Discussion

The recruitment and function of the Rvs complex in the last scission stage of endocytosis has been explored in this work, as well as some previously untested proposals for how membrane scission could be effected in yeast endocytosis.
I propose that Rvs localizes by interactions of the BAR domains of the Rvs complex with invaginated membranes, and that the SH3 domain is required for efficient and timely recruitment of Rvs to sites. Arrival of Rvs on membrane tubes then scaffolds the membrane tube and prevents membrane scission, in a manner that depends on recruitment of a critical number of Rvs molecules, till actin forces rupture the membrane, causing vesicle scission.

Here I discuss the main findings of this thesis in support of these propositions.

4.1 Recruitment of Rvs to endocytic sites

Rvs is relatively short-lived protein at endocytic sites, recruited in the last stage, to membrane tubes[1–3]: timing and position appear to be tightly regulated. FCS measurements have shown that the cytoplasmic content of Rvs167, as well as that of Rvs161 is quite high compared to other endocytic proteins[4]: many early proteins, and several of the later WASP/ Myosin module like Las17, Vrp1, type1 myosins, are measured at 80-240nM, while cytoplasmic intensity of 161 is 721nM, and 167 is measured at 354nM. In spite of this, relatively few numbers of Rvs are recruited to endocytic sites, suggesting that cytoplasmic concentration alone does not determine recruitment dynamics. Comparison between FCS measurements of cytoplasmic concentration for different endocytic proteins, and their recruitment to the endocytic sites indicates low correlation between the two, perhaps unsurprisingly, requiring that other directed mechanisms recruit proteins in a timed and efficient manner. In the case of Rvs, both timing and efficiency appear crucial to its function, the question is now what confers both.

4.1.2. Timing of localization and efficiency of recruitment of Rvs

4.1.2.1 The BAR domain senses membrane curvature.

CLEM has shown that when Rvs arrives at endocytic sites, the membrane is already tubular[2]. The curved structure of the BAR dimer has suggested that it is recruited by its preference for some membrane shapes over others. In the absence of membrane curvature, in sla2del cells, the Rvs BAR domain does not localize to cortical patches. This demonstrates for the first time that this BAR domain does indeed sense and requires membrane curvature to localize to cortical patches. Work on BAR domains propose that electrostatic interactions between positive charges at the concave surface and tips of the curved BAR domain structure and negatively charged lipids mediate membrane binding. Mutations in these lipid-binding surfaces would clarify the interaction with underlying lipids, and test if Rvs relies on similar interactions.

4.1.2.2 BAR domain times recruitment of Rvs

Without the SH3 domain, that is in BAR cells, Rvs167 is able to localize to endocytic sites, and has a similar lifetime. In Fig3.4 A-B, Abp1 and Rvs167 in WT and BAR cells are aligned in time to the peak of the respective Abp1 fluorescent intensities. While WT Rvs arrives about 4 seconds after the arrival of Abp1, Rvs in BAR cells arrives only 6 seconds after Abp1 arrives. There is a time delay between Abp1 and Rvs167 recruitment in BAR cells, confirmed by the TIRF measurement in 3.4D.

This delay could occur either because the membrane has not acquired the required membrane structure, or because enough forces have not been generated by the reduced Abp1 recruitment. That this delay is from the lack of tubular structure is supported by the fact that Sla1 moves inwards at a slower rate in BAR cells, which would mean that it takes longer for the membrane to reach the same dimensions as the WT. Also in support of this, BAR arrives at Sla1 at when it has moved inwards 25-35nm, which is also the Sla1 distance moved when WT Rvs arrives. To be noted is that Sla1 is not directly at the plasma membrane, and the centroid of Sla1 sits about 20nm higher on the flat plasma membrane than Sla2[1]. Therefore, a 25-35nm distance of Sla1 would correspond to 45-55nm of membrane invagination, by which point the membrane is already tubular[1,2], consistent with Rvs arrival at invaginated tubes. This suggests Rvs recruitment is timed to particular membrane invagination length, and that this timing is provided by the BAR domain.

