**Discussion**

Recruitment and function of the Rvs complex has been studied in this work, and several existing models for membrane scission have been tested. We propose that Rvs is recruited to endocytic sites via interactions between the Rvs BAR domains and invaginated membrane, and that SH3 mediated protein-protein interactions are required for efficient recruitment of Rvs. We found that arrival of Rvs at the membrane invagination scaffolds the membrane and prevents membrane scission. WT invagination lengths depend on recruitment of a critical number of Rvs molecules. Both timing and recruitment efficiency appear crucial to Rvs function.

**BAR domains sense *in vivo* membrane curvature and time recruitment of Rvs**

The curved structure of Endophilin and Amphiphysin BAR dimers (Peter et al., 2004; Mim et al., 2012) has supported the idea that Rvs is recruited by its membrane interaction, although it has not been conclusively shown that yeast BAR domains interact with endocytic sites via membrane curvature.

In the absence of membrane curvature- in *sla2*Δ cells- BAR-GPA domains do not localize to endocytic sites (Fig.3b). This demonstrates for the first time that the BAR domain senses and requires membrane curvature to localize to endocytic sites. BAR-GPA has a similar average lifetime at endocytic sites as full length Rvs167 (Fig5b). However, time alignment with Abp1 shows that there is a delay in the recruitment of BAR-GPA (Fig5b). Sla1 moves inwards at a slower rate in *bar-gpa* cells, so it takes longer for the membrane in these cells to reach the same invagination length as in WT. We propose that Rvs recruitment is timed to specific membrane invagination length- therefore to a specific membrane curvature- accounting for the delay in recruitment. The timing of recruitment is therefore provided by the BAR domain.

**SH3 domains allow efficient and actin independent recruitment**

BAR-GPA accumulates to about half the WT number (Fig.5b), even though the same cyto-  plasmic concentration is measured (Fig5 supplement), indicating that loss of the SH3 domain decreases the efficiency of recruitment of Rvs. In *sla2*Δ cells, full-length Rvs167 forms patches on the membrane (Fig.3b). Since BAR-GPA does not localize to the plasma membrane in *sla2*Δ cells, localization of the full-length protein must be mediated by the SH3 domain. That full-length Rvs167 is able to assemble and disassemble at cortical patches in *sla2*Δ cells without the curvature- dependent interaction of the BAR domain (Fig.3b, supplement?) indicates that the SH3 domain can mediate both the recruitment and disassembly of Rvs at endocytic sites. In *sla2*Δ cells treated with LatA (Fig.3b), both membrane curvature and actin-interacting proteins are removed from endocytic sites. Full-length Rvs167 in these cells still shows transient localizations at the plasma membrane: the SH3 domain is able to localise the Rvs complex in an actin and curvature independent manner.

**Loss of SH3 domain disrupts endocytic actin network dynamics**

In WT cells, the Abp1 and Rvs167 fluorescent intensities reach maxima concomitantly (Fig5b), and the consequent decay of both coincide. Coincident disassembly indicates that upon vesicle  scission, the actin network is immediately disassembled. Membrane scission occurs  around the intensity peak of the two proteins (ref Wanda, Andrea). This coincident peak is lost in *bar-gpa* cells: BAR-GPA average fluorescent intensity peaks several seconds after Abp1 intensity starts to drop, and the decay of  Abp1 is prolonged, taking nearly double the time as in WT. Although it is not clear what the decoupling of  Abp1 and BAR-GPA peaks means, the changes in Abp1 dynamics suggests a strong disruption of the actin  network dynamics.

**Rvs acts as a membrane scaffold preventing membrane scission**

Invaginations in *rvs167*Δ cells undergo scission when the Sla1 centroid has moved about 80nm (Fig.1f), compared to the WT lengths of 140nm. This shows that enough forces are generated at 80nm to cause scission. Since invagination lengths of *rvs167*Δ cells are increased by overexpression of the BAR-GPA domains (Fig.6), we think that localization of Rvs BAR domains to the membrane tube stabilizes the  membrane (Boucrot et al., 2012; Dmitrieff and Nedelec, 2015). This allows the invagination to grow until actin polymerization produces enough forces to sever the membrane. The requirement for Rvs scaffolding cannot be removed by reducing turgor pressure (Fig.6 supplement? or 7?),  so the function of the scaffold is not to counter turgor pressure. There is a limit to the stabilizaiton by BAR domains: in diploid strains with 4 copies of each RVS gene, the same amount of actin is recruited before scission. The invaginations lengths are the same as in the other strains even though more Rvs is recruited. It is possible that the nature of the Rvs complex interaction with the membrane changes after a certain amount of Rvs is recruited.

