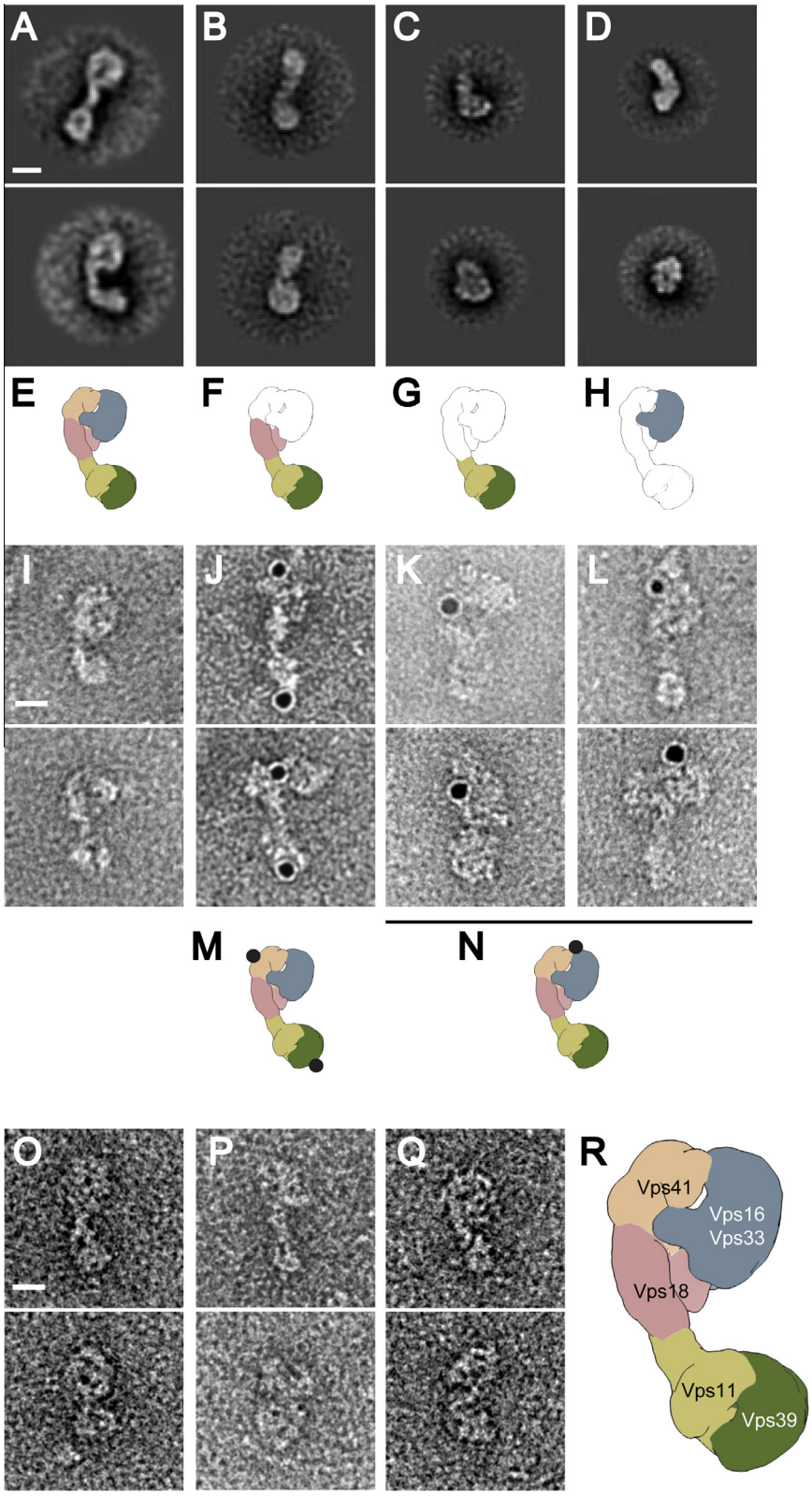

Fig. 2. Negative stain EM of different HOPS constructs, conditions and with known interactors. (A–D) The class sums of HOPS (A) and the subcomplexes, Vps39–11–18 (B), Vps39–11 (C), and Vps16–33 (D). Two extreme conformations are depicted in the upper and lower panel, respectively. (E–H) Models corresponding to class sums shown in (A–D) [34]. (I–L) Raw particle images of HOPS (I), HOPS-Ypt7 (J), HOPS-$H_{abc}(Q_a)$ (K), and HOPS-Ykt6(R-SNARE) (L). The respective interactors were immuno-gold labeled (black dots). Addition of diverse factors destabilizes the complex. (M-N) Models corresponding to raw particle images shown in (J–L). (O–R) Addition of lipids as PI(3)P (O), PI(4,5)P_2 (P) or cardiolipin (Q) has no effect on HOPS flexibility. Two extreme conformations are depicted in the upper and lower panel, respectively. (R) Subunit organization within the HOPS complex. All scale bars are 10 nm.