4.1.2.3 SH3 domain times affects actin dynamics

In WT cells, the Abp1 and Rvs167 fluorescent intensity reach maxima at the same time, and the consequent decay of both coincide. Both decay indicates the disassembly of the actin network and Rvs scaffold upon membrane scssion. Membrane scission essentially occurs at the intensity peak of the two proteins. This coincident peak is lost in BAR cells. Rvs in these cells peaks several seconds after Abp1 intensity starts to drop, and the decay of Abp1 is prolonged, to take nearly double the time. As we see in Fig.3.4C, the number of Abp1 molecules recruited is also decreased to about two thirds the WT number. Although it is not clear what the decoupling of Abp1 and Rvs peaks mean, the changes in Abp1 dynamics suggests a strong disruption of the actin network. As SH3 domains are known actin regulators, this is effect is not very surprising, but study of other components of the actin network is required to understand how exactly this has changed the endocytic machinery.

4.1.1.2 The SH3 domain makes Rvs recruitment efficient

Cellular expression alone does not determine how much Rvs gets recruited: as has been shown in Fig.3.4C, Rvs in BAR cells accumulates to about half its wild-type number, even though the same cytosolic concentration is measured (see methods). This indicates that the SH3 domain increases the efficiency of recruitment of Rvs to invaginated tubes. Likely this is via interactions with its so-far-unknown binding partner. As the membrane tube presents a relatively small interaction surface (compared to that covered the actin network, measured in Kukulski et al[2]., as the ribosome exclusion zone), it would be more efficient to first recruite Rvs via and SH3 domain interaction, that would cluster Rvs near the membrane tube, and this would then increase the likelihood of membrane binding via the BAR domain.

4.1.1.3 The SH3 domain can assemble and disassemble Rvs molecules independent of the BAR domain and actin interactions

In the absence of membrane curvature in sla2del cells, full-length Rvs is able to localize to cortical patches without the curvature-dependent interaction of the BAR domain (Fig3.3D-F). The independent ability of the SH3 domain to localize and disassemble protein is unexpected. This indicates that the SH3 domain is able to mediate recruitment of a cluster of Rvs molecules, and then disassemble this cluster.

In sla2del cells treated with Lat (Fig.3.3G-H), actin-based membrane curvature, as well as actin-binding proteins are removed from the plasma membrane. Full-length Rvs167-GFP in WT cells show transient localizations at the plasma membrane when treated with LatA (Fig.2A). In BAR cells with LatA treatment, this localization is lost, suggesting that the former localizations are dependent an interaction mediated by the SH3 domain.

What does the SH3 domain interact with?

SH3 interaction with an endocytic binding partner could help recruit Rvs to sites. Many such interaction partners have been proposed; Abp1 interaction with the Rvs167 SH3 domain has been shown[5,6], Las17[7,8], Cmd1[9], type I myosins[10], and Vrp1[5]- which recruits the myosins, are being studied as potential targets of the Rvs167 SH3 domain. Since the SH3 is able to localize to endocytic sites in an actin independent manner, the interaction candidate could be one that does not require actin. All of the suggested binding partners localize to the base of the invagination[1,11], and do not follow the membrane into the cytoplasm. If one of these was the SH3 interaction partner, SH3 domains are recruited at the base of the invagination, and then pushed up with membrane as the tube grows longer, explaining the small movement of the Rvs167 centroid in the early portion of its lifetime. Centroid tracking however, suggests that Rvs is accumulated all over the membrane tube without bias towards the base of the invagination. If this was recruited to the base, the centroid would move continuously upwards rather than remain relatively non-motile before the jump at scission time. It is possible for the SH3 to drive early recruitment of the protein, which is then "switched off" as the membrane invaginations grow long enough to provide binding surfaces for the BAR domain interactions.

4.1.3 Arrangement of Rvs

No solved structure for the Rvs complex exits. That Rvs is a hetero- rather than homodimer suggests that the structure need not resemble that of Amphiphysin or Endophilin homodimers, and a high-resolution structure will be necessary to clarify the interaction and arrangement of Rvs on endocytic tubes. It is therefore unclear how Rvs is arranged on the membrane tube, although there are some indications from the experiments in this work of the interaction with the membrane.