If enough forces are generated at 80nm, why is scission efficiency decreased in *rvs167*Δ compared to WT? Forces from actin may be at a threshold when the invagination is at 80nm. There could be enough force to sever the membrane, but not enough to sever reliably. The Rvs scaffold then keeps the network growing to accumulate enough actin to reliably cause scission. Controlling membrane  tube length could also be a way for the cell to control the size of the vesicles formed, and therefore the amount of cargo packed into the vesicle.

**What causes membrane scission?**

We looked for changes in the dynamics of Sla1 and Rvs167 that would indicate a scission defect in various mutant strains: longer invaginations than in WT, so Sla1 centroid movements of over 140nm, and a bigger inwards jump of Rvs167 centroid, indicating that a longer invagination has been cut. In *vps1Δ* cells, no major changes are seen in Sla1 or Rvs167 dynamics. We conclude that even if Vps1 is recruited to endocytic sites, it is not necessary for Rvs localization or function, and is not necessary for scission.

In the lipid hydrolysis model, synaptojanins hydrolyze PIP2 molecules that are not covered by BAR domains, resulting in a boundary between hydrolyzed and non- hydroplyzed PIP2. Interfacial forces generated at this lipid boundary causes scission (Liu et al., 2006). Deleting synaptojanins Inp51 and Inp52 should increase invagination lengths if scission was driven by lipid hydrolysis. Sla1 and Rvs centroid dynamics shows that deletion of neither Inp51 nor Inp52 result in scission delay. In *inp51*Δ cells, Rvs assembly is slightly slower than that in WT: Inp51 could play a role in Rvs recruitment. In the *inp52*Δ strain, about 12% of Sla1-GFP tracks retract, this could suggest a moderate influence of Inp52 on scission. Rvs centroid persists after scission in *inp52*Δ cells: disassembly of Rvs after scission is delayed. Sla1 signal also persists for longer after scission in the *inp52*Δ than in WT cells, suggesting that post-scission disassembly of proteins from the vesicle is inhibited in *inp52*Δ cells. Inp52 likely plays a role in recycling endocytic proteins from the vesicle to the plasma membrane.

A protein-friction model has proposed that BAR domains induce a frictional force on the membrane, causing scission (ref!). If more BAR domains were added to the membrane tube at a faster rate, the frictional  force generated as the membrane is pulled under it should increase, and the membrane should rupture faster. That is, membrane scission should occur as soon as WT forces are generated on the tube. In Rvs duplicated cells, adding up to 1.6x the WT amount of Rvs at faster rates to membrane tubes does not affect the length at which the membrane undergoes scission (Fig.6). We think that protein friction does not contribute significantly to membrane scission in yeast endocytosis.

We observed that the maximum amount of Abp1 measured in all the diploid strains is about 220 molecules (Fig.6g/h). Since only one allele of Abp1 is fluorescently tagged in these strains, the total amount of Abp1 recruited is  about 440±20 molecules. In WT haploid cells, the maximum number of Abp1 measured is 460±20 molecules. We propose that recruitment of a similar amount of Abp1 before scission in all these strains indicates that scission is dependent on the amount of Abp1, and correspondingly, on the amount of actin recruited. We propose that actin supplies the forces necessary for membrane scission. The membrane invagination continues until the “right” amount of actin is recruited. The amount of force necessary is determined by the physical properties of the membrane like membrane rigidity, tension, and proteins accumulated on the membrane (Dmitrieff and Needeelec, 2015). Vesicle scission releases membrane-bound Rvs, resulting in release of the SH3 along with BAR domains. Release of the SH3 domains could indicate to the actin network that vesicle scission has occurred, beginning disassembly of actin components.

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**Model for membrane scisison**

We propose that Rvs is recruited to sites by two distinct mechanisms. SH3 domains cluster Rvs at endocytic sites, increasing the efficiency with which the BAR domains sense curvature on tubular membranes. BAR domains bind to endocytic sites by sensing tubular membrane. Membrane shape is stabilized by BAR-membrane interaction against fluctuations that could cause scission. This prevent actin forces from rupturing the membrane, and the invaginations continue to grow in length as actin continues to polymerize. As actin continues to polymerize,, enough forces are generated to overcome the resistance to membrane scission provided by the BAR scaffold. The membrane ruptures, and vesicles are formed. Synaptojanins may help recruit Rvs at endocytic sites: Inp51 and Inp52 have proline rich regions that could act as binding sites for Rvs167 SH3 domains. They are involved in vesicle uncoating post-scission, likely by dephosphorylating PIP2 and inducing disassembly of PIP2-binding endocytic proteins. Eventually phosphorylation regulation allows endocytic proteins to be reused at endocytic sites, while the vesicle is transported elsewhere into the cell.