processes and – as recruiting factors for tethering proteins and their associated SM proteins - of essential need for the assembly of SNAREs, which drive lipid bilayer mixing. To date, 11 Rabs are known in yeast and about 60 in humans (reviewed in [4]). They function as molecular switches by cycling between the active GTP- and inactive GDP-bound form. The release of GDP and thereby the binding of GTP is catalyzed through the action of guanine nucleotide exchange factors (GEFs) (reviewed in [4,30]). Rabs associate reversibly with organelles via their C-terminal lipid prenyl-anchor [2] and allow, once activated, the specific recruitment of various effector molecules, like tethering factors, to organelle surfaces [1–3]. These can bridge the distance between vesicle and organelle membrane prior to SNARE-driven fusion [4]. The most prominent Rab on late endosomes and lysosomes is Rab7 (Ypt7 in yeast, [31]). A major role of Ypt7 is to support HOPS association with vacuole membranes during fusion [32], and only the presence of HOPS allows the fusion of Ypt7- and SNARE-containing vesicles [9]. Ypt7-GTP directly binds selectively to HOPS at two opposing ends, Vps39 and Vps41 ([10,33,34], Fig. 2J and M). The initial idea that Vps39 also functions as a GEF [10] was disproven by the identification of the Mon1-Ccz1 complex as the GEF of Ypt7 [35].

Phosphorylation of HOPS by the casein kinase Yck3 is required for its function in the AP-3 pathway [36–38]. Yck3 targets the ALPS (amphipathic lipid-packaging sensor) motif, an α-helical segment, within the N-terminal domain of Vps41 [38]. Consequently, phosphorylated Vps41 is then able to bind to the AP-3 complex (discussed below). *In vitro* phosphorylation of purified HOPS by Yck3 makes HOPS binding to Ypt7 strongly dependent on GTP [32,39], likely by lowering the intrinsic membrane affinity after phosphorylation of the ALPS motif. All these findings led to the hypothesis that HOPS bridges membranes by binding the activated (GTP) Ypt7 [34]. Although we expected that binding of the Rab would decrease the flexibility of HOPS, negative stain EM showed that this is not the case. Instead the overall conformation of the head and tail domains seem to change by a rearrangement of the respective subunits altering their relative distance ([34], compare Fig. 2I and J). It is possible that a Ypt7-induced conformational change results in the exposure of respective binding sites for other HOPS interactors (Fig. 3).

In human cells, Rab7 is also essential for the localization of the small GTPase Arl8 to phagosomes, and loss-of-function mutants in the HOPS subunits Vps39 or Vps41 lead to accumulation of Rab7 phagosomes. GTP-bound Arl8 physically interacts with HOPS via Vps41 in *Caenorhabditis elegans* and mammalian cells [40,41]. HOPS might also promote fusion between phagosomes and lysosomes by being recruited to Rab7-positive lysosomes as well as Arl8 containing lysosomes in higher eukaryotes [40]. Recent data cast doubt on the Rab7-HOPS connection in metazoan cells. Taking *Drosophila* as a model system to monitor Rab-interactions, Munro and colleagues detected only weak binding of HOPS to Rab7, whereas Rab2 was identified as the preferred interaction partner in pull-down assays [42]. The authors argue that Rab2, as well as Arl8, are highly conserved in higher eukaryotes, but got lost in budding yeast [42], which might have changed the Rab preference of the HOPS complex. Future studies have to unravel if the tethering mechanism promoted via Rab proteins is conserved between different species, and if different Rabs are involved in distinct substeps of this process.

3.2. HOPS recruitment of SNAREs

Subsequent to tethering the fusion of donor and acceptor membrane has to be catalyzed. This requires the assembly of a ternary SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) complex composed of subunits from both opposing membranes. SNARE-mediated fusion requires four complementary domains termed R, Q_a, Q_b, and Q_c, depending on the