4.1.3.1 Rvs does not form a tight scaffold on membrane tubes

In-vitro helical arrangements of BAR domains on membrane tubes have suggested that the Rvs might form a similar helical scaffold. Correlating CLEM and centroid movements has proposed that an Rvs scaffold covers the entire membrane tube upto the base of the future vesicle. In diploid Rvs overexpression strains, a lot more Rvs can be recruited, at a much faster rate than in WT cells (Fig.3.9 B-C). Disassembly dynamics, however, is the same as in WT. The sharp decay of fluorescent intensity of Rvs indicates that all of the protein is suddenly released, consistent with a BAR scaffold that breaks upon vesicle scission, releasing all the membrane-bound protein at once. This decay in the 4x Rvs strain suggests that all the Rvs here is also bound to the membrane. Since the membrane is able to accommodate 1.4x the amount of BAR protein as the WT, it would suggest that at lower protein amounts, like in the WT, a tight helix that covers the entire tube was not likely. Adding molecules to such a tube would result in a change in at least disassembly dynamics. That the membrane surface area does not change in the 4x Rvs compared to WT is assumed from the identical movement of Sla1 in both. Sla1 movements in diploids and haploids are also identical. It is possible that a wider tube is formed, that would increase the surface area of binding. This would, however, require the BAR domains to interact with a lower radius of curvature than in WT.

4.1.1.1 A limit for how much Rvs can be recruited to the membrane

In the case of 2x Rvs in haploids, a change in disassembly dynamics is seen. Here, the maximum number of molecules recruited is 178 +/- 7.5 compared to 113.505 +/- 5.2. Nearly 1.6x the WT amount of protein is recruited to membrane tubes. The Rvs167 centroid here shows a delay in disassembly, suggesting that the excess protein is not directly on the membrane. The excess Rvs either interacts with the actin network via the SH3 domain, or interacts with other Rvs dimers. Since the concave surface of the BAR domains, which are known to interact with the membrane are positively charged, and the convex surface is negatively charged, multiple layers of BAR domains are unlikely, and have not been reported to my knowledge.

Whatever the arrangement of the Rvs complex on the membrane, disassembly dynamics is changed in the case of 2x RVS - haploid, compared to all the other haploid and diploid strains. Since the number of Rvs molecules is highest in this strain, this suggests that there is a limit to how much Rvs can assemble on the tube without altering interaction with the endocytic network.

There is a difference in the rate of Rvs recruitment in WT between haploids and diploid strains. Why this exists is not clear. Diploid cells do not double in volume compared to haploids: in normal growth conditions, the volume of the diploid cell is around 1.57x that of the haploid cell, and the average cell surface area increases to 1.4x[12]. In diploids, the genome that contains four copies of Rvs in principle should express twice the amount of Rvs as one that contains four copies. Cytoplasmic quantification, however, shows that the increase is 1.4x in the duplicated diploid case compared to the WT diploid case, and recruitment to endocytic sites increases proportionately. There is then 1.4x the protein in nearly 1.6x the cellular volume, resulting in a dilution of the protein content per unit volume of the cell, which could then explain the decreased recruitment. Recruitment of Rvs is proportionate to gene copy number, but as detailed earlier, is not only dependent on protein concentration, but also on the SH3 domain.

4.2 What causes membrane scission?

4.2.1.Dynamin does not not drive scission

Some studies have suggested that Vps1 localizes to endoytic sites, and affects the scission mechanism: Nannapaneni et al[13]., find that the lifetimes of Las17, Sla1, Abp1 increase in the absence of Vps1. Rooij et al[14].,. find that Rvs167 lifetimes increase, and are recruited in fewer patches to the cell cortex. On the other hand, vps1Δ did not increase the scission failure rate of rvs167Δ in other studies[15], and did not co-localize with endocytic proteins[16]. If Vps1 was to affect scission, the number of failed scission events should increase in vps1Δ cells, but we do not find so, confirming other studies[15]. Vp1 tagged with super-folded GFP and imaged in TIRF does not form cortical patches that co-localize with Abp1-mCherry (data

from Andrea Picco, not shown). GFP-tagging could affect the recruitment of Vps1 to endocytic sites while maintaining its role in other cellular processes like vesicular trafficking. Membrane movement and scission dynamics are however, unchanged in the absence of Vps1. If loss of Vps1 prevented or delayed scission, the membrane would continue to invaginate past the WT lengths, and Sla1 movements of over 140nm would be measured. The Rvs centroid movement would likely also be affected: a bigger jump inwards would indicate that that a longer membrane has been cut. That there are no changes in the coat or scission dynamics indicate that even if Vps1 is recruited to sites, it is not necessary for Rvs localization or function, and is not necessary for scission.

