﻿**Introduction:**

In Clathrin-mediated endocytosis, a flat plasma membrane is pulled into a tubular invagination that eventually forms a vesicle. Forces that drive the transition from invagination to spherical vesicle in mammalian cells are provided by constriction of the GTPase Dynamin. Dynamin is now known to act in concert with the crescent-shaped N-BAR proteins Endophilin and Amphyphysin (ref. Dynamin papers). Proline-rich motifs on the Dynamin. In yeast cells, what causes membrane scission is unclear, although the yeast N-BAR protein complex Rvs has been identified as an important component of the scission module. In yeast, the Amphiphysin and Endophilin homologue Rvs is a heterodimeric complex composed of Rvs161 and Rvs167 (Friesen et al., 2006). Deletion of Rvs reduces scission efficiency by nearly 30\% and reduces the invagination lengths at which scission occurs (ref Marko, wanda). Apart from a canonical N-BAR domain which forms a cresent-shaped structure, Rvs167 has a Glycine-Proline-Alanine rich (GPA) region and a C-terminal SH3 domain. Rvs161 and Rvs167 N-BAR domains are 42\% similar, and 21\% identical, but are not interchangeable (Sivadon, Crouzet and Aigle, 1997). The GPA region is thought to act as a linker with no known other function, while loss of the SH3 domain affects budding pattern and actin morphology. Most Rvs deletion phenotypes can however, be rescued by expression of the BAR domain alone (Sivadon, Crouzet and Aigle, 1997), suggesting that the BAR domains are the main functional unit of the Rvs complex. Homology modelling has shown that the BAR domain of Rvs167 is similar to Amphiphysin and Endophilin (Youn et al., 2010), and is therefore likely to function similarly to the mammalian homologues. In keeping with this theory, Rvs has been shown to tubulate liposomes in vitro (Youn et al., 2010). The Rvs complex arrives at endocytic sites in the last stage of the endocytosis, and disassembles rapidly at the time of membrane scission (Picco et al., 2015), consistent with a role in membrane scission. While it is known to be involved in the last stages of endocytosis, a mechanistic understanding of the influence of Rvs on scission however, remains incomplete.

We used quantitative live-cell imaging and genetic manipulation in *S.cereviciae* to investigate the influence of Rvs and several Rvs interacting proteins that have been suggested to have a role in scission. We found that arrival of Rvs to endocytic sites is timed by interaction of its BAR domain with a specific membrane curvature. The Rvs167 SH3 domain affects localization efficiency of the Rvs complex and also influences invagination dynamics. This indicates that both BAR and SH3 domains are important for the role of Rvs as a regulator of scission. We tested current models of membrane scission, and find that deleting yeast synaptojanins or dynamin does not change scission dynamics. Interfacial forces at lipid boundaries are therefore unlikely to be sufficient for scission, and forces exerted by dynamin are not required. Furthermore, invagination length is insensitive to overexpression of Rvs, suggesting that the recently proposed mechanism of BAR-induced protein friction on the membrane is not likely to drive scission. We propose that recruitment of Rvs BAR domains prevents scission and allows invaginations to grow by stabilizing them. We also propose that vesicle formation is dependent on forces exerted by a different module of the endocytic pathway, the actin network. Preventing premature membrane scission via BAR interaction could allow invaginations to grow to a particular length and accumulate enough forces within the actin network to reliably cut the membrane.

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**Results:**

Rvs167 deletion results in reduced coat movement

Deletion of the Rvs genes have resulted in the scission-specific phenotype of membrane retraction: inward movement and consequent retraction of the invaginated membrane back to the cell wall (ref.Marko). Since Rvs161 and Rvs167 form dimers (ref.Dominik), deletion of Rvs167 effectively removes both proteins from endocytic sites. We therefore quantified the effect of deletion of Rvs167 on membrane invagination. To follow the membrane invagination we used Sla1, an endocytic coat protein. The endocytic coat is pulled along with the membrane as it invaginates, and thus acts as a proxy for the behavior of the plasma membrane (Fig.1b). We endogenously tagged Sla1 at the N-terminus with eGFP in WT and \textit{rvs167$\Delta$} cells (Fig.1a), and tracked the dynamics of Sla1.