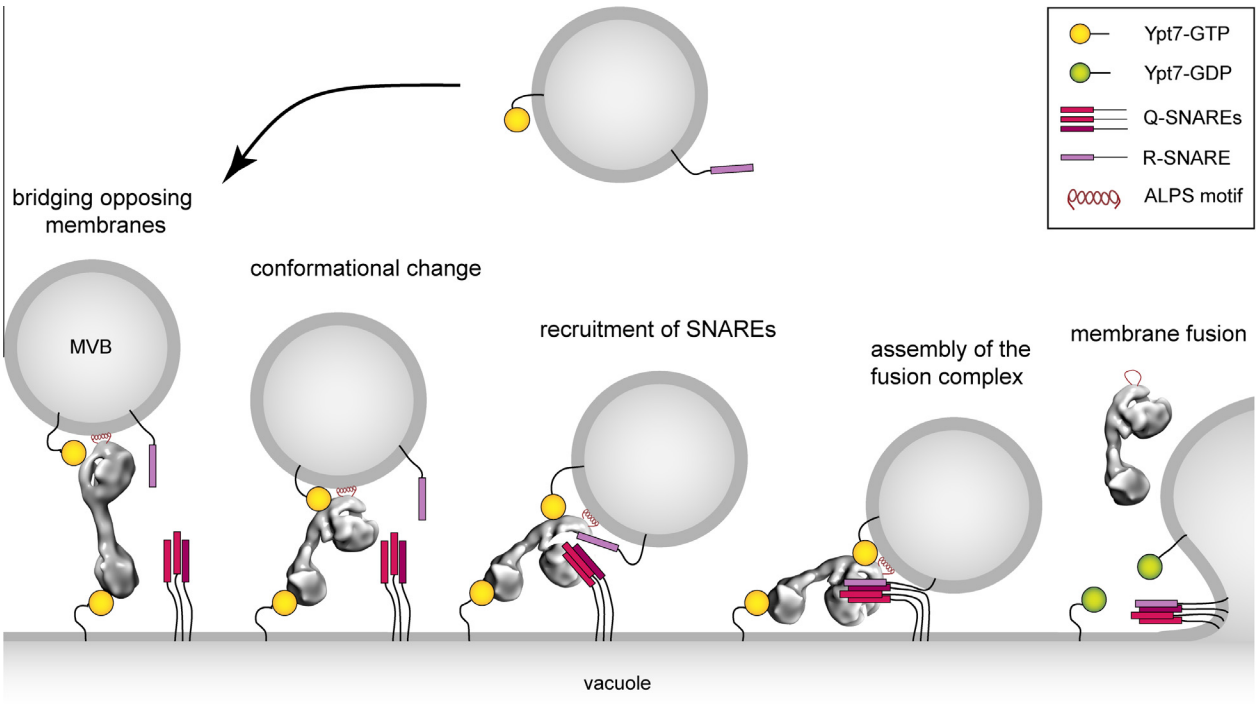

Fig. 3. Model of HOPS flexibility during the tethering process in yeast. HOPS assembles at the vacuole [157]. It can bind on Ypt7-GTP positive and highly curved membranes of multivesicular bodies (MVB). In its stretched conformation it could bridge Ypt7 positive membranes selectively, connecting late endosomes and vacuoles/lysosomes. Structural bending could allow guiding the vesicle closer to the target membrane. In the presence of Q-SNAREs HOPS can bind these to the head part and the vesicular R-SNARE is brought close to the SNARE binding site. HOPS catalyses the formation of the ternary SNARE complex. SNARE assembly induces vesicular fusion with the target membrane and HOPS can dissociate from this side supported by Rab-deactivation (Rab-GDP).

presence of Arg or Gln within the central '0-layer' of the SNARE domain [43]. The assembly of the membrane-bound proteins to a quarternary complex is supposed to function in *trans* like a zipper that eventually results in lipid bilayer mixing [43–46]. There are 25 SNAREs annotated in *S. cerevisiae*, 36 members in humans and 54 variants in *Arabidopsis thaliana* (reviewed in [43]). Like Rab-GTPases SNAREs vary depending on the sort of vesicle they localize to [47,48]. It could be shown that yeast HOPS associates with SNAREs [32,49], and also prevents the disassembly of a formed SNARE complex [50]. Preincubation of HOPS with individual vacuolar SNAREs can inhibit binding to the SNARE complex, suggesting that its affinity to the single subunits might be higher than for the assembled ternary complex [51]. The SM-like Vps33 subunit is responsible for not only binding but also mediating SNARE assembly and stabilizing the SNARE complex [50–52]. In contrast to class I SM proteins such as the metazoan Munc18-1, the class II SM protein Vps33 does not bind to the Q_a-SNARE domain [53], but only the ternary Q- and quaternary Q/R-SNARE complex [51,52]. Human Vps33A as well as the *C. thermophilum* Vps33 lack the pocket for binding the N-terminal peptide of the Q_a-SNARE [27,28]. Most likely this is due to the position of domain 1, and amino acids in the binding pocket are different to the one found in rat Munc18 [27]. With the help of structural information it was possible to model SNARE proteins into the *Ct*Vps33 binding pocket of the open and closed Vps33 domain 3 ([27], Fig. 1C and D). The core helices of domain 3, 3a and 3b, are conserved between different eukaryotic species like unicellular flagellates, fungi, insects and vertebrates including the residues that bind to the closed conformation of the remaining SNARE including the H_{abc}-domain [22,27,54]. Pull-down experiments, vesicular fusion assays, and negative stain electron microscopy data provided evidence that also yeast HOPS interacts with the Q_a-SNARE Vam3, assembled Q-SNAREs consisting of the SNARE domains of Vam3 (Q_a), Vam7 (Q_c), and Vti1 (Q_b), and the R-SNARE Ykt6 [55]. All SNAREs are required for fusion of vesicles with the vacuole membrane [56–58]. Interestingly, HOPS binds efficiently to the N-terminal H_{abc}-domain of Vam3, but not the SNARE domain [59]. All interactions occur in the large head part of the complex, where Vps33 and Vps16 are localized ([34], Fig. 2K, L and N, Fig. 1C). The binding of the Vam3 H_{abc}-domain seems to happen selectively via Vps16, whereas Vps33 binds the assembled Q-SNAREs and the quarternary SNARE complex [52,59]. *In vitro* binding of SNARE components to HOPS resulted in structural destabilization rather than reduced flexibility (Fig. 2K and L). How HOPS binding to the SNARE complex and the individual H_{abc} domain are coordinated is not yet clear. We suggest that HOPS serves as an assembly platform to promote SNARE function in fusion.

