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REGULATION OF MEMBRANE SCISSION IN YEAST ENDOCYTOSIS

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Abstract

Some other stuff that happened

Clathrin-mediated endocytosis is an essential cellular process to take up cargo molecules from the cell surface. More than 50 proteins form the underlying macromolecular machinery, which is highly conserved among eukaryotes. Understanding its structural organization is key to unraveling the architectural principles that allow the endocytic machinery to form vesicles with high efficiency and regularity. However due to its small size, complexity and fast dynamics, it is still mostly unknown how endocytic proteins are arranged *in situ*.

I have used single-molecule localization based superresolution microscopy to image endocytic sites in fixed budding yeast *Saccharomyces cerevisiae* with high spatial resolution, and found an intricate structural organization of proteins within the endocytic machinery.

Using high-throughput localization microscopy, I imaged tens of thousands of endocytic sites and revealed a striking radial organization of endocytic proteins, where each endocytic functional module occupies a distinct radial zone. Over the endocytic time line, protein assemblies increase in size as well as in regularity. The initial phase of endocytosis is characterized by variable structures, while in the late phase proteins assemble with a higher degree of radial order. I discovered that actin polymerization, which is essential for endocytosis in yeast, is spatially pre-determined by nucleation promoting factors to occur exclusively around the coat, and that proteins, which couple actin to the plasma membrane, are located at the center of the coat. This circular pre-patterning provides an elegant explanation for how the actin machinery is able to efficiently generate and transfer force to invaginate the plasma membrane. In the outermost radial zone, an endocytic myosin possibly pulls actin filaments outwards to facilitate membrane invagination.

I established a novel approach to reconstruct temporal information of fixed endocytic sites by integrating the images with centroid trajectories from living cells, and obtained a time-resolved superresolution visualization of the highly dynamic mobile phase of endocytosis. This shows that the actin network emerges from a nucleation zone at the base of the plasma membrane, which is determined by nucleation promoting factors.

I therefore propose that the endocytic machinery undergoes a structural transition after an initial phase with variable timing and structures, toward the regular late phase, in which endocytic proteins are radially organized around the coat. A robustly pre-patterned actin nucleation zone prepares the endocytic machinery for the onset of actin polymerization, which represents a key mechanistic step

and marks the transition point into the mobile phase, where the coat is rapidly internalized and a vesicle is formed.

Zusammenfassung

Clathrin-vermittelte Endozytose ist ein essenzieller zellulärer Prozess, um Moleküle von der Zelloberfläche aufzunehmen. Mehr als 50 Proteine bilden die zugrundeliegende makromolekulare Maschinerie, welche in Eukaryoten höchst konserviert ist. Um ihre Konstruktionsweise zu verstehen, welche eine Vesikelbildung mit hoher Effizienz und Regelmäßigkeit erlaubt, ist es notwendig zu untersuchen, wie endozytotische Proteine strukturell organisiert sind. Aufgrund der kleinen Größe, Komplexität und Dynamik der endozytotischen Maschinerie ist die Anordnung dieser Proteine *in situ* jedoch größtenteils unbekannt.

Ich habe Einzelmolekül-Lokalisationsmikroskope verwendet, um endozytotische Strukturen in fixierten Zellen von Bäckerhefe *Saccharomyces cerevisiae* mit hoher räumlicher Auflösung abzubilden. Dabei habe ich eine komplexe Organisation der endozytotischen Proteinen festgestellt.

Mithilfe von Hochdurchsatz-Lokalisationsmikroskopie konnte ich zehntausende endozytotische Strukturen untersuchen. Dabei habe ich eine bemerkenswerte radiale Ordnung gefunden, in welcher die funktionalen Module festgelegte radiale Bereiche besetzen. Je weiter Endozytose fortgeschreitet, desto größer und regelmäßiger werden die Strukturen. Zu Beginn sind die Anordnungen vielfältig in Größe und Form, während sie später einen hohen radialen Organisationsgrad aufweisen. Ich habe entdeckt, dass Aktin-Polymerisation, welche in Hefe zur Endozytose benötigt wird, nur in einem durch Aktin-Nukleirungsfaktoren bestimmten, ringförmigen Bereich auftritt. Dieser Bereich bildet sich um eine Proteinschicht herum, in welcher Proteine die Plasmamembran mit dem Aktinnetzwerk verknüpfen. Durch dieses Ringmuster kann die notwendige Kraft, um die Plasmamembrane einzustülpen, durch die Bildung eines Aktinnetzwerkes effizient erzeugt und auf die Membran übertragen werden. In einem äußeren Ring zieht ein endozytotischer Myosin-Motor möglicherweise das Aktin-Netzwerk auseinander, um den Einstülpungsprozess zu unterstützen.

Ich habe ein neues Konzept entwickelt, um den endozytotischen Zeitpunkt von fixierten Strukturen direkt aus den hochauflösten Bildern zu bestimmen, indem sie mithilfe von Fluoreszenz-Partikelverfolgungs-Daten aus lebenden Zellen ausgewertet werden. Dadurch konnte ich die höchst dynamische mobile Phase der Endozytose mit zeitlicher und hoher räumlicher Auflösung darstellen. Diese Visualisierung zeigte direkt, dass sich das Aktin-Netzwerk aus einer Nukleierungszone auf der Plasmamembran bildet, welche durch Nukleirungsfaktoren gebildet wird.

Ich schlage deshalb ein Modell vor, wie sich die endozytotische Maschinerie organisiert. Nach einer Initiierungsphase mit variablem Zeitablauf und vielfältigen Strukturen gibt es einen Übergang hin zu regelmäßigen, radial organisierten

Proteinanordnungen, welche sich um die zentrale Proteinschicht bilden. Durch diese Organisation wird ein Bereich robust vorbestimmt, in welchen sich später das Aktin-Netzwerk bilden kann. Der Beginn der Aktin-Polymerisierung ist ein wichtiger mechanistischer Schritt, der den Übergang hin zur mobilen Phase der Endozytose bewirkt, in welchem schließlich das Clathrin-umhüllte Vesikel gebildet wird.

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List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
5-FOA	5-fluoroorotic acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AF	Alexa Fluor
ANTH	AP180 N-terminal homology
CP	Capping protein
CME	Clathrin-mediated endocytosis
ConA	Concanavalin A
CLEM	Correlative light and electron microscopy
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
EDTA	Ethylene diamine tetraacetate
EE	Early endosome
EH	Epsin homology
EM	Electron microscopy
EMCCD	Electron multiplying charge-coupled device
ENTH	Epsin N-terminal homology
ER	Endoplasmic reticulum
FRAP	Fluorescence recovery after photobleaching
LB	Lysogeny broth
MVB	Multivesicular body
NPF	Nucleation promoting factor
ORF	Open reading frame
OD₆₀₀	Optical density (600 nm)
PFA	Paraformaldehyde
PBS	Phosphate buffered saline
PIP₂	Phosphatidyl inositol (4,5) diphosphate
PEG	Poly ethylene glycol
PM	Plasma membrane
PCR	Polymerase chain reaction
RFP	Red fluorescent protein

ROI	Region of interest
RNA	Ribonucleic acid
RT	Room temperature
SH3	Src homology 3
SC	Synthetic complete
TIRF	Total internal reflection fluorescence
UIM	Ubiquitin interaction motif
UV	Ultra violet
VPS	Vacuolar protein sorting
WH	WASP homology
WASP	Wiskott-Aldrich syndrome protein
YPD	Yeast extract peptone dextrose
YPAD	Yeast extract peptone dextrose plus adenine

1 | Introduction

1.1 Endocytosis and cell trafficking pathways

The plasma membrane serves as the defining barrier between the internal and external cell, thus creating cellular identity, and facilitating evolution out of the primordial soup into a defined structure that can regulate entry of signals into the cell. In eukaryotes, and with increasing complexity, in multicellular eukaryotes, tuning cellular response to external signals has resulted in a complex network of signaling pathways, and tight coupling of these pathways with the process of endocytosis. Endocytosis is defined as the uptake of molecules too big to pass through the plasma membrane. It involves the invagination of the plasma membrane into a cargo-filled tube, and culminates in the severing of this tube to form a cargo-filled vesicle, whose components and contents are then targeted to other cellular organelles for either degradation or recycling.

Apart from internalizing cargo, endocytosis allows regulation of the plasma membrane itself: its lipid and protein composition, and therefore many physical and biochemical properties like tension, rigidity, surface-receptor composition and localization. Cargo taken up by endocytic pathways include these surface-receptors and their ligands, that are transported across the cell, taking part in a signaling cascade, and forming the link between cell signaling and endocytosis.

