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.

\section{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.

\subsection{Timing of localization and efficiency of recruitment}

\subsubsection{The BAR domain senses membrane curvature}

It has been shown before that Rvs localizes to membrane tubes after they are 60nm long2. From the expected curved structure of the BAR dimer, it has so far been assumed 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. Rvs167 recruitment has been correlated along the endocytic timeline against the smallest angle between the two membrane sides2, which is 180 when the membrane is flat, and goes to 0 as the membrane becomes tubular. At the time of the recruitment of Rvs, this angle is 0, indicating that Rvs is recruited only to tubular membrane shapes. BAR recruitment by sensing membrane curvature would allow for this specificity. Work on BAR domains have so far not uncovered a specific interaction with a lipid subtype, and have suggested that hydrophobic interactions mediate this interaction. It is not clear how the Rvs BAR domains interacts with the membrane, and mutations of the lipid-binding surfaces are necessary to clarify the interaction with underlying lipids.

\subsubsection{BAR domain times recruitment of Rvs

A change in disassembly dynamics is seen, however, 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 before scission is 178 +/- 7.5 compared to 113.505 +/- 5.2 yields a 1.57x recuitment of protein to membrane tubes. Here, the disassembly of Rvs after scission is delayed, and would suggest 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. Currently, I am not able to distinguish between the two, since the SH3 interaction partner is currently undetermined, and the arrangement of Rvs on the membrane is currently unknown. BAR-BAR interactions have been observed for other BAR proteins, albeit via lateral interactions at the tips of the curved structure, between apposed BAR domain. The concave face of BAR domains has been shown to interact with the membrane, and interactions that allow concentric arrangement of BAR domains are not seen before and are unlikely, but perhaps still possible.

Whatever the arrangement of the Rvs complex on the membrane, that the disassembly dynamics is changed in the case of 1.6x Rvs, compared to WT, and 1.4x Rvs 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 1.4x. 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 that this is likely not the only factor that influences recruitment.

\subsubsection{The SH3 domain makes Rvs localization efficient}

Cellular expression alone does not determine how much Rvs gets recruited. Unexpectedly, the lack of SH3 domain affects the recruitment of Rvs to the plasma membrane, even though the amount of protein expressed is the same (from analyzing cytoplasmic signal in Rvs167-GFP vs rvs167Δ SH3-GFP cells, see methods), the amount of protein recruited to endocytic sites is nearly halved. This indicates that the SH3 domain increases the efficiency of recruitment of Rvs to invaginated tubes. Transient cortical patches in LatA treated cells expressing Rvs167-GFP that are absent in LatA cells expressing rvs167Δ SH3-GFP (Fig.1A) suggests that the former patches are caused by an interaction mediated by the SH3 domain. These patches suggest that the SH3 domain is able to cluster protein to the cell cortex, although the mechanism by which this occurs is not known.

Full-length Rvs is able to localize to cortical patches without the membrane-dependent interaction of the BAR domain (Fig. Full-length RVS in sla2del and LatA treatment shows cortical patches). This indicates that the SH3 domain is able to mediate recruitment of a cluster of Rvs molecules, and then disassemble this cluster. The independent ability of the SH3 domain to localize and disassembly protein complexes is surprising, since SH3 domains are known so far to mediate protein-protein interaction and self-regulation of activation states.

\subsection{Timing of Rvs}

\subsection{The BAR- membrane curvature interaction}

It has been shown before that Rvs localizes to already invaginated membrane tubes, suggesting that its role in the last stage is not to drive or significantly influence membrane curvature. From the expected curved structure of the BAR dimer, it has so far been assumed 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 demonstrate for the first time that the Rvs BAR domain does indeed require membrane curvature to localize to cortical patches. Work on other BAR domains have so far not discovered a specific interaction with a lipid subtype, and have suggested that hydrophobic interactions are mediate this interaction. It is not so far clear how the Rvs BAR domains interacts with the membrane, and mutations of lipid interacting surfaces will be necessary to determine the type of interaction with the underlying lipids. It is also unclear how Rvs is arranged on the membrane tube. Although solved structures of BAR domains show high structural similarity in spite of low sequence similarity, no structure for the Rvs complex exits. The fact that this is a hetero- rather than homodimer suggests that the structure does not necessarily 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.

\subsubsection{The SH3 domain affects timing of Rvs recruitment: scisssion and actin disassembly are likely uncoupled}

\subsubsection{What recruits the SH3 domain?}

SH3 interaction with the 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 shown27,28, Las1729,30, Cmd131, type I myosins32, Vrp127, which recruits the myosins, have all emerged as potential candidates, and are being studied as potential targets of the Rvs167 SH3 domain. Since the SH3 localizes to sites in an actin independent manner, the interaction candidate is likely one that does not require actin to localize, leaving Vrp1, Las17, Myo3/5. Las17 and type 1 mysoins localize to the base of the invagination, and do not move into the cytoplasm a significant amount during invagination. If one of these was the SH3 interaction partner, SH3 domains are then recruited at the base of the invagination, and then pushed up with membrane as the tube grows longer. Centroid tracking however, suggests that Rvs is accumulated all over the membrane tube without bias towards the base of the invagination: if this was the case, the centroid would move upwards rather than remain non-motile. It is possible for the SH3 to drive early recruitment and localization, which is then "switched off" as Rvs is clustered by the SH3 domain, and targeted recruitment via an interaction partner is no longer necessary.

\subsection{Arrangement of Rvs}

\subsubsection{Rvs does not form a tight scaffold on yeast membrane tubes}

Cryo EM structures of mammalian BAR proteins have suggested that the BAR dimers of Rvs might form a similar helical scaffold with lateral interactions between adjacent BAR domains on invaginated membrane tubes. In the Rvs overexpression strains in diploids, Rvs can be recruited in much higher numbers, and at a much faster rate to the membrane than in WT cells, but appears to have similar disassembly dynamics as in the WT (Fig.5). The atypical, sharp decay fluorescent signal indicates that all of the Rvs on the membrane is suddenly released, consistent with the idea of a scaffold that breaks upon vesicle scission, releasing all the membrane-bound BAR protein. The decay in the 4x Rvs strain suggests that all the Rvs is also bound to the membrane, and since the membrane is now able to accommodate about 1.6x the amount of BAR protein as the WT on the same amount of membrane, it appears that in the WT case, a tight helix that covers the entire tube was not likely to be formed: adding molecules to such a tube would result in a change in at least disassembly dynamics.