3.3. Further interactors of HOPS

Beside the Rab GTPase Ypt7 and SNARE proteins additional interactors of the HOPS complex could be identified. One prominent example is the Apl5 motif of the AP-3 delta subunit, which directly binds Vps41 [60,61]. The interaction depends on the conserved hydrophobic residues L100 and L101 within Vps41 [38]. This points toward HOPS function in the recruitment of AP-3 coated vesicles to the vacuole [62]. Whether AP-3 binding to HOPS alters HOPS flexibility is not yet clear, but unlikely, considering the previous experiences. Furthermore, yeast HOPS specifically binds certain lipids [49]. Extensive screens have shown that the complex preferably binds phosphoinositides and acidic lipids [49,63,64]. The Vps41 subunit contains an helical ALPS motif, which is likely present within a loop region the putative β-propeller, and which is known to bind to highly curved membranes [65,66]. This feature is also known from Golgins [67]. As long as the membrane is highly curved, the ALPS motif is hidden in the lipid bilayer. At the less curved vacuolar membrane, the Vps41 ALPS motif is exposed and can be phosphorylated by the casein kinase Yck3. Conformational changes now allow Apl5-mediated binding of AP-3 vesicles [6,38]. The binding of HOPS to AP-3 is not dependent on the phosphorylation state, but the phosphorylation of the ALPS motif most likely prevents reverse binding to the lipid bilayer. During the tethering of AP-3 vesicles HOPS needs to be as flexible as in recruiting Ypt7-positive late endosomes. Therefore, it is likely that lipids and AP-3 binding do not decrease the flexibility of HOPS. Electron microscopy images indeed confirmed that the addition of lipids had no significant effect on HOPS flexibility (Fig. 2O, P and Q). Further studies have to shed light on the mechanism in detail.

We would like to note that some studies suggest a role of Vps41 independent of its function within HOPS. Yeast Vps41 was also identified on Golgi-derived AP-3 vesicles [60]. More recently, human Vps41 was shown to localizes to a vesicle population named LAMP carriers that eventually fuse with late endosomes or lysosomes [68]. Moreover, metazoan Vps41 can self-assemble into a putative coat, and might thus provide the missing clathrin-like layer that cooperates with AP-3 adapter complex [69]. Future studies will be necessary to address the physiological relevance of the observed lattice formation of Vps41.

4. Molecular function of other tethering factors

Based on the knowledge we gained on the HOPS complex so far, we conclude that binding of lipids, Rab and SNARE proteins as well as its flexibility we observe *in vitro* might also be of importance during the tethering process *in vivo*. Other tethering factors probably exhibit similar features to allow vesicle recruitment to the target membrane, but since they differ in their structure, the underlying mechanisms might be different from the one observed for HOPS.

4.1. Function of coiled-coil tethering factors (CCT)

The most prominent coiled-coil tethers are golgins, rabaptin or GM130, as well as giantin. Although they belong to the same class of proteins their mode of action is quite different (reviewed in [70]).

Golgins are composed of very long homodimeric coiled-coil dimers. For the yeast Uso1, this length extends about 150 nm [71]. Its human orthologue p115 can bind to giantin, extending its length about 200–250 nm, based on sedimentation analysis and structural models [72–74]. Golgins are anchored to the Golgi-membrane via the interaction of their GRAB/GRIP domain with Arf or Arl GTPases ([75,76], Fig. 4A). It was also shown that Rabs like Rab1 can bind coiled-coil tethers [77]. Some CCTs even contain multiple binding sites for small GTPase [78–80]. Vice versa, one Rab can also bind to different golgins, although this spectrum is rather limited [81–83]. By forming a meshwork, they are proposed to prevent vesicles from escaping while trafficking between the cisternae of the Golgi stack. It has been proposed that sequential binding events then bring the vesicle close to the target membrane to permit SNARE binding and ternary complex assembly [80]. The golgin GMAP-210 uses a different feature for the tethering process [67]. It is also anchored to the membrane via its GRAB-domain that binds Arf-GTP, but seems to bind vesicles via an APLS motif on the opposite end of the molecule, which has a preference for highly curved membranes ([65], Fig. 4A, introduced in Section 3.1). Thus, GMAP-210 needs to undergo a conformational change to recruit vesicles to the Golgi membrane.