Somewhat dramatically, endocytosis “constitutes the major communications infrastructure of the cell. As such, it governs almost all aspects of the relationships of the cell with the extracellular environment and of intracellular communication. Its evolution constitutes, arguably, the major driving force in the evolution of prokaryotic to eukaryotic organisms”¹. Plasma membrane regulation and internalization of signaling molecules are critical for the function of the cell. Among the vast array of important cargo that are taken up via endocytosis are cholesterol^{2,3}, insulin⁴, and other hormones. Not surprisingly, many human diseases have been linked to defects in the endocytic pathway like familial hypercholesterolemia^{2,3} -the study of which established the field of endocytosis-,

Alzheimer's⁵, and some types of cancer⁶. The importance of the endocytic machinery as the entry portal of the cell is evident in the fact that it is hijacked by pathogens like viruses and bacteria to enter host cells⁷. Other components of the cellular signaling pathway transmit signals across the cell and between various organelles like the Golgi apparatus and endoplasmic reticulum. These membranes undergo similar transitions of the bounding membrane, and have mechanistic and biochemical similarities^{8,9}.

Although many early discoveries relating to endocytic pathways were identified in mammalian cell types^{10,11}, description of endocytosis in *S.cerevisiae*¹² marked the beginning of important findings that were made in the yeast and later verified in mammalian cells. The ease of genetic manipulation, availability of the completed sequence of the yeast genome, and relative simplicity of endocytic pathways- there is only one- drove several discoveries that established yeast as a powerful model organism^{13,14}.

1.2 Clathrin-mediated endocytosis

Many different endocytic pathways that facilitate the internalization of cargo at the plasma membrane exist, as depicted in Fig.2, all differing in the size and type of cargo. Of them, Clathrin-mediated endocytosis (CME), is universal among eukaryotes and contributes to 90% of cargo trafficked into the cell¹⁵. First identified by studying yolk uptake in mosquitos, ultrastructural studies of their oocytes (where the concentration of uptake events is high enough to be easily studied) identified a bristly coat formation on the cell membrane and similarly bristly vesicles, that then lost this coat and fused to eventually form yolk bodies in the mature oocyte¹⁶. The bristle was noted in several cell types, and was later identified as a lattice of a single highly conserved protein¹⁷. This protein was named Clathrin, derived from the latin word for lattice. Clathrin is formed of light and heavy chains incorporated into a triskelion¹⁸ that assembles into closed hexagonal and pentagonal structures on the inner leaflet of the plasma membrane. Clathrin-mediated endocytosis has, since four decades ago, been recognized has an ubiquitous mechanism of membrane uptake in cell types ranging from the frog presynaptic membrane¹⁹ to rat vas deferens²⁰.

Clathrin and associated proteins do not only interact with the plasma membrane. It has also been observed localizing to the trans-golgi network (TGN); these clathrin-coated vesicles mediate traffic from the TGN to the endosome. Specification of vesicle target to different cellular compartments is achieved by Clathrin interaction with specialized adaptor proteins like the adaptor protein complexes (AP), which specify Golgi-to-early endosome traffic, while Golgi-localized gamma-adaptin (GGA) complexes specify Golgi-to-late endosome traffic. These membrane associations, among others form components of the cellular

signaling pathway that transmit signals across the cell and between various organelles like the Golgi apparatus and endoplasmic reticulum. These membranes undergo similar transitions of the bounding membrane, and have some mechanistic and biochemical similarities^{13,14}.

1.3 CME in mammalian and yeast cells

1.3.1 Clathrin is required for mammalian CME

That the clathrin lattice is responsible for remodeling the plasma membrane and selecting cargo was speculated in the first papers that noted the “bristly” coat^{16,21}. In multicellular organisms like *C.elegans*, clathrin depleted by RNAi results in decreased endocytic uptake in oocytes and dead progeny²², in *D.melanogaster*, deletion of clathrin heavy chain results in embryonic lethality²³. In Hela cells, knock-down of the heavy chain by RNAi results in decrease in endocytosis by 80%²⁴; essentially, endocytosis fails in the absence of clathrin. The exact contribution of clathrin in the progression of endocytosis has been heavily debated, but its involvement itself has not. Although several genes involved in CME in yeast were found to be homologues of the mammalian machinery, however, early work in yeast revealed that clathrin is not necessary for endocytosis²⁵. Loss of clathrin changes the size of the vesicles formed at scission, and leads to decrease in the number of established endocytic sites^{26,27}: it appears to affect establishment of sites and regulation of scission. It became apparent that though the mammalian and yeast systems were mechanistically similar and most of the yeast endocytic proteins had mammalian homologues²⁸, there are some significant differences.

1.3.2 Actin forces are required for yeast CME

Cortical actin patches were first seen in *S.cerevisiae*, that were later established as endocytic sites from the colocalization of other endocytic proteins. While the mammalian CME uptake is heavily dependent on clathrin, the yeast system relies on actin and its proper organization for endocytosis²⁹. Not only is actin itself necessary for the initiation of plasma membrane deformation³⁰, coupling the endocytic coat to actin are necessary for internalization^{31,32}. The cell wall surrounding the plasma membrane in yeast cells induces a high intracellular turgor pressure³³, which could explain the high force requirement for membrane deformation in yeast.

1.3.3 CME in yeast is highly regular

In yeast, over fifty proteins are recruited, interact, and disassembly during this process. In mammals as well as in yeast, the proteins that arrive at an endocytic site can be distributed into different modules according to their relative time of recruitment and function. A variable initiation phase assembles coat proteins

on the plasma membrane and establishes an endocytic site. While the later proteins show relatively high variability in both recruitment as well as time spent at sites in mammalian cells, in yeast this initiation is followed by a very stereotypic sequence of events that assembles coat proteins, nucleates actin, organizes the actin network, invaginates a membrane tube, and finally severs the membrane to produce cargo-filled vesicles²⁶. Coat proteins arrive upon initiation of endocytic sites, the actin and WASP modules arrive next, and includes actin nucleating proteins, actin, actin-binding proteins that organize the actin network and produce forces that begin to pull the membrane into the cytoplasm. The scission module arrives last, and regulates the final shape transitions of the endocytic site from tubular membrane to vesicle.

Sterotypicity of the post-initiation stages of yeast endocytosis has allowed averaging the behavior of various proteins from multiple endocytic events. Tracking and averaging the behavior of these proteins has led to understanding the spatial and temporal regulation of endocytosis in remarkable detail^{26,34,35}. The multiple stages of endocytosis are discussed below.

1.3.3.1 Early initiation phase

A variable initiation phase establishes endocytic sites and selects cargo³⁶. The earliest proteins to arrive at sites, Ede1 and Syp1 are not required to form endocytic sites. Deletion of an entire seven protein set of early endocytic proteins (Ede1, Syp1, Yap1801/1802, Apl1, Pal1, Pal2) does not prevent endocytosis. It seems that the initiation of endocytosis in yeast is independent of the recruitment of any one protein, and is likely a result of several different cooperative or independent factors³⁶, that could give the process robustness in the absence of alternate pathways for uptake of essential nutrients and signals. The variability in this phase could also provide a “check-point”, to ensure that sufficient cargo is loaded²⁸ before later (energy consuming) phases are triggered.

1.3.3.2 Coat module

Coat proteins serve to template later proteins³⁵, as well as form the link between the actin module³², ingressing membrane, and cargo associated with it. Unlike in mammalian cells, as mentioned earlier, clathrin adaptors and the clathrin triskeleion are not necessary for the progression of sites, although deletion of clathrin introduces a high variability in the timing of scission²⁷.

Deletion of coat proteins Sla2 and Ent1 results in a particular phenotype in which actin polymerization is achieved, but the membrane is decoupled from actin forces, resulting in actin “flames” without membrane bending^{32,37}. The complex between proteins Sla1, Pan1 and End3 links the early coat to other coat proteins and polymerized actin, is involved in actin regulation itself, and

connects vesicles to actin cables and endosomes^{38–40}. The arrival of Sla1 is a strong predictor of successful endocytosis^{26,41}. These coat proteins are pulled upwards into the cytoplasm, and follow the moving membrane.

1.3.3.3 Actin module

Once the coat proteins are assembled, proteins that nucleate and organize the actin machinery are recruited. Actin filaments are nucleated by the Arp2/3 complex, and act in concert with other actin nucleation promoting factors (NPFs), such as the yeast WASP homologue Las17, type 1 myosins Myo3 and Myo5, Pan1, and actin binding protein Abp1. Apart from Pan1, which moves inwards upon membrane movement and forms part of the coat module, the remaining NPFs are recruited to the base of endocytic sites and do not move inwards with the membrane³⁴. Las17 is a potent actin nucleator, without which endocytosis essentially fails⁴². Myo3/5 are non-processive motors that interact with and can translocate actin filaments, but whose mechanistic contribution is unknown. Deletion of either Myo5 or Myo3 has subtle phenotypes, but deletion of both effectively blocked endocytosis⁴². Abp1 binds actin filaments and activates the Arp2/3 complex.

Bbc1, F-BAR protein Bzz1, and Vrp1 are other actin associated proteins that are recruited within the actin module. Bbc1 is known to inhibit Las17 NPF activity, its deletion accumulates actin at endocytic sites⁴³. Bzz1 relieves Las17 of NPF activity inhibition by Sla1⁴². Vrp1 stimulates the Arp2/3 complex, recruits myosins, and interacts with Las1^{44,45}.