4.2.2 Lipid hydrolysis is not the primary cause of membrane scission

The synaptojanin-mediated scission model predicts that forces generated by a lipid phase-boundary causes scission[17]. Synaptojanin-like Inp51 is not seen to localize to the cellular cortex, but cytoplasmic concentration measured by FCS is low[4], suggesting low levels of expression that are likely undetected by our imaging method. Inp52 localizes to the top of invaginations right before scission, consistent with a role in vesicle formation. Some predictions of the lipid model do not match our observations, howerver.

First, vesicle scission is expected to occur at the interphase of the hydrolyzed and non-hydrolyzed lipid. Since the BAR scaffold covers the membrane tube, this interphase would be at the top of the area covered by Rvs. Kukulski et al[2]., have shown that vesicles undergo scission at 1/3 the invagination length from the base: that is, vesicles generated by lipid hydrolysis-based line tension would be smaller than have been measured. Second, removing forces generated by lipid hydrolysis by deleting synaptojanins should increase the invagination lengths, since scission is delayed or failed without those forces. Deletion of Inp51 and Inp52 does not change the invagination lengths: Sla1 movement does not increase. That the position of the vesicle formed is also unchanged compared to WT is indicated by the magnitude of the jump into the cytoplasm of the Rvs centroid.

There are some changes, however, in the synaptojanin deletion strains. In inp51del strains, Rvs assembly is slightly slower than that in WT. It is currently unclear why this occurs. Rvs centroid persists after scission for about a second longer than the WT does, indicating that

disassembly of Rvs on the base of the newly formed vesicle is delayed. In the inp52del strain, about 12\% of Sla1-GFP tracks do not undergo scission. Although this is low compared to the failed scission rate of the rvs167del cells (close to 30\%), this data could suggest a moderate influence of Inp51 and Inp52 on Rvs dynamics and therefore on scission.

In the inp5152del strain, Rvs is accumulated at patches, but majority of Rvs patches do not show the sharp jump into the cytoplasm. Membrane morphology is hugely aberrant in these cells, complicating interpretation of this data[18]. Electron microscopy shows long, undulating membrane invaginations, with multiple endocytic sites that are assembled and disassembled, but fail to undergo scission[18,19]. Where on these long membranes Rvs localizes could be clarified by CLEM or superresolution microscopy. Large clusters of Rvs seen in the inp5152del strain could be multiple patches on same invaginated tube. This would influence the molecule numbers acquired by our analysis, and yield a higher number than at a single site. Rvs does, interestingly, assemble and disassemble. If no vesicles are formed at these membranes, it would indicate that Rvs disassembly is not caused by membrane scission.

4.2.3 Protein friction does not drive membrane scission

Protein-friction mediated membrane scission proposes that BAR domains induce a frictional force on the membrane, causing scission. In Rvs duplicated haploid strain, adding upto 1.6x the WT amount of Rvs to membrane tubes does not affect the length at which the membrane undergoes scission. The model introduced in Section 3.2.3 would suggest that if more BAR domains were added to the membrane tube, the frictional force generated as the membrane is pulled under it would increase, and the membrane would rupture faster, that is, as soon as WT forces are generated on the tube. Since BAR domains are added at a faster rate in the duplicated strain, these forces would be reached at shorter invagination lengths. In the haploid duplication strain, the WT amount of Rvs is recruited at nearly -2 seconds, but scission does not occur at this time. In diploid strains, adding 1.4x the WT amount of Rvs in the 4x Rvs case also does not change length of membrane scission. Decreasing the amount of Rvs from WT amounts, like in the 1x Rvs strain, however reduces the scission efficiency, and slightly reduces the inward movement of the membrane (supplemental). Scission efficiency decreases with decrease in Rvs recruitment, but does not increase with adding

excess Rvs. Protein friction does not appear to contribute significantly to membrane scission.