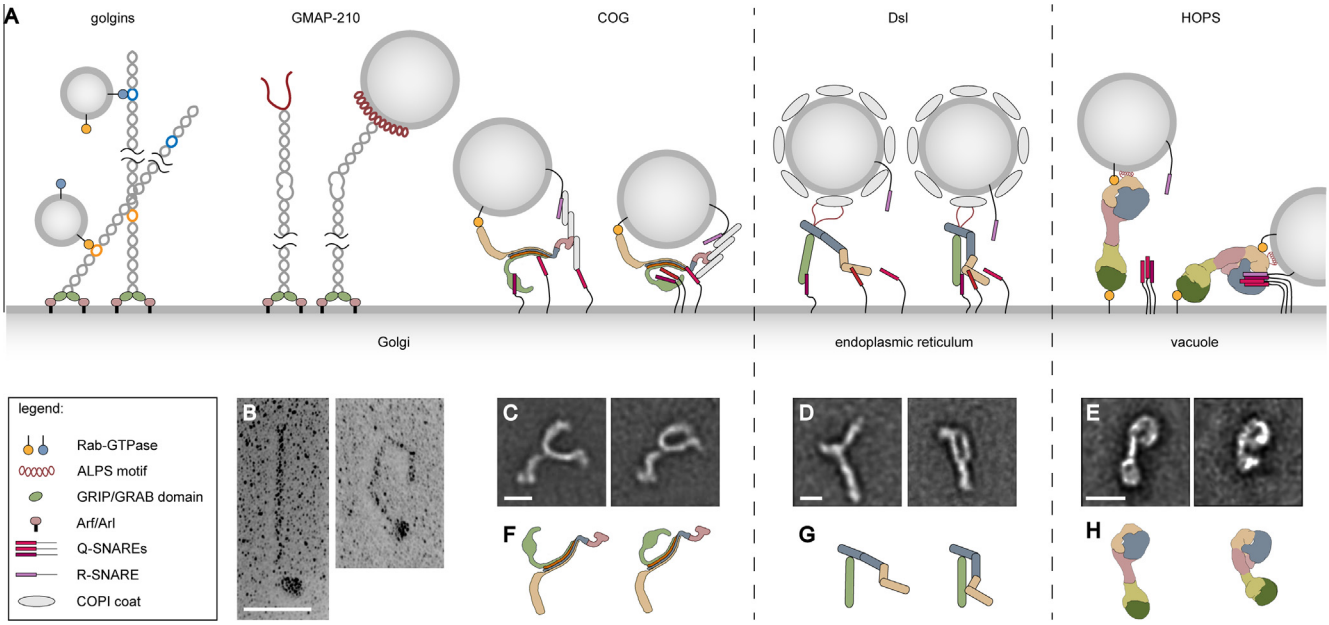

Fig. 4. Intrinsic flexibility of different tethering factors. (A) Simplified models for the influence of flexibility during membrane tethering. Golgins sequentially recruit vesicles to their specific Rab binding sites (orange and blue circles) whereas GMAP-210 uses hinge regions to guide vesicles close to the Golgi (figures adapted from [70]). There they can be taken over e.g. by COG (Cog1 blue, Cog2 brown, Cog3 sandy brown, Cog4 green, Cog8 pink, Cog5–6–7 light grey based on [116]). COG is able to bind Q- and R-SNAREs with Cog4, 6 and 8 as well as the Rab Ypt1 at Cog2/3 to tether membranes [87,126]. The Dsl complex (Sec39p (green), Dsl1p (blue) and Tip20p (sandy brown)) uses flexibility to recruit the SNARE proteins Use1 and Sec20 at the ER membrane and COPI on coated vesicles via its flexible loop. HOPS (subunits are colored as described in Fig. 2R) bridges opposing membranes via Ypt7 and brings them close enough together to catalyze SNARE complex assembly. (B–E) The proposed mechanisms are partially based on rotary shadowing or negative stain electron microscopic data that show the different tethering factors Cog1–2–3GFP-4 (C, [116]), Dsl1–Sec39 (D, [132]) and HOPS (E, [34]) in either open (left) or closed (right) conformation. Scale bars are 50 nm. (F–H) Models corresponding to the class sums depicted in (C–E).

Sequence analysis show that the coiled-coil domains of golgins are not as continuous as predicted, but are interrupted by unstructured parts that allow strong bending and kink formation [84]. This was also nicely shown for Uso1 by rotary shadowing electron microscopy (Fig. 4B, [71]). At the N-terminal head domain an armadillo-like, tether repeat, α-helical tripod motif interrupts the structure, which can also bind Rab-GTPases [85,86]. These features might also apply to other golgins. Taken together, golgins seem to catch GTPase-coated vesicles at long distance with the help of multiple binding sites along their coiled-coil domains. These can either be achieved by the same or by coiled-coil domains of neighboring golgins. Vesicles might get close to the Golgi-membrane by bending of the golgin-rod [71,84]. At the Golgi, the vesicle will be released from golgins by either changes in membrane curvature after fusion [66], or by release from the Rab with the help of a GAP protein. There they could be taken over from other tethering factors like the COG complex that catalyzes SNARE complex formation (see also Chapter 4.2; Fig. 4 A, C and F; [87]).