Once NPFs and WASP/Myo proteins are recruited, Arp2/3 is recruited and actin polymerization begins. Along with Arp2/3, actin crosslinkers like Sac6 and Scp1, capping protein complexes like Cap1/Cap2, Aip1/Cofilin, Abp1/Aim3 are recruited. This begins the invagination of membrane, along with the coat proteins. Actin monomers are added at the base of the invagination, and coupled into the membrane via coat proteins, so as actin polymerization progresses, the entire actin network is pushed upwards, taking the membrane along with it³⁴.

1.3.3.4 Scission module

While the role of the yeast dynamin-like Vps1 is unclear, relatively few copies of the Rvs complex are recruited in a time window that spans only a few seconds, and membrane scission occurs when the invagination is about 140nm long, indicating tight regulation of this transition^{30,34}. Coat proteins and the actin network are rapidly disassembled by phosphorylation and dephosphorylation of the components. What actually regulates scission in yeast is not yet determined (see 1.3.5).

1.3.4 Membrane scission in mammalian cells

1.3.4.1 Scission is dependent on dynamin

In mammalian cells, membrane scission in endocytosis is primarily effected by the GTPase dynamin. Dynamin was discovered as a microtubule interacting protein 46, and since has been shown to have a pivotal role in membrane scission and fission at many different organelles across the cell. The importance of dynamin in endocytosis was demonstrated in a temperature sensitive mutant of the *Drosophila shibire* gene, which results in paralysis of flies at the non-permissive temperature. These flies fail to form synaptic vesicles 47–49. *Shibire* codes multiple isoforms of dynamin that are differentially expressed across the organism 50. Knock-down of dynamin isoforms results in initiation of clathrin-coated pits, but vesicle formation is disrupted, resulting in accumulation of a large number of long membrane tubes 51.

1.3.4.2 Dynamin is an oligomeric GTPase

Dynamins consist of a GTPase domain, a stalk region, a bundle signalling element that acts as the linker between the GTPase domain and stalk, a PIP2-binding pleckstrin homology domain (PH) domain and a proline rich domain (PRD) that extends beyond the GTPase domain 52. In-vitro, dynamin oligomerizes into helical structures with the PH domain apposed against the membrane, and the GTPase domain facing away from the membrane 53,54. Dynamin within the helical structure undergoes conformation changes upon GTP hydrolysis that constricts the helix as well as the membrane tube under it, collapsing the inner leaflet of the bilayer membrane into a hemi-fused state, resulting in membrane fission 55. Disruption of its GTPase activity results in membrane tubes that accumulate dynamin, as well as the BAR domain proteins endophilin and amphiphysin 56–58

1.3.4.3 Dynamin interacts with BAR proteins to cause scission

Dynamin arrives at clathrin-coated pits via interaction with BAR proteins endophilin and amphiphysin 51. BAR domain proteins form intrinsically curved protein dimers named for the conserved module contained in their founding members, metazoan BIN/ Amphiphysin and yeast proteins Rvs167, Rvs161. In addition to the BAR domain, most BAR proteins have additional motifs that mediate their interaction with membranes or other proteins: some BAR proteins have an N-terminal amphiphatic helix (N-helix) that is inserted into the membrane, phosphoinositide binding motifs like phox or pleckstrin homology (PH) domains direct BAR proteins to specific lipids within membranes, some BAR proteins have Src homology 3 (SH3) domains that mediate protein-protein interaction. These SH3 regions act as a scaffold for the proline-rich domains of dynamin 59.

1.3.4.4 Dynamin and BAR proteins interact via PRD and SH3 regions

Dynamin's PRD interacts with the SH3 domains of BAR proteins endophilin and amphiphysin^{59–62}. Endophilin recruitment is reduced in the absence of dynamin, and appears to inhibit the GTPase action of dynamin^{60,62,63}, while dynamin recruitment is decreased without endophilin. Amphiphysin levels are unchanged in absence of dynamin, while deletion of amphiphysin results in increased recruitment and prolonged lifetimes of dynamin and absence of membrane scission⁶⁰. These results suggest a role for amphiphysin for disassembly of dynamin involving GTP hydrolysis, and a role for endophilin in dynamin assembly, although the mechanistic interplay between the two BAR proteins with dynamin is still debated, and the sequence of events is not clear^{63,64}. Dynamin localization to localize to clathrin-coated pits is not dependent on BAR proteins, but both GTP hydrolysis and interaction with BAR proteins is necessary for efficient vesicle scission^{60,65}.

1.3.5 Membrane scission in yeast

1.3.5.1 Yeast dynamin-like proteins

In yeast, three dynamin-like large GTPases have been identified: Vps1, Dnm1, and Mgm1. Dnm1 and Mgm1 are involved in mitochondrial fission and fusion⁶⁶. Vps1 is essential for vacuolar protein sorting⁶⁷, is involved in fission and fusion of vacuoles⁶⁸ and peroxisomes⁶⁹, is required for regulation of golgi to endosomal trafficking⁷⁰, and may arrive at early endocytic events⁷¹. None of the three yeast dynamins have the typical PH domain^{72,73} that in mammalian interacts with the lipid bilayer. Instead, an “InsertB” region likely performs the same function. Although yeast dynamins also do not have PRDs that could interact with the SH3 domains of yeast BAR proteins, Vps1 has been shown to interact with the clathrin and other endocytic proteins^{71,74,75}, though other work has failed to observe localization of Vps1 at endocytic sites^{41,74}. The role of Vps1 in endocytosis is not clear, but it is a candidate for the role of the canonical dynamin in CME.

1.3.5.2 Yeast BAR domain proteins Rvs161/167 regulate scission timing

In yeast, the Amphiphysin/ Endophilin homologue is the heterodimeric complex Rvs161/167⁷⁶ (Rvs), of which Rvs167 has an SH3 domain. Rvs arrives at endocytic sites in the last stage of the endocytosis, and disassembles rapidly at the time of membrane scission³⁴. Deletion of Rvs results in failure of membrane scission in nearly 30% of endocytic events²⁶. Scission failure is identified by the movement inwards of the plasma membrane into the cytoplasm, followed by its retraction back towards the cell wall, indicating a failure to form vesicles. No mutation of known endocytic proteins exhibits this phenotype, while some mutations like that of the yeast Syndapin Bzz1 and Synaptojanin Inp52, in the

background of *rvsΔ*, exacerbates the retraction phenotype⁴¹. This unique profile suggests that although Rvs is not necessary for scission, localization of the complex makes scission more efficient, and Rvs likely acts in concert with other proteins to effect this efficiency.

1.3.5.3 What causes scission?

How Rvs may affect scission has not been determined. Since yeast dynamins do not have a PRD, there is likely no interaction with Rvs, so a mechanism that does not involve PRD-SH3 interactions like in mammalian cells is likely necessary. Yeast cells are under high turgor pressure that makes forces from actin polymerization necessary for invagination^{77,78}. There is therefore likely to be some interplay between scission-stage proteins and the actin network that could modulate the final shape transitions.

Proposed scission mechanisms

Several scission models have been presented in the literature so far. Yeast dynamin is the obvious solution to membrane scission. Although none of the three dynamin-like proteins has a proline-rich domain, Vps1 has been suggested to play a role in endocytosis⁷¹. *vps1Δ rvs167Δ* double mutant has been shown to increase membrane retraction rates after invagination⁷⁹, an indication of scission failure. Another hypothesis has proposed that lipid hydrolysis by yeast synaptojanin-like proteins can cause vesicle scission⁸⁰. Synaptojanins dephosphorylate PIP2, a lipid subtype enriched at endocytic sites. In this model, Rvs would form a scaffold on the membrane tube, protecting the underlying PIP2 and causing a boundary between BAR-protected PIP2 at the tube and hydrolyzed PIP2 at the bud tip. This lipid boundary produces a line tension at the interphase that could generate enough force to pinch off a vesicle.

In-vitro experiments have proposed protein friction as a mechanism by which membrane scission could occur⁸¹. In this model, a BAR domain scaffold exerts a frictional force on a membrane that is pulled under it. Such a friction-dependent membrane scission model would predict that if more BAR proteins are added to the membrane, frictional force would increase, and scission should occur at shorter invagination lengths. *In-vivo*, this pulling force is generated by actin polymerization.

Recently, steric pressure exerted on the membrane by disordered protein domains that typically follow the BAR region has been proposed as a mechanism for scission⁸². In these experiments, the amphiphysin BAR domain is able to drive scission, but scission efficiency increases three to four-fold because of the disordered protein domains, and these domains do not need to have any specific biochemical properties: they can be replaced by any other disordered protein

region. It has also been proposed that BAR domain scaffold membrane tubes and stabilize them, preventing scission^{83,84}. For a more detailed discussion on these models, see /refresults

Many of these theories are contradictory, based on in-vitro data, using mammalian BAR proteins, at concentrations of protein orders of magnitude higher than physiological levels, and without the context of interaction partners, relevant membrane tension, native lipid composition and intra-cellular turgor pressure: a mechanism for membrane scission in yeast is yet to be determined.