27% of Sla1 patches that begin to move inward retract in \textit{rvs167$\Delta$} cells (Fig.1d), consistent with retraction rates measured in other experiments (Kaksonen, Toret and Drubin, 2005), and suggesting failed scission in 27% of endocytic events (Fig.1d). Movement of the remaining 73% Sla1 patches were quantified (Fig.1e) as described in Picco et. al, 2015. In brief, yeast cells expressing fluorescently-tagged endocytic proteins are imaged at the equatorial plane. Since membrane invagination progresses perpendicularly to the plane of the plasma membrane, proteins that move into the cytoplasm during invagination do so in the imaging plane. Centroids of Sla1 patches- each patch being an endocytic site- are tracked in time. Between 40-50 Sla1 centroids are averaged. This provides an averaged centroid that can be followed with high spatial and temporal resolution. When different endocytic proteins are simultaneously imaged with Actin Binding Protein Abp1, Abp1 provides a frame of reference to which all the other proteins can be aligned. Abp1 is used because it is an abundant at endocytic sites and is easily imaged. Time=0 is established as the peak of the Abp1 fluorescence intensity in respective co-tagged strains strains (Fig.1f). Abp1 fluorescent intensity maxima in wild-type cells is concomitant with the peak of Rvs167 fluorescent intensity and is time window in which scission occurs (ref2andrea, refwanda).

Sla1 centroid movement in \textit{rvs167$\Delta$} cells and WT look similar up to about 60nm (Fig.1e), after which Sla1 centroid movement slows dramatically, with a total inward movement of about 80nm. CLEM has shown that Rvs167 localizes to endocytic sites after the invaginations are about 60nm long (Kukulski et al., 2012). Sla1 movement in \textit{rvs167$\Delta$} shows therefore that membrane invagination is unaffected till Rvs is supposed to arrive. Sla1 in \textit{rvs167$\Delta$} then continues to move at a much slower rate to about 80nm. That membrane scission occurs at shorter invagination lengths than in WT is corroborated by the smaller vesicles formed in \textit{rvs167$\Delta$} cells (Kukulski et al., 2012). In WT cells, Sla1 continues to move inward to 140nm. This indicates that first, membrane scission can occur at invagination lengths of 80nm. Then, that the arrival of Rvs prevents membrane scission at 80nm and allows further membrane invagination.

Vps1 does not influence coat or scission dynamics

Endocytic membrane scission in mammalian cells is understood to be driven by constriction of the tubule neck by the Gtpase Dynamin (Grigliatti et al., 1973; Poodry and Edgar, 1979; van der Bliek and Meyerowrtz, 1991). In yeast, the Dynamin-like protein Vps1 is reportedly recruited to endocytic sites (refAyscough). To test whether Vps1 influences scission, endocytic dynamics are observed in cells lacking Vps1 and compared against WT cells. In Fig.1d, retraction of Sla1 in \textit{vps1$\Delta$} and wild-type cells is quantified, suggesting that retraction rates no not increase in the absence of Vps1.

In Fig.e, the averaged centroid trajectory of Sla1-eGFP is tracked in ~50 endocytic sites in \textit{vps1$\Delta$} and WT cells. Centroid movement of Sla1-eGFP in WT cells shows a linear movement to about 150nm. Sla1 movement in \textit{vps1$\Delta$} cells has the same magnitude of inward movement to about 140nm, and has the same speed of movement as in WT. This indicates that plasma membrane dynamics remain uninfluenced by the absence of Vps1.

Centroid tracking has shown that the number of molecules of Rvs167 peaks at the time of scission, and is followed by a rapid loss of fluorescent intensity, simultaneous with a sharp jump of the centroid into the cytoplasm (ref.Andrea). This jump, also seen in Rvs167-GFP kymographs (Fig.1c), is interpreted as loss of protein on the membrane tube, causing an apparent spatial jump to the protein localized at the base of the newly formed vesicle. Kymographs of Rvs167-GFP (Fig.1c), as well as Rvs167 centroid tracking (Fig.1e) in Vps1 deleted cells show the same jump, indicating that vesicles are formed in the same position in Vps1 deletion cells as in WT cells. From the absence of change in coat as well as scission dynamics in Vps1 deleted cells, we conclude that Vps1 does not influence the endocytic process.

Needs:

Abp1, Sla1 fluorescence intensity for \textit{rvs167$\Delta$}

Molecule numbers for all the Abp1, Sla1

Error bars for histogram

Recheck alignment, with Abp1-mCh in higher time resolution

Split Rvs and Sla1 movement into 2 plots

All references (need a latex friendly bibliography)

**Fig.1**