To complete the section on coiled-coil tethering factors we will finally focus on Rabaptin-5, which binds Rab5-GTP via its C-terminal part, and the Rab5 GEF Rabex-5 by its C2-1 domain, and the Rab4 and Rab8 via its N-terminal domain [3,88–93]. Since Rab4 is required for the transport of endosomes to cell-surface, and Rab5 is needed for endocytosis, rabaptin-5 likely coordinates between both pathways [89]. Interestingly, Rabaptin-5 also contains a large linker between its two predicted coiled-coil domains [93]. This linker could not be crystallized and is likely very flexible. Movement of this part could guide Rab-loaded vesicles to the target membrane. Rabaptin-5 as an effector of Rab5 forms a complex with Rabex-5, the GEF of Rab5. Upon binding to Rab5, the C2-1 domain of Rabaptin-5 switches from the V-state to an elongated form, which facilitates access of the bound GEF Rabex-5 to Rab5 and promotes further Rab5 recruitment [93]. Future studies have to show, if conformational changes in other tethering factors also recruit GEF-proteins to enhance Rab binding in the active state [93].

4.2. Function of other MTCs than HOPS (CATCHR families)

The second group of tethering factors is formed by the MTCs. Known representatives are composed of three (Dsl) to eight (exocyst) subunits with a molecular mass ranging from 250 kDa to 1 MDa. In contrast to the dimeric coiled-coil tethers they are spanning distances of less than 50 nm and therefore most likely rather control specific vesicle recognition and membrane fusion than their long distance recruitment. The known MTCs are grouped into the CATCHR (complex associated with tethering containing helical rods) family and the already discussed class C tethering factors HOPS and CORVET. For a long time the TRAPP complexes, which function as GEFs, have been discussed to also exhibit tethering activity, though it is likely that their proposed tethering activity is due to the activation of their target Rab, which then cooperates with its tether such as p115 [4,94,95].

Members of the CATCHR family (also known as DCGE subfamily), as the Dsl (transport from Golgi to endoplasmic reticulum (ER)), COG (intra-Golgi trafficking), GARP (endosomes to the trans-Golgi network) and exocyst complexes (post-Golgi vesicles to the plasma membrane), have originally been clustered due to their sequence homology [96,97]. Later, structural data demonstrated that they share common tertiary structure motifs [98,99]. Until today, (partial) structures of different subunits have been published containing one (Cog2), two (Sec15, Exo84), three (Sec6) or four (Exo70) helix bundle domains [82,98,100–105]. Another two subunits of the Dsl complex, Tip20 and Dsl1, have similar structures like subunits of the exocyst complex [99].

The model for the octameric 750 kDa exocyst complex (composed of Sec3, 5, 6, 8, 10, 15; Exo70 and Exo84) resembles a Y-shaped structure, with a central bundle of rods forming a

scaffold and arms, which bridges different GTPase-containing membranes [106]. The exocyst complex interacts with the GTP-bound form of two small GTPases, Rho3 and Cdc42 [107,108]. The single subunits consist of tandem repeats of α-helix bundles [101–106]. Interestingly, it can form two stable subcomplexes. The subunits Exo70 and Sec3 are anchored to the plasma membrane via Rab and Rho GTPases and the phosphoinositide PI(4,5)P_2, whereas the hexameric subcomplex of the other six subunits is thought to bind via Sec4 to the vesicle [109–111]. Therefore, it was postulated that assembly of both subcomplexes to the holocomplex leads to tethering of secretory vesicles and control of SNARE assembly [112–114]. The exocyst Sec6 interacts with Sec9 Q_{bc}-SNARE [113]. The tethering mechanism of exocyst thereby seems to be similar to the HOPS-recruitment of the Vam7-(Q_c)SNARE, even though Vam7 is not part of the HOPS complex. For exocyst, the interacting class II SM protein Sec1 may fulfill the same function as Vps33 in HOPS [27,114]. Interestingly, the Exo70 subunit exhibits flexibility [102]. Its domain 1 can bend up to 14,6° relative to domain 2 and some residues in loop regions between helices could not be resolved in the crystal structure (residues 63–72, 224–235 and 528–543) and are likely disordered or flexible. How these features are of functional relevance has to be examined in the future.

The COG (conserved oligomeric Golgi) complex has a central role in intra-Golgi transport. With its three flexible legs COG seems to be more like a coat, but could also span membranes with its length of about 40 nm ([115,116], Fig. 4A, C and F). Depending on the species, the octameric core complex has a size of 590–750 kDa [117]. While the subunits Cog1–4 form a stable functional unit with their absence leading to lethality [96,118–122], a Cog5–8 quadruple knockout caused no severe growth defect in yeast [96,116]. The expression of single subunits failed for all but for Cog2. Only a partial crystal structure could be obtained for human Cog4 (residues 537–785; [98]) and an NMR structure of yeast Cog2 (residues 109–262; [100]). In contrast, coexpression of Cog2–4 and Cog1–4 gave suitable amounts of stable monodispersed complex for analysis via negative stain electron microscopy [116], which showed both complexes forming a Y-shape structure with moving arms (see also Fig. 4C). The highest flexibility can be found in the joints linking these arms. Mapping of the C- and N-termini of each subunit by fusion to GFP-protein resolved that one arm is formed by the C-terminus of Cog4 and the second one by the C-terminus of Cog3. Remarkably, in the third arm the N-terminal parts of Cog1–4 overlap in a 10 nm segment without increased thickness compared to the other arms. This indicates that they most likely form a bundle of antiparallel α-helices ([44], Fig. 4F). Cog8 seems to connect Cog1–4 with the Cog5–8 network, though it has not been possible to reconstitute the octameric complex (Fig. 4A and F). Lees at al. [116] mention that the human Cog2 sequence is significantly longer than its yeast homolog, and thus predict that the shape of human COG tetrameric complex is rather H-shaped. So far, detailed structural data of most parts are still missing, but shared homology on the ternary structural level could be found with exocyst.