1.4 BAR domain proteins

The BAR protein superfamily have a highly conserved BAR domain structure across eukaryotes and are involved in a range of cellular processes including endocytosis, actin organization, cell polarity, transcription and tumor suppression^{85,86}. Of the mammalian isoforms of the founding members, Bin1 (Amphiphysin II) and Bin3 are ubiquitously expressed, while Amphiphysin I is expressed only in neurons. The conserved portion of these proteins, as well as of Rvs167 and Rvs161, is an N-terminal region that forms the BAR domain. This domain typically forms dimers with other BAR domains, and have an intrinsic curvature defined by the dimerization angle. This curvature categorizes BAR proteins to classical BAR, Fer-Cip4-homology-BAR (F-BAR, shallow curvature), and I-BAR (inverted curvature). Membrane-binding is mediated by cationic clusters that bind via non-specific electrostatic interactions to anionic lipids like phosphatidyl serine (PS) or phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P₂ „ henceforth PIP₂).

BAR dimers are able to oligomerize and scaffold large areas of membrane. These scaffolds can tubulate and generate curvature across membrane regions much larger than the dimensions of a BAR dimer^{62,87}. BAR scaffolds can also bind membranes in a curvature-dependent manner. Correlation between the membrane shapes that they bind in-vivo and their intrinsic curvature has been shown for many BAR proteins: they may induce, stabilize, or generate specific curvature within cells.

1.4.1 NBAR proteins and membrane shapes

Classical BAR domain proteins form a crescent-shaped structure. Some of them have an N-terminal amphiphatic helix (N-helix), forming a subclass of classical BAR called NBAR domains. The two significant endocytic BAR proteins, Endophilins and Amphiphysins, are NBAR proteins. The 35-40 residue N-helix acts as an amphiphatic wedge that is unstructured until it is inserted into the upper leaflet of a membrane bilayer⁸⁷. The insertion causes displacement of lipids,

resulting in bending of the membrane, indicating that N-helix insertion into a membrane bilayer could favor membrane scission both energetically and kinetically^{84,88}. BAR domains lacking this helix are not able to efficiently tubulate vesicles⁸⁹. The N-helix also increases efficiency of binding to liposomes⁶² in a curvature sensitive manner, and confers salt sensitivity⁸⁹.

High resolution structural data has shown that NBAR proteins can hold form helical scaffolds on tubular membranes^{87,90,91}. An energetically favorable arrangement of BAR domains consist of dimers parallel to each other, apposed to the membrane, supporting membrane tubulation and preventing scission by stabilizing the membrane tube⁸⁴. Hybrid N-helix and BAR scaffolds can therefore allow coexistence of both vesicles and tubules, with preference for one or the other depending on the ratio between number of N-helices that favor vesiculation, and BAR generated scaffold stability⁸⁴.

Both BAR proteins implicated in CME , Amphiphysin and Endophilin are shown to tubulate membranes in-vitro^{87,89,91} and form a helical scaffold. The tubulation diameter resembles the diameter of the proteins themselves, and involve lateral interactions of the neighboring BAR domains⁹². Both BAR domains are able to form mixed helices in the presence of dynamin^{62,93}.

1.4.2 NBAR protein in endocytosis: Amphiphysin

Two mammalian isoforms of Amphiphysins (Amph) are found. AmphI is enriched in neurons in mammals, while AmphII (Bin1) is expressed in other tissue types, with one isoform enriched in muscle T-tubule junctions⁹⁴. The only Amphiphysin (d-Amph) in flies is expressed in various tissues, and enriched at muscle T-tubule junctions in flies. The d-Amph dimer forms a coiled coil, with each BAR domain made of three long, kinked alpha-helices⁸⁷. In-vitro, liposome tubulation activity of Amphiphysin is concentration dependent, at very high concentrations, it is also able to sever tubular membrane to form vesicles⁸⁷.

Amph I and II both have BAR domains, a proline rich region, and C-terminal SH3 domain. Amphiphysin I, but likely not II binds Clathrin and its adaptors⁹⁵ and can polymerize clathrin into invaginated lattices in a BAR domain dependent manner⁸⁷, while both bind dynamin, and the lipid phosphatase Synaptjanin⁶¹.

1.4.3 NBAR protein in endocytosis: Endophilin

Endophilins A1-A3 (EndoA) were discovered as SH3 domain containing proteins⁹⁶ that co-localized with dynamin, and interacted with Synaptjanin⁵⁸ and amphiphysin⁹⁷: all already identified as important regulators of synaptic vesicle recycling by endocytosis. A second mammalian protein was later discovered

as related, and then termed EndophilinB (EndoB). Other sequenced eukaryotes have a single isoform of EndoA and B.

EndoA1-3 isoforms are found in neurons, ubiquitously, and enriched in the brain and testes respectively. All three are found at presynaptic membranes. Crystal structure of EndoA1 shows essentially the same structure as that of amphiphysin, with an additional amphiphatic helix similar to the N-helix, located at the centre of the crescent-shaped dimer^{99,98}. This helix is thought to insert into the membrane in the same way as the N-helix, potentially inducing faster tubulation of membranes. EndoA1 and 2 may interact with calcium channels at synapses, and may be involved in lipid modification^{99,100}, suggesting different roles for the two BAR domain proteins in membrane interaction. Endophilin interacts with dynamin, NWASP and Synaptojanin proteins via its SH3 domain^{61,63,101}.

1.4.4 NBAR protein in yeast endocytosis: the Rvs complex

RVS167 and RVS161 (reduced viability upon starvation) genes were discovered in a screen that tested for survival under starvation conditions¹⁰². Rvs167 and Rvs161 are both NBAR domain proteins that thought to form obligate heterodimeric complexes (Rvs) in-vivo^{103,104}. Although there is evidence of heterodimerization: loss of one destabilizes the other, deletion phenotypes of Rvs167 is the same as that of Rvs161, and FCCS measurements indicate that they dimerize^{26,103,105}, it has also been reported that Rvs161 has some functions that do not match that of Rvs167. Rvs161 for instance, interacts with Fus2 in cell-cell fusion, while Rvs167 does not¹⁰⁶. It is consistent however, that at endocytic sites they function together as heterodimers.

Rvs161 and Rvs167 are similar in structure at the N-terminus, both contain NBAR domains that are 42% similar, and although share 21% identity, are not interchangeable¹⁰⁷. In addition to the BAR domain, Rvs167 has a Glycine-Proline-Alanine rich (GPA) region and a C-terminal SH3 region. 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 recapitulated by expression of the BAR domain alone¹⁰⁴, suggesting that the BAR domains are the main functional unit of the complex.

Deletion of the genes show abnormal actin morphology, confer salt sensitivity, as well as amino-acid and lipid sensitivity, and have abnormal budding pattern^{103,108–110}. Homology modelling has shown that the BAR domain of Rvs167 is similar to Amphiphysin and Endophilin, and is therefore also likely to

function similarly to the mammalian homologues. In keeping with this theory, Rvs has been shown to tubulate liposomes in-vitro¹¹¹.

Averaged centroid tracking of the Rvs complex has shown that Rvs arrives in the scission stage of endocytosis. When maximum number of Rvs is recruited, that is, at peak fluorescent intensity, the centroid jumps inwards, concomitant with a sharp decay in fluorescent intensity. This behavior is unique among endocytic proteins, and since similarity in structure with Amphiphysin/ Endophilin BAR domains is expected, has led to the proposition that Rvs may also form a helical scaffold on the membrane tube, whose sudden disassembly either leads to or is caused by membrane scission. The sharp movement into the cytoplasm of the Rvs centroid is then caused by the disassembly of the scaffold, and a jump in the centroid position to the remaining Rvs on the base of the newly formed vesicle³⁴. How Rvs is recruited to endocytic sites, and the cause of the scaffold disassembly are not known, and are the major questions addressed in this work.

2 | Aims of the study

More than 50 different proteins are involved in clathrin-mediated endocytosis. At endocytic sites, they assemble into a small, complex and dynamic macromolecular machinery. Although individual components have been identified and well-characterized during decades of research, their structural organization is poorly understood. In my PhD project, I proposed that single-molecule localization based superresolution microscopy provides both the molecular specificity and necessary spatial resolution to study how proteins are arranged *in situ* within the endocytic machinery. More specifically, I addressed the following questions:

- How is Rvs recruited to endocytic sites?

The recently developed technique of localization microscopy critically depends on dense and specific fluorescent labeling of the cellular structure of interest with a dye suitable for localization microscopy. In the first part of my project, I established an optimized sample preparation pipeline to enable high quality dual-color localization microscopy of yeast cells. These efforts are described in section ??.

- How does it regulate scission

3 | Results

3.1 Recruitment of Rvs to endocytic sites

Curvature sensing or generation?

Cellular membrane shape is a result of properties like rigidity, tension, intracellular pressure, and are influenced by the lipid composition and the proteins embedded in it^{1,2}. Since tension, pressure, and rigidity all oppose membrane deformation, energy is required to deform and bend it. BAR domains can generate curvature if the energy required to deform the membrane is less than the energy spent in binding flat membrane.

