**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 tubes1–3: 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 proteins4: 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 tubular2. 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 Sla21. Therefore, a 25-35nm distance of Sla1 would correspond to 45-55nm of membrane invagination, by which point the membrane is already tubular1,2, 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 al2., 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 shown5,6, Las177,8, Cmd19, type I myosins10, and Vrp15- 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, 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 yeast membrane tubes**

High-resolution structures of mammalian BAR domains 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 in WT Rvs indicates that all of the Rvs on the membrane is suddenly released, consistent with a BAR scaffold that breaks upon vesicle scission, releasing all the membrane-bound protein at once. A similar 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 on the same membrane surface area, it appears that in the WT case, 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 strain is assume from the identical movement of Sla1 in the 4x strain as in the WT. Sla1 movements in diploids and haploids are identical. It is possible that a wider tube is formed, that would increase the surface area of binding, since we do not control in these experiments for tube radius. This would, however, require the BAR domains to interact with a lower radius of curvature than the WT.

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

A change in disassembly dynamics mentioned in the above section is seen in Rvs duplication in haploid cells. In this case, an even higher amount of Rvs is recruited to cells than in the WT: the maximum number of molecules recruited is 178 +/- 7.5 compared to 113.505 +/- 5.2. Nearly 1.6x amount of protein is recruited to membrane tubes than in WT. 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, that the disassembly dynamics is changed in the case of 1.6x Rvs- 2x RVS strain in haploid, compared to both WT and 1.4x Rvs- 4x RVS in diploids suggests that there is a limit to how much Rvs can assemble on the tube without a change in protein-protien or protein-membrane interaction. Why there is a difference between recruitment of Rvs in the diploid and haploid case is uncertain. Diploid cells do not double in volume compared to haploids: in normal growth conditions, the volume of the diploid cell various between 1.57x that of the haploid cell, and the average cell surface area increases by 1.4x11. In the gene duplication case, we have two copies of Rvs that in principle should expressed at twice the haploid level. Cytoplasmic quantification, however, shows that the increase is 1.4x in the duplicated diploid case compared to the WT diploid case, as does the recruitment to endocytic sites. 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.

It appears that the recruitment is then proportionate to gene copy number, and protein content in the cell, but as detailed earlier, this is also influenced by the SH3 domain.

**4.2 What causes membrane scission?**

**4.2.1.Dynamin does not not drive scission**

Some studies have suggested that Vps1 does localize to endoytic sites, and affects the scission mechanism: Nannapaneni et al12., find that the lifetime of Las17, Sla1, Abp1 increase in the absence of Vps1. Rooij et al13.,. 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 studies14, and did not colocalize with other endocytic proteins15. We see a slight but insignificant increase in Rvs167 lifetimes in vps1Δ cells. If Vps1 was to affect scission, the number of failed scission events should increase in vps1Δ cells, and increase the lengths of invaginated tubes, but we do not find so. The lack of influence of Vps1 on coat and scission dynamics shows that membrane scission is not dependent on a dynamin interaction. 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). That tagging does not cause the slight growth defect of the vps1Δ cells is seen in dot spot assays. GFP-tagging could however, affect the recruitment of Vps1 to endocytic sites while maintaining its role in other cellular processes like vesicular trafficking. Outside of potential issues recruiting the protein to endocytic sites, membrane movement and scission dynamics are unchanged in the absence of Vps1, suggesting that even if it recruited to sites, it is not necessary for Rvs localization or function.

**4.2.2 Lipid hydrolysis is not the primary cause of membrane scission**

Inp51 is not seen to localize to the cellular cortex, but cytoplasmic concentration measured by FCS is low, suggesting low levels of expression that are likely undetected by our imaging protocol. Inp52 localizes to the top of invaginations right before scission, consistent with a role in vesicle formation.

However, the synaptojanin-mediated scission model19 predicts that first, vesicle scission would occur at the top of the invaginated tube, at the interphase of the hydrolyzed and non-hydrolyzed lipid. Kukulski et al2., have shown that vesicles undergo scission at 2/3 the invagination depths: that is, vesicles generated by lipid hydrolysis based line tension would be smaller than have been seen. Second, removing forces generated by lipid hydrolysis by deleting synaptojanins should then increase the invagination lengths measured. Deletion of Inp51 and Inp52 do not change the invagination depths at which scission occurs, as measured from the maximum movement of Sla1. That the position of the vesicle formed is also unchanged is indicated by the magnitude of the jump into the cytoplasm of the Rvs complex.

There are some changes, however in the synaptojanin deletion strains. FIrst, in the inp51del strain, Rvs assembly is slightly slower than that of the WT. Thus far, it is unclear what this means. 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 move into the cytoplasm and undergo scission. Although this is low compared to the failed scission rate of the rvs167del cells (close to 30\%), it could suggest a moderate influence of inp52 on scission dynamics. In the inp5152del strain, Rvs is accumulated at patches, but most Rvs patches do not show the sharp jump into the cytoplasm. Membrane morphology is hugely aberrant in these cells, complicating the interpretation of this data.

Electron microscopy shows long, undulating membrane invaginations, with multiple endocytic sites that are assembled and disassembled. Where the Rvs complex localizes in these cells could be clarified by CLEM or superresolution microscopy. Large clusters of Rvs on the same invaginated tube would influence the molecule numbers acquired by this kind of analysis, and yield a higher number than at a single site (although what is actually happening at these patches is not actually clear). Rvs does, interestingly, assemble and disassemble. If there is no vesicle at these membrane, it would indicate that Rvs disassembly is decoupled from membrane scission.

**4.2.3 Protein friction does not drive membrane scission**

In Rvs duplicated strains, adding upto 1.6x the WT amount of Rvs to membrane tubes does not affect the length at which the membrane undergoes scission. The protein friction model introduced in Section 4.2.3 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. In the haploid duplication strain, the WT amount of Rvs is recruited at nearly -2 seconds, but scission does not occur at this time point. In the diploid strains, meanwhile, adding 1.4x the WT amount of Rvs 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.

does not appear to contribute significantly to membrane scission, since invagination depths do not change if more BAR protein is localized to membrane tubes.

**4.2.4 Actin polymerization generates forces required for membrane scission**

**4.3 Function of the Rvs complex**

**4.3.1 A critical amount of Rvs is required to allow invaginations to grow**

Sla1 in rvs167Δ cells undergoes scission at short invagination lengths of about 60nm (Fig.4) , compared to the WT lengths of 140nm16; 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, as well as with Rvs167 stabilizing the membrane invagination via membrane interactions of the BAR domain33. Since WT invagination depths are reproduced by overexpression of the BAR domain alone, we propose that localization of Rvs to the membrane tube stabilizes the membrane pore, and allows deep invaginations to grow until actin polymerization produces enough forces to sever the membrane and cause scission. Here, the forces are generated entirely by actin polymerization, and the amount of force necessary is determined by the physical properties of the membrane.

**4.3.2** Rvs scaffolds membrane pore

This then begs the question, why does Sla1 move inwards less? The SH3 domain reduces the efficiency of Rvs recruitments, and loss of Rvs on the membrane tube

**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 do patches of Rvs167, 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.

**4.5 Model for membrane scisison**