Initially, COG was localized to the rims of the Golgi cisternae as well as to vesicular structures of the cis- and trans-Golgi network [84,96,115,123–125]. COG is important for the retrograde intra-Golgi trafficking, but may also function as a tether in trafficking between Golgi and endosomes, and anterograde ER-to-Golgi transport [115,118,126–129]. Also for COG some essential interaction partners could be identified. Yeast Cog2 interacts with γ-COPI in a yeast-two-hybrid assay [126], while antibodies to human Cog3 co-precipitate β-COPI [82,127]. The COG complex also interacts with SNAREs; Cog4 binds the cis-Golgi t-SNARE Sed5 and enhances the stability of intra-Golgi SNARE complexes [87]. Cog4 also interacts via its N-terminal part with syntaxin-5 partner Sly1, a member of the Sec1/Munc18 (SM) protein family [130]. The mode of action is most likely comparable to the one proposed for exocyst, where the flexibility could be a feature to facilitate the recruitment of its interactors.

In comparison to the previously described tethering complexes, the Dsl complex (dependence on SLY1–20) is with 250 kDa the smallest of the known MTCs. It consists of three subunits Tip20 and Sec39/Dsl3, which are most likely bridged by Dsl1 based on the interaction of the CATCHR domains from Dsl1 and Tip20 [131–133]. Dsl also interacts with the COPI coat of Golgi-derived vesicles by Dsl1 binding the δ-COP [134] and α-COP [135] subunits of the COPI vesicle coat. Sec39 and Tip20 bind the ER-SNAREs Use1 and Sec20 [99,132,136]. Interestingly, Tip20 binds the regulatory part of Sec20, but not the SNARE-motif, which undergoes dramatic conformational changes during SNARE assembly. The Dsl complex interacts with ER standing Q-SNARE proteins and allows formation of a stable heptameric complex, Tip20–Dsl1–Sec39–Use1–Sec20–Ufe1–Sec22 [131,132,137,138]. This assembly is dependent on the whole intact Dsl complex. Together with Sly1, it then seems to chaperone the assembly of a SNARE complex, thus presumably facilitating membrane fusion. Electron microscopy data showed that the Dsl complex has a stiff, rod-like conformation with flexible arms [132], Fig. 4D and G). Hughson and colleagues were able to obtain structural data for parts of Dsl1 and Dsl1 in complex with Sec39 as well as Tip20. The whole complex mainly consists of α-helices. Crystallographic experiments unraveled that Dsl1 is organized like other MTC subunits as Exo70 or Tip20 [98,100–105]. It interacts with Sec39 via two helices. Destruction of the interface affects cell viability, whereas interference of the Dsl1–Tip20 interface had only mild effects [99,131,132]. Dsl1 contains a region that is not well-folded (*S. cerevisiae* residues 378–488, *Kluyveromyces lactis* amino acids 367–423, [99,132,139]), and does not result in interpretable electron density. Since the region was previously described to bind to vesicular coats [135,140,141], it is interpreted to catch COPI vesicles like a "lasso", being able to extend for 10 nm or more (Fig. 4A). Beside this feature, Dsl1 also shows flexibility between amino acids 333 and 355. This part resembles a kind of hinge region, which was nicely visualized by single particle electron microscopy ([132], Fig. 4D). The position of Dsl1 N-terminal part varies dramatically at its position between open and closed conformation, and it has been suggested that the "lasso" region could influence this movement upon COPI binding. Since Tip20 and Sec39 do not interact directly, it is likely that the movement of both Dsl1 domains is physiologically relevant in the functional cycle during vesicle tethering. There are additionally hints that also the Dsl1–Tip20 linkage might be bendable [99]. Although Exo70 and Tip20 contain a similar architecture, they lack these flexible regions and appear to be comparably stiff [101–103,132]. Forming a tower-like structure of approximately 20 nm in height, the Dsl complex could bridge up to 30 nm by extension of the flexible loop. The movement of Dsl1 N-terminal part (amino acids 1–339) with Tip20 however is probably needed to recruit Sec20 to catalyze SNARE complex formation. Thus, Dsl flexibility is essential for its function as a tethering complex.