Scaffolding as a curvature-generation mechanism has been extended to various types of BAR proteins, (Arkhipov et al., 2009; Frost et al., 2008; Henne et al., 2007; Itoh et al., 2005; Pykalainen et al., 2011; Saarikangas et al., 2009; Shimada et al., 2007; Yu and Schulten, 2013). In order for BAR scaffolds to impose membrane curvature, some requirement have to be met³: they have to have present a large membrane-interacting surface that can mediate membrane binding, have intrinsic curvature that can be imposed on the surface, and have a rigid structure that can overcome bending resistance of the membrane. Because of their shape (Peter 2004, Gallop 2006, Weissenhorn 2005), and their capacity to oligomerize into large assemblies on tubes (Mim 2012, Mizuno 2010, Takei 1999, Yin 2009), it has been suggested that BAR domains impose their shape on the membrane, and generate membrane curvature in cells. Further, tubulation both in-vivo and of liposomes is dependent on the rigidity of the central crescent-shaped domain⁴. The N-helix of NBAR domains can generate curvature independently of the BAR scaffold (Varkey 2010, Westphal and Chandra 2013).

For endophilin, the BAR domain is relatively far from the membrane, suggesting a mechanism dependent on the N-helix (Jao 2010). Different BAR domains thus likely employ different mechanisms to interact with the membrane for generating vesicles, and tubes (Ambroso 2014). For endophilin, for example, the N-helix is necessary for liposome binding⁵, while that of amphiphysin is important, but not necessary⁶.

Curved BAR proteins that can induce curvature are also able to sense curvature: in-vitro, BAR domains show a preferential-binding to vesicles based on their intrinsic curvature. Curvature-generation and sensing seem to intrinsically couple mechanisms. That BAR domains are able to generate curvature does not imply that this is their function, at least in endocytosis: in-vivo, the significance of curvature-generation is not determined. Tracking over thirty different endocytic proteins in NIH-3TC cells (derived from mouse fibroblasts), TIRF imaging shows that Endophilin2 and Amphiphysin1 arrive late in the endocytic time-line right before scission⁷, suggesting they arrive when membrane tubes are already formed.

In the case of Rvs, centroid tracking and averaging shows that the complex similarly localizes to sites late in the endocytic timeline, close to scission⁸. CLEM studies have further shown that Rvs localizes to sites after the membrane invaginations are about 60nm deep into the cytoplasm: Rvs localizes once membrane curvature is established. Whether this localization is dependent on membrane curvature, recognized by the BAR domain has not been shown.

Rvs localization in yeast endocytosis As has been shown before, Rvs localizes to endocytic patches at the yeast plasma membrane in the late scission-stage. When imaged at the plasma membrane, rather than a slow inward movement typical for coat proteins like Sla1, Rvs167-GFP shows a sharp jump into the cytoplasm, concomitant with scission^{8,9}. The average lifetime of Rvs167-GFP is about 7secs, as measured by epifluorescence microscopy at the equatorial plane of a haploid yeast cell.

3.1.1 BAR domain senses membrane curvature in-vivo

Rvs167 without the BAR domain, that is, Rvs167-delsh3 localizes to cortical patches (Fig.2.3). In order to test whether this localization is because of membrane curvature, I studied its dynamics in sla2del cells. Sla2 is a coat protein that acts as a linker between the membrane and the actin cytoskeleton, and allows forces generated within the actin network to be transmitted to the membrane¹⁰. In sla2del cells, rather than cortical actin patches, actin “flames” appear inside the cytoplasm. Electron microscopy shows that the membrane is flat in these cells: although actin is able to polymerize and recruit other actin-binding proteins, actin forces are decoupled from the membrane (Fig.2.2 schematic).

In sla2del cells, Rvs167-GFP is recruited to the plasma membrane (Fig.2.3), while in sla2del cells expressing Rvs167-delsh3GFP (henceforth BAR-GFP), localization is removed, except for rare transient patches at the plasma membrane.

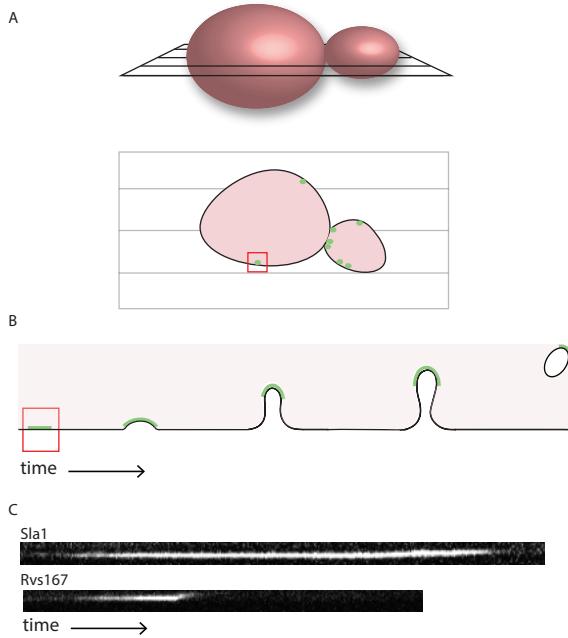
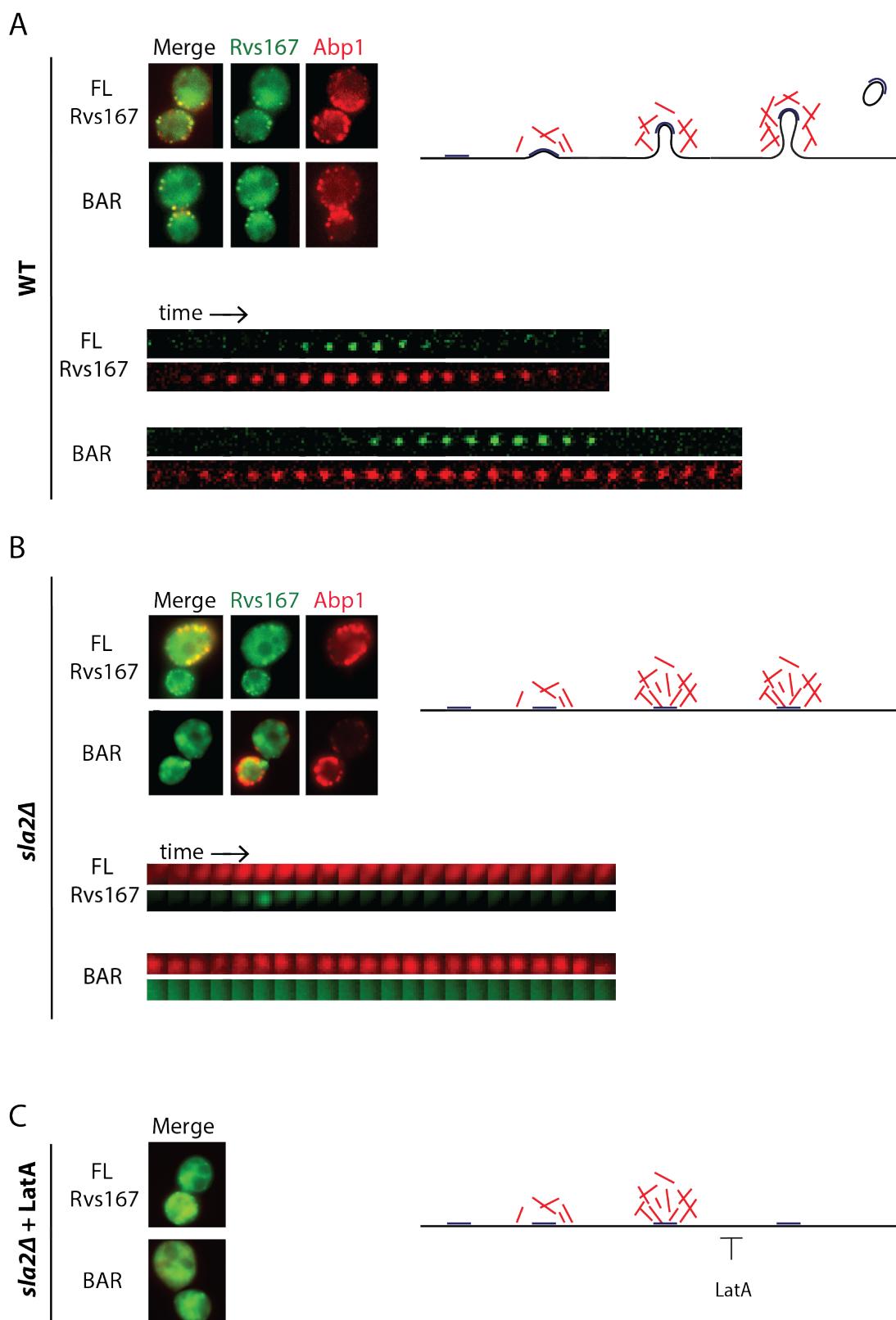


FIGURE 3.1: The Unit Circle

3.1.2 SH3 domain is able to recruit Rvs in an actin-independent manner

Full-length Rvs is able to localize to cortical patches in Sla2del cells. This localization must come from the SH3 domain, since BAR alone does not localize in these cells. We expected that the SH3 domain must interact with WASP or actin-binding proteins: an interaction with Abp1 has been shown, as well as with Las17, type I Myosins, and Vrp1. In order to prove this, I imaged BAR-GFP in cells treated with the actin sequestering agent LatrunculinA (LatA). LatA is a sea-sponge toxin that binds monomeric actin, and prevents incorporation of actin into filaments. Since a high actin turnover is required at endocytic sites, LatA effectively disassembles WASP components and other actin-binding proteins of the endocytic machinery, and blocks endocytosis.