4.2.4 Actin polymerization generates forces required for membrane scission

Maximum amount of Abp1 measured in all the diploid strains is about 220 molecules. In this case, only one allele of Abp1 is tagged, so half the amount of Abp1 recruited is measured. The maximum amount of Abp1 recruited is then double that measured, which is about 440 +/- 20 molecules. In WT haploid cells, the maximum number of Abp1 measured is 460 molecules, +/- 20 molecules. That the same number of molecules of Abp1 is recruited in all cases before scission indicates a dependence on the amount of Abp1, and hence, the amount of actin recruited. This data is consistent with actin forces supplying the forces necessary for membrane scission. The membrane ingression continues until the "right" amount of actin is recruited. At this amount of actin, enough forces are generated to rupture the membrane. In the BAR strains, however, a lower amount of actin is recruited. It is clear that in the absence of the SH3 domain the actin network is severely perturbed, and the effect of this on scission dynamics is still unclear.

4.3 Function of the Rvs complex

4.3.1 Rvs scaffolds the membrane pore, preventing scission

Sla1 in rvs167Δ cells undergoes scission at short invagination lengths of about 60nm (Fig.4), compared to the WT lengths of 140nm; Rvs167 is required at membrane tubes to prevent premature scission. This is consistent with the SH3 domain mediating actin forces to the invagination neck, causing scission. This can also be explained by Rvs stabilizing the membrane invagination via membrane interactions of the BAR domain33. Since WT invagination depths are reproduced by overexpression of the BAR domain alone, I propose that localization of Rvs BAR domains to the membrane tube stabilizes the membrane. This allows deep invaginations to grow until actin polymerization produces enough forces to overcome this stabilization and sever the membrane. Here, the forces are generated by actin polymerization, and the amount of force necessary is determined by the physical properties of the membrane.

4.3.2 A critical amount of Rvs is required to stabilize the membrane

Scission efficiency decreases with decreased amounts of Rvs: in diploids, lowering the amount of Rvs by 20 molecules decreasing the scission efficiency by xxx. This indicates that a particular coverage of the membrane tube is required for effective scaffolding by BAR domains. In this casse, some endocytic events recruit the criticial number of Rvs, in which case the membrane grows to near WT lengths.

If enough forces are generated at 60nm, why is there a lower scission efficiency?
Forces from actin at the this are maybe at a threshold. There is enough to sever, but not to sever reliably. The Rvs scaffold keeps the network growing to accumulate enough actin, and perhaps also to control vesicle size.

4.4 Role of other scission-stage proteins

4.4.1 Inp52 is likely involved in uncoating vesicles after scission

Deletion of Synaptojanin-like Inp52 does not affect the invagination depths of Sla1, but Sla1 patches persist for longer after scission in the inp52Δ than in WT cells, as does Rvs167 centroid, indicated by the arrows in Fig.2. Both delays suggest that rather than the scission time-point, post- scission disassembly of proteins from the vesicle is inhibited by the deletion, and that Inp52 plays a role in recycling endocytic proteins to the plasma membrane. The slower assembly of Rvs in inp51del and the increase in scission efficiency could indicate that there is a slight effect on Rvs recruitment, and that lipid hydrolysis could play a small, although non-major role in scission.

4.5 Model for membrane scission

I propose that Rvs is recruited to sites by two distinct mechanisms. The SH3 domain recruits rapidly Rvs to sites via interaction with some endocytic protein(s). This increases the efficiency with which the BAR domain senses membrane curvature, and the BAR domain then binds the membrane tube by this interaction. BAR domains interact with the entire membrane tube, without the helical arrangement seen from in-vitro experiments of other

BAR domains. BAR-membrane interactions prevent actin forces from causing membrane scission, and the invaginations continue to grow in length, and actin continues to polymerize and exert forces on the membrane. BAR recruitment to endocytic sites is restricted by the surface area of the membrane tube: after a certain amount of Rvs, the excess interacts with endocytic sites via the SH3 domain. As actin continues to polymerize, at a certain amount of actin, enough forces are generated to overcome the resistance to membrane scission provided by the BAR scaffold, the membrane ruptures, and vesicles are formed.

Synaptojanins might help the recruitment of Rvs at endocytic sites: Inp51 and Inp52 have proline rich regions that could act as binding sites for SH3 domains. They are involved in vesicle uncoating post-scission, likely by phosphorylation regulation of endocytic proteins remaining on the vesicle, as has been proposed by other work.

Autoregulation of BAR domains by the SH3 domain has been proposed for other BAR domains. Activation of the BAR domain itself has not been tested in this work, neither has the function of the N-helix of both 161 and 167, or the GPA region present in 167.