The fourth CATCHR complex, GARP (Golgi-associated retrograde protein), is composed of four subunits, Vps51, 52, 53 and 54, with a size of 125–320 (Vps51) or 700–1700 amino acids (Vps52, 53, 54) [142–144]. The 360 kDa holocomplex interacts via Vps52 with the Rab Ypt6-GTP and with Vps53 with Arl1-GTP [145–147]. Vps51 specifically binds the H_{abc} domain of the t-SNARE [143,146]. For human Vps53 and Vps54 it was found that they can also bind the SNAREs Syntaxin6, Syntaxin16 and VAMP4 [148]. The single subunits contain alternating short coiled-coils connecting α-helical stretches [96,142,143,146,149–151] with homology to other CATCHR members as Dsl, COG or exocyst [96,97], and their N-terminal regions are responsible for complex assembly

[146,148,150,152,153]. GARP is thought to form a core with four arms built of the C-termini interacting with Rab or SNARE proteins [144]. For *S. cerevisiae* Vps53 [154] and *Homo sapiens* Vps54 [150] crystal structures could be resolved, resembling α-helical bundles with similarity to the D- and E-like domains of exocyst Sec6 [104]. Structure predictions for Vps51 and 52 also suggest this CATCHR-fold [155]. Due to this one proposes a function similar to Dsl and COG complex [144].

5. Concluding remarks

Although being structurally quite different tethering factors have a lot in common. Some subunits form active complexes, while others seem to have rather regulatory functions, since their deletion only mildly impairs the targeted transport pathway (e.g. COG; [96]). They all specifically bind small GTPases, mostly Rabs, in their GTP form, sometimes as a prerequisite to interact with Arl GTPases, and may also recruit GEFs. This could allow selective binding of GTPases by controlling their affinity based on the nucleotide status. At least for HOPS it was shown that the Rab Ypt7 affects the area of the complex that it binds to. This could cause disassembly of the complex, but might theoretically also allow binding of the equivalent GAP. In principle, this could deactivate the Rab-GTP to prevent its rebinding to the tethering factors once the complex has been released. All MTCs are also able to bind SNARE proteins and for some the catalytic effect on formation of the ternary SNARE complex could be shown. For HOPS, it was possible to show that structural flexibility is important for binding SNARE components [27].

Single subunits of MTCs are very often unstable or cannot be expressed, whereas expression of subcomplexes is possible for some of them. Interaction between subunits depend very often only on a few amino acid residues (see HOPS Vps33–Vps16, Dsl Dsl1–Sec39). Although it is yet unclear if they form a functional unit on their own, for some tethering factors stable subcomplexes have been identified. For instance, HOPS falls apart into Vps16–33 and Vps39–11–18, once Vps41 has been depleted, and for exocyst tethering is proposed to be catalyzed by the assembly of the hexameric complex. There is still no evidence for increased affinity amongst the subcomplexes, once they are bound to Rabs, coat proteins or assembled SNAREs. At least for the HOPS complex, binding of any other protein tested had exactly the opposite effect. Even though the different subcomplexes could reflect possible dynamics of the HOPS complex during the fusion cycle, we currently consider subcomplexes as intermediates in the assembly of the holocomplex with no functional role on their own.

There are different modes of intrinsic molecular flexibility. On the one hand flexible loops like the "lasso" found in Dsl1 could be useful to interact with potential binding partners such as coat proteins [99,132,135,139–141]. This is suitable in some cases since most tethering factors are already anchored at least on one side, limiting their ability to move towards their opposing target. It has to be studied whether some of the flexible loops found in other tethering factors might also be directly responsible to recruit interactors.

A second feature are hinge regions within complexes and subunits. Here we distinguish between the ones functioning at rather local/short distance and the ones causing large global movements. The first ones resemble clamp-like movements within or between single subunits. Opening up of clefts may allow binding of SNAREs or Rabs as found for HOPS complex. These movements may allow for the accessibility of binding sites. However, an induced flexibility/instability of the complex upon binding could also ease the release of associated factors, once the tethering process continues. One could think of the tethering factors as a flexible platform that allows for binding of SNAREs to catalyze their assembly, whereas it is released after that to promote the progression of the fusion process.

As already mentioned some of the observed movements cause quite large, global conformational changes. This could be observed for almost all tethering factors. Although it is not likely, it cannot be ruled out that some of these effects are caused by the lack of components, binding partners or artifacts caused by the detection method (in most cases negative stain electron microscopy). However, such features could nicely explain the fishing and recruitment of vesicles to the target membrane. The accordion-like shape of Uso1 and structural features of other golgins allows strong bending and kink formation. Movement of arms as observed for the Dsl and COG complexes could allow assembly of components into close vicinity. Smaller movements as observed for HOPS (Fig. 4E and H) could catalyze SNARE assembly and membrane fusion.

In summary, structural flexibility is not a mere side effect, but of essential need during membrane tethering. This is supported by the fact that induced rigidity causes lack of function [34]. All of the known tethering complexes show one or more of the mentioned features, which could explain how they reach for partners, bind them selectively, recruit them to the potential fusion locus, catalyze the formation of fusion complexes, and possibly dissociate to give way for subsequent membrane fusion. Although extensive work was done to obtain structural data and information on interactors, to date models for the fusion process are incomplete and time-resolved experiments are missing. It will be very exiting to unravel the detailed mechanisms in the future.

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