Surprisingly, full-length Rvs is localized to the plasma membrane in spite of the LatA treatment, suggesting that the SH3 domain is able to recruit Rvs to the plasma membrane. This recruitment occurs in the absence of a BAR-membrane interaction, since BAR-GFP localization is completely removed in LatA treated cells. Rvs167-GFP patches are transient, so an assembly-dissassembly mechanism is mediated by the SH3 domain outside of its BAR domain interaction. Localization of Rvs161, which does not have an SH3 domain, is also removed by LatA treatment, supporting the conclusion that the BAR domain senses membrane curvature in-vivo.

FIGURE 3.2: Rvs localization in *sla2* deletion

3.1.3 What does the SH3 domain interact with?

3.1.3.1 Vrp1

3.1.3.2 Type 1 myosins

3.1.3.3 Las17

3.1.4 Other potential mechanisms of assembly/ disassembly of Rvs

3.1.4.1 Interaction with Calmodulin

3.1.4.2 FBAR protein Bzz1

3.2 Role of the SH3 domain

Is likely that SH3 domains, are involved in modulating oligomerization (5, 14) and MC-S, G (15, 82).

3.3 Role of the N-helix

3.4 Scission mechanisms

3.4.1 Membrane scission is not dependent on Vps1

Yeast dynamin is the obvious solution to membrane scission. Although none of the three dynamin- like proteins has a proline-rich domain, one of the yeast dynamins, Vps1 has been suggested to be involved in endocytosis^{11,12}. Rooij et al., suggest that Vps1 localizes to endocytic sites in the late scission stage, and that the vps1 Δ rvs167 Δ double mutant increases membrane retraction rates after invagination, an indication of scission failure. Vps1-GFP does not localize to endocytic sites in Gadila et at.¹³, but localizes to the golgi body and to vacuoles. Kishimoto et al, do not find a colocalization between Vps1 and Abp1 localization, and also report that the vps1 Δ rvs167 Δ double mutation does not affect membrane retraction rates. Vps1 tagged with both GFP as well as superfolded GFP, and imaged by TIRF microscopy fails to colocalize with Abp1 (data not shown, personal communication with Andrea Picco). The debate concerning the involvement of Vps1 in membrane scission in yeast has been compounded by the possibility that the GFP tag at the Vps1 C-terminal could interfere with its localization to endocytic sites, or its interaction with the Rvs complex.

In order to exclude the possibility of interference from the GFP tag, I investigated the role of Vps1 by studying coat and scission proteins in vps1 Δ cells. The late coat protein, Sla1 is used as a marker for coat movement, and Rvs167 marks scission time. Centroid tracking and averaging is performed as described in Picco et al., and inward movements of the both in wild-type and vps1 Δ cells are compared.

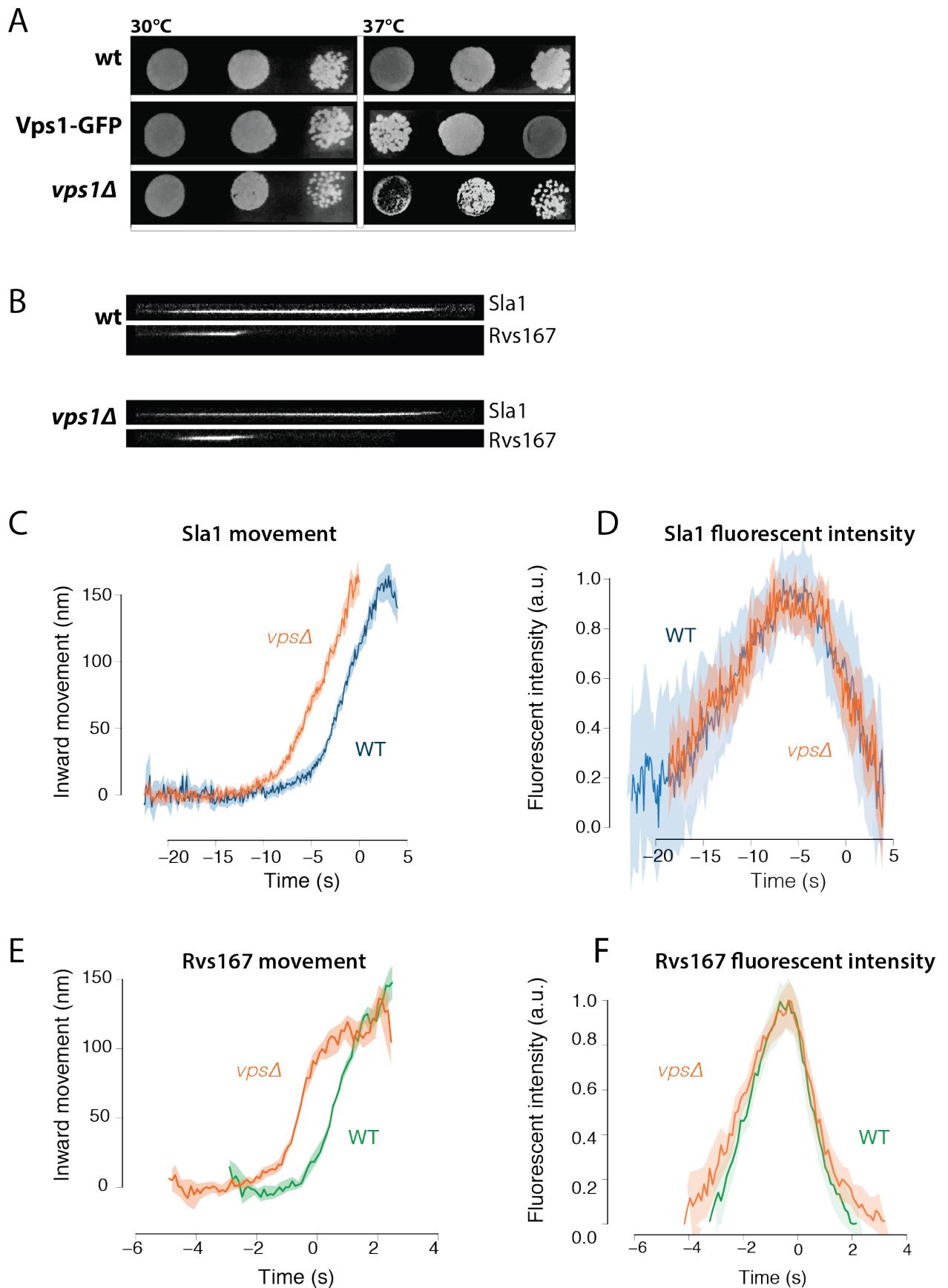


FIGURE 3.3: Rvs localization in vps deletion

vps1Δ cells exhibit a growth defect at 37C, as has been reported¹¹. Sla1-GFP accumulates at the plasma membrane, moves inwards, and disassembles like in WT. The average lifetime of Sla1-GFP at endocytic patches remains unchanged. In WT cells, Sla1 moves into the cytoplasm about 140nm before membrane scission occurs. If Vps1 was required for membrane scission, Sla1 would be expected to undergo delayed or failed scission. However, *vps1Δ* does not increase the rate of membrane retraction. Inward movement of Sla1 is also not changed: it moves inward at the same rate, and to similar maxima of 140nm. Further, the averaged centroid of Rvs167 would not show the sharp jump into the cytoplasm if scission failed. If scission was delayed, the average lifetime of Rvs167 would increase. The inward movement of Rvs167, and its average lifetime, however, remains the same as in WT. I conclude that if Vps1 does localize to endocytic patches in *S.cerevisiae*, it is not involved in regulating membrane scission.

3.4.2 Lipid hydrolysis and membrane scission

Can lipid hydrolysis drive membrane scission? Another hypothesis has proposed that regulated lipid hydrolysis can cause vesicle scission¹⁹. Phosphatidylinositol (PIs) and their derivatives play important roles in many cellular processes including membrane trafficking and cell signalling. Conversion between lipid types is driven by kinases, lipases, and phosphatases and controlled throughout the membrane trafficking pathway.

Phosphatidylinositol(4,5)-biphosphate (PI (4,5) P₂) is an important lipid type found at the cell surface, and is enriched and depleted from endocytic sites at the plasma membrane in concert with the assembly and disassembly of the endocytic machinery. Synaptojanins form a subset of inositol polyphosphate 5-phosphatases that hydrolyze PI(4,5)P₂ to PI(4)P by removing the phosphate at the 5' position of the inositol ring, and play a role in CME and intracellular signalling, as well as in modulating the actin cytoskeleton¹⁴. Synaptojanins localize to endocytic sites, and in mammalian cells, disruption of Synaptojanin genes results in cellular accumulation of PI(4,5)P₂ and coated vesicles at the plasma membrane, suggesting a role for lipid hydrolysis in releasing coat proteins from nascent vesicles. Syaptojanins contain an N-terminal homology domain with the cytoplasmic domain of the yeast SAC1 gene, that is implicated in lipid metabolism, actin morphology, and vesicle transport in the secretary pathway¹⁵. A central catalytic domain is followed by a proline-rich C-terminal regions that are the canonical interaction partners of SH3 domains: they are known to interact with actin binding proteins and BAR domain proteins, potentiating also a role in membrane invagination and scission.

The yeast encodes three Synaptojanin-like proteins- Inp51, Inp52 and Inp53- that regulate phospholipid metabolism. Double deletion of Inp51 and Inp52

has been shown to increase the lifetime of endocytic proteins and produce aberrant membrane invaginations that could indicate scission failure and defective endocytosis, although uptake of extracellular membrane appears to proceed in spite of the morphological aberrations^{16,17}. Deletion of Inp52 along with Rvs167 increases scission failure rate, supporting a possible role in membrane scission¹⁸. Loss of inp51 mutation shows a increase in bulk PIP2 level, although changes in PIP2 levels have not been reported for mutations of inp52, and are not measured locally at the endocytic sites^{19,20}.

Loss of yeast synaptojanins does not significantly affect coat and Rvs dynamics

In a model proposed by Liu et al, Synaptojanins and BAR proteins interact to regulate PI(4,5)P₂ hydrolysis, which in turn drives membrane scission. Here, the Rvs scaffold on the membrane tube protects the underlying PIP2 from hydrolysis. Synaptojanin arrives at sites, and hydrolyses unprotected PIP2. This generates a boundary between BAR-protected PIP2 at the tube and PIP at the bud tip. The lipid boundary produces a line tension at the interphase that would generate enough force to pinch off a vesicle.

I tested this model by investigating the effect of synaptojanin deletion on coat and scission proteins. First, of the three yeast Synaptojanins, only Inp52-GFP localizes to cortical patches. Dual-color imaging and time alignment with Abp1 as described in Picco et al., shows that Inp52 localizes to endocytic sites at the late stage of scission along with Rvs. The centroid of Inp52-GFP can be localized to the tip of the invaginated tube, consistent with the Liu theory of membrane scission: spatial and temporal localization is consistent with influence on scission. Inp51-GFP exhibits a diffuse cytoplasmic signal, while Inp53 localizes to patches within the cytoplasm, likely to the trans-golgi network, as has been noted in other work.

Deletion of Inp52 does not affect the speed of membrane invagination, as reported by the movement of the Sla1 centroid. Sla1-GFP patches are assembled and disassembled, as are Rvs167-GFP patches. All Sla1-GFP patches in the movies analysed ($n=13$ cells) move inwards. Dense clusters at bud neck are not considered. 72.9% of Rvs167-GFP patches move inwards into the cytoplasm ($n=4$ cells, 37 patches). Remaining patches are disassembled without apparent inward movement. Dense clusters at bud neck are not included in the statistics. Vesicle scission appears to occur similar to wild-type, since the Rvs167 centroid moves inwards to approximately the same distance into the cytoplasm, indicating that the base of the vesicles are likely at the same position as in wild-type. Both Sla1 and Rvs167 centroids however, persist post-scission (arrowheads in figure) instead of disassembling immediately like in the WT. Since majority of the patches move inwards, and the increase in the lifetime of Rvs is post-scission, I find that

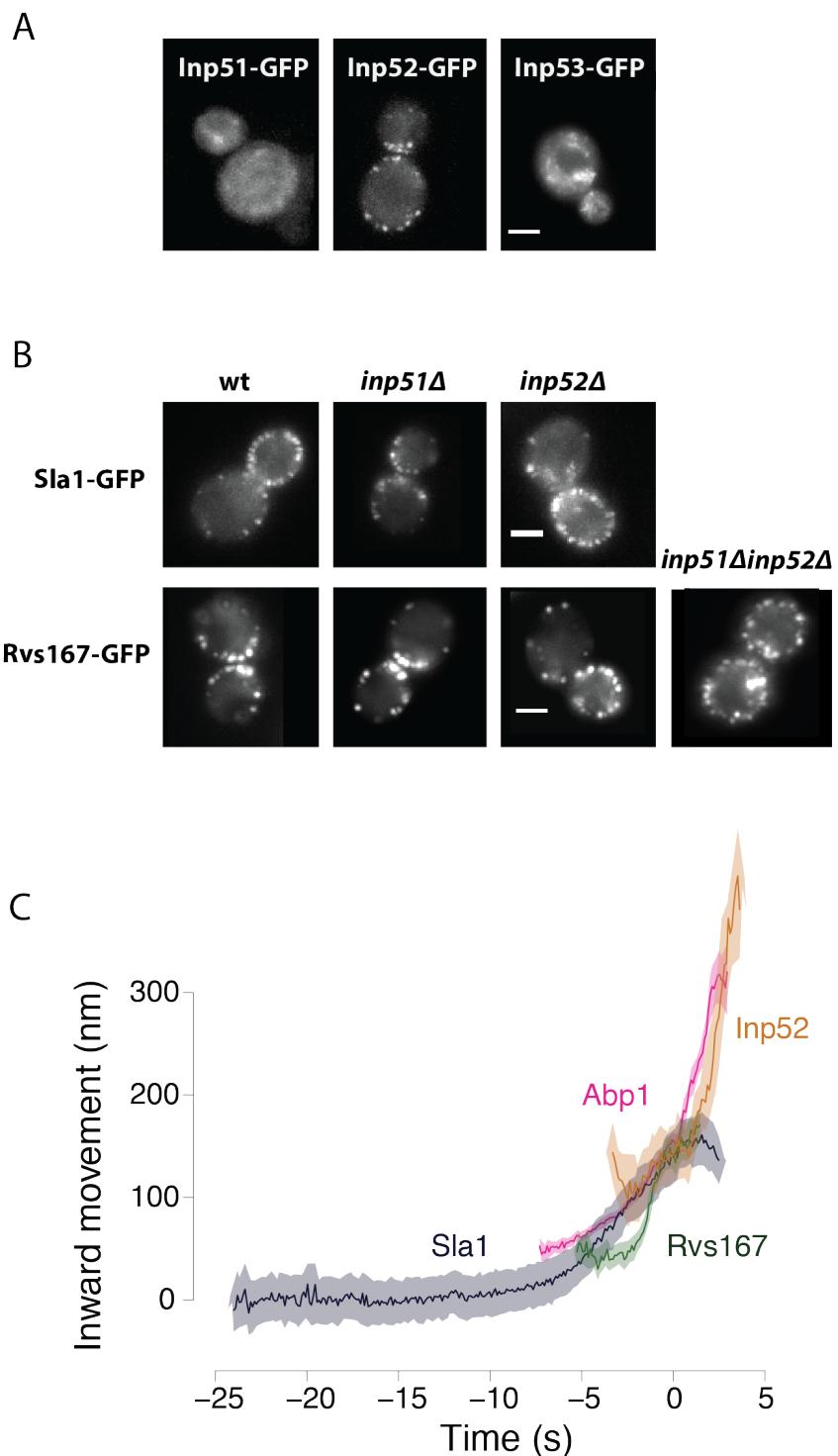


FIGURE 3.4: Yeast synaptojanins

the data is consistent with a role for Inp52 in vesicle uncoating, rather than a primary role in membrane scission, with the aberrations in plasma membrane morphology consequent of failure to recycle components, rather than scission.

Deletion of Inp51 does not affect Rvs167 or Sla1 centroid movement. All Sla1-GFP patches move inward (n=19 cells). 93% of Rvs167-GFP patches move inward (n=3 cells, 44 patches), similar to WT. Assembly of Rvs167 in the Inp51del is slowed, the implication of this delay is not thus far clear.

3.4.3 Protein friction does not induce membrane scission

Yeast dynamin is the obvious solution to membrane scission. Although none of the three dynamin- like proteins has a proline-rich domain, one of the yeast dynamins, Vps1 has been suggested to be involved in endocytosis^{11,12}. Rooij et al., suggest that Vps1 localizes to endocytic sites in the late scission stage, and that the vps1Δ rvs167Δ double mutant increases membrane retraction rates after invagination, an indication of scission failure. Vps1-GFP does not localize to endocytic sites in Gadila et at.^{,13}, but localizes to the golgi body and to vacuoles. Kishimoto et al, do not find a colocalization between Vps1 and Abp1 localization, and also report that the vps1Δ rvs167Δ double mutation does not affect membrane retraction rates. Vps1 tagged with both GFP as well as superfolded GFP, and imaged by TIRF microscopy fails to colocalize with Abp1 (data not shown, personal communication with Andrea Picco). The debate concerning the involvement of Vps1 in membrane scission in yeast has been compounded by the possibility that the GFP tag at the Vps1 C-terminal could interfere with its localization to endocytic sites, or its interaction with the Rvs complex. Yeast dynamin is the obvious solution to membrane scission. Although none of the three dynamin- like proteins has a proline-rich domain, one of the yeast dynamins, Vps1 has been suggested to be involved in endocytosis^{11,12}. Rooij et al., suggest that Vps1 localizes to endocytic sites in the late scission stage, and that the vps1Δ rvs167Δ double mutant increases membrane retraction rates after invagination, an indication of scission failure. Vps1-GFP does not localize to endocytic sites in Gadila et at.^{,13}, but localizes to the golgi body and to vacuoles. Kishimoto et al, do not find a colocalization between Vps1 and Abp1 localization, and also report that the vps1Δ rvs167Δ double mutation does not affect membrane retraction rates. Vps1 tagged with both GFP as well as superfolded GFP, and imaged by TIRF microscopy fails to colocalize with Abp1 (data not shown, personal communication with Andrea Picco). The debate concerning the involvement of Vps1 in membrane scission in yeast has been compounded by the possibility that the GFP tag at the Vps1 C-terminal could interfere with its localization to endocytic sites, or its interaction with the Rvs complex.

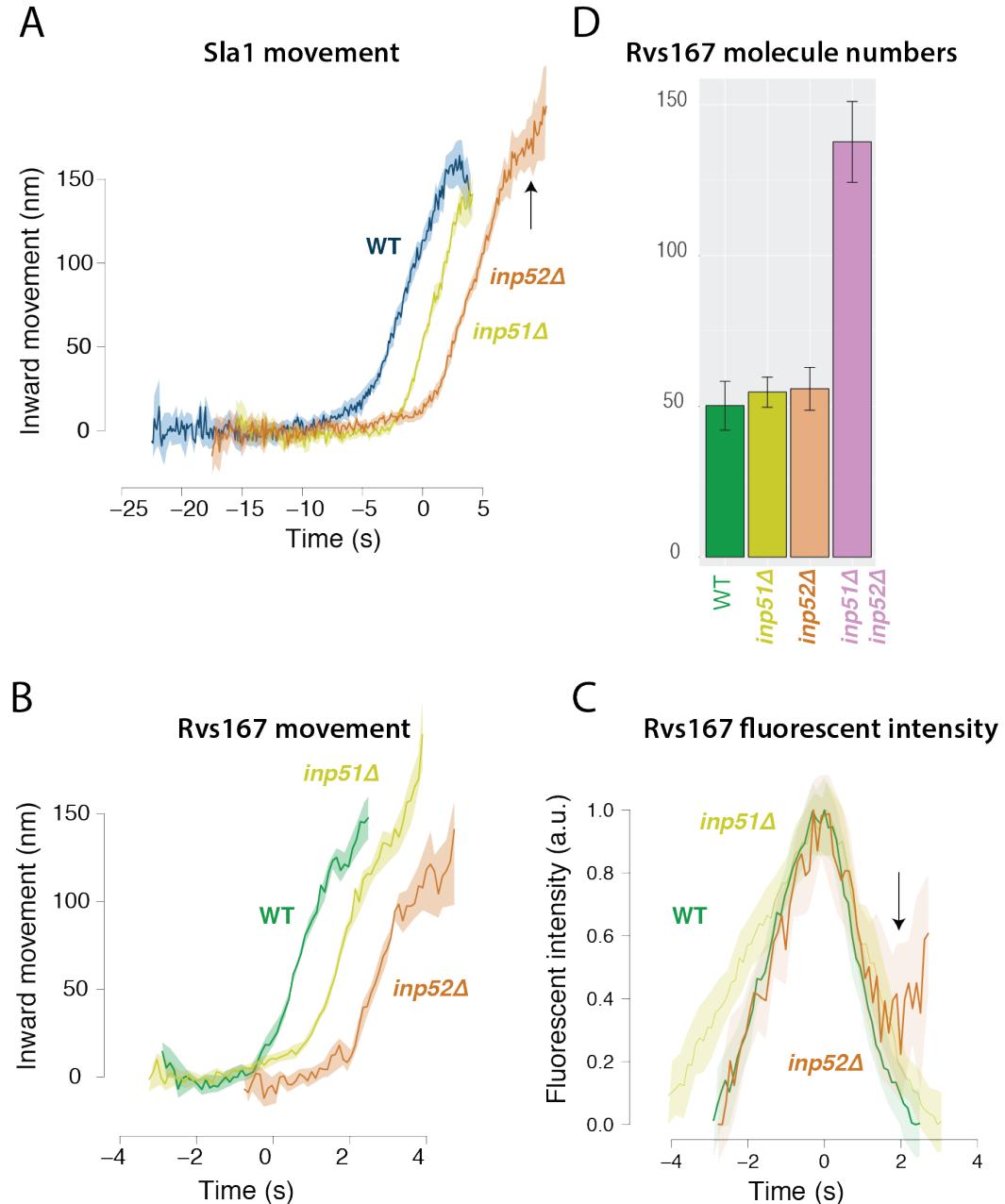
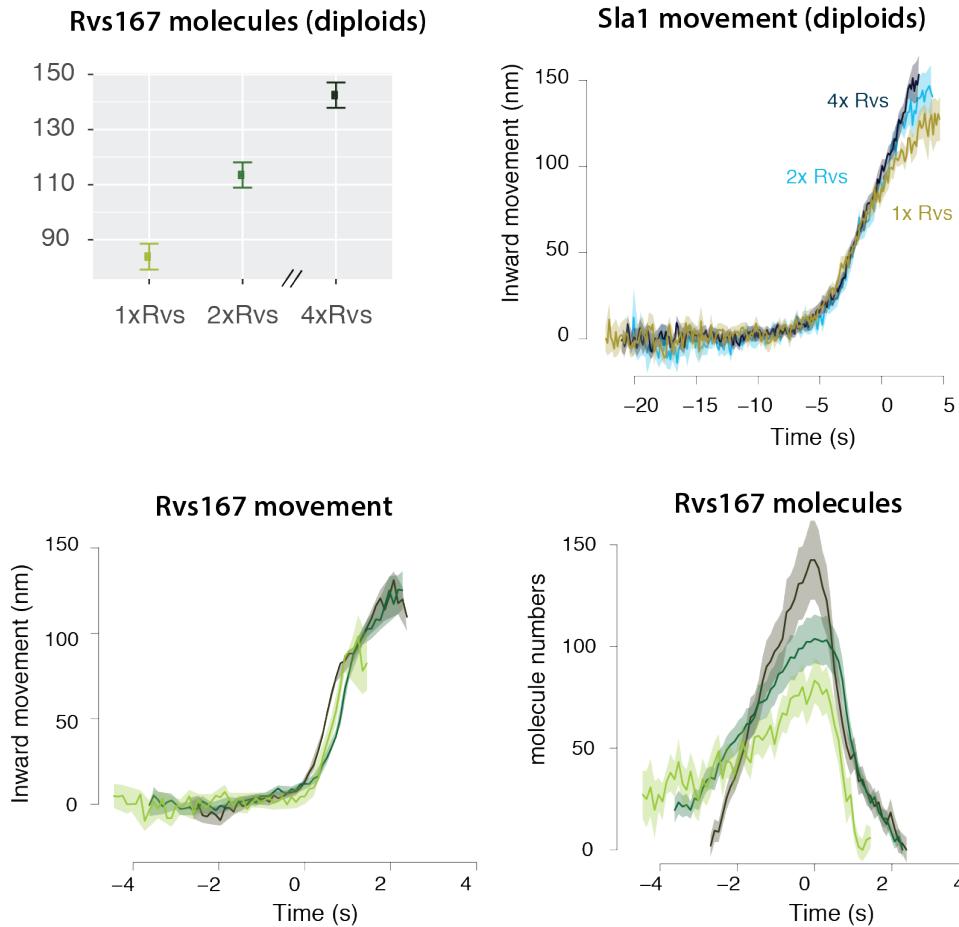


FIGURE 3.5: Sla1 and Rvs167 in Synaptosomal deletion

Rvs copy number titration in diploids



Rvs copy number titration in haploids

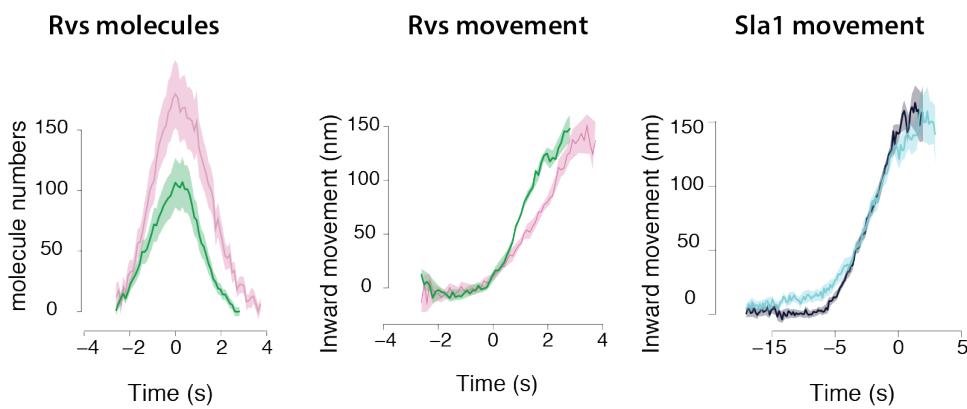


FIGURE 3.6: Sla1 and Rvs167 in Synaptosomal deletion

**3.4.4 Scission timing is determined by actin forces,
BAR domains prevent scission**