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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 hochaufgelösten 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
BFP	Back focal plane
CP	Capping protein
CME	Clathrin-mediated endocytosis
ConA	Concanavalin A
CLEM	Correlative light and electron microscopy
DNA	Deoxyribonucleac acid
DMSO	Dimethyl sulphoxide
dSTORM	Direct STORM
DTT	Dithio treitole
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
fPALM	Fluorescence photo-activation localization microscopy
FRAP	Fluorescence recovery after photobleaching
GPU	Graphics processing unit
GSDIM	Ground state depletion and individual molecule return
LB	Lysogeny broth
mRNA	Messenger ribonucleic acid
MVB	Multivesicular body
MWCO	Molecular weight cutoff
MEA	Monoethylamine
NB	Nanobody

NMR	Nuclear magnetic resonance
NPC	Nuclear pore complex
NPF	Nucleation promoting factor
NA	Numerical aperture
ORF	Open reading frame
OD_{600}	Optical density (600 nm)
PFA	Paraformaldehyde
PBS	Phosphate buffered saline
PIP_2	Phosphatidyl inositole (4,5) diphosphate
PAFP	Photoactivatable fluorescent protein
PALM	Photoactivated localization microscopy
PS-CFP2	Photoswitchable cyan fluorescent protein 2
PAINT	Point accumulation for imaging of nanoscale topology
PSF	Point spread function
PEG	Poly ethylene glycol
PM	Plasma membrane
PCR	Polymerase chain reaction
RFP	Red fluorescent protein
ROI	Region of interest
RESOLFT	Reversible saturable optical fluorescence transitions
RNA	Ribonucleic acid
RT	Room temperature
SMAP	Single-molecule analysis platform
SDS	Sodium dodecyl sulphate
SH3	Src homology 3
STED	Stimulated emission depletion microscopy
STORM	Stochastic optical reconstruction microscopy
SIM	Structured illumination microscopy
SC	Synthetic complete
TH	Tail homology
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 cellular signalling

The plasma membrane serves as the defining barrier between the internal and external cell, thus creating cellular identity, facilitates 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 a 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. Apart from internalizing cargo, it 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, that are transported elsewhere in the cell for degradation or as part of a signaling cascade, 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. Other components of the secretary pathway like the Golgi apparatus and endoplasmic reticulum undergo similar transitions of the bounding membrane to transmit signals and other cargo across the cell, and have mechanistic similarities. Although many early discoveries relating to endocytic pathways was first identified in mammalian cell types2,3, description of endocytosis in S.cerevisiae4 marked the beginning of important discoveries being made in the yeast system that would then be verified in mammalian cells. The ease of genetic manipulation, establishment of the yeast genome, and relative simplicity of endocytic pathways- there is only one - drove several discoveries using yeast as a model system that were later verified in mammalian cells.

1.2 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME). Many different endocytic pathways that facilitate the internalization of cargo at the plasma membrane exist, as depicted in Fig.1. Of them, Clathrin-mediated endocytosis (CME), is universal among eukaryotes and contributes to 90% of cargo trafficked into the cell2. 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 oocyte3. The bristle is seen on several cell types, and was later identified as a lattice of a single highly conserved protein4. This protein was named Clathrin, derived from the latin word for lattice. Clathrin is formed of light and heavy chains incorporated into a triskelion that assemble 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 plasma membrane uptake in cell types ranging from the frog presynaptic membrane5 to rat vas deferens6.

1.3 Clathrin mediated endocytosis in Mammalian vs Yeast

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” coat6,11. In multicellular organisms like C.elegans, clathrin depleted by RNAi result in decreased endocytic uptake in oocytes and dead progeny12, in D.melanogaster, deletion of Clathrin heavy chain results in embryonic lethality13. In Hela cells, knock down of the heavy chain by RNAi results in decrease in endocytosis by 80%14; essentially, endocytosis fails in the absence of clathrin. The role of clathrin in the progression has been heavily debated, but its involvement itself has not. Although several genes involved in Clathrin-mediated endocytosis in yeast were found to be homologues of the mammalian machinery, early work in yeast revealed that clathrin was not necessary for endocytosis15. It became apparent that though the mammalian and yeast systems were mechanistically similar and most of the yeast endocytic proteins had mammalian homologues16, there are some significant differences.

1.3.2 Actin forces are required for yeast CME

Cortical actin patches were first seen in S.cerevisae, that were later established as endocytic sites from the colocolization 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 endocytosis17. Not only is actin itself necessary for the intiation of plasma membrane deformation18, coupling the endocytic coat to actin are necessary for internalization19,20. The cell wall surrounding the plasma membrane in yeast cells has meant that the yeast cells are under high turgor pressure21, which would explain the high force requirement for membrane deformation in yeast.

1.3.3 Clathrin mediated endocytosis in yeast is modular

CME in yeast involves the recruitment, interaction, and disassembly of over fifty proteins. This process involves a flexible initiation phase that establishes endocytic sites and selects cargo15, then a very stereotypic sequence of events nucleates actin, organizes the actin network, invaginates a membrane tube, and finally severs the membrane to produce cargo-filled vesicles16. Work on yeast has shown that the initiation of endocytic sites is independent of the recruitment of any one protein15, and revealed how the the components17 and organization of the actin machinery allows the formation of a membrane invagination18–20. However, the mechanism of the final stage of membrane scission that leads to vesicle formation remains unclear.

The early phase

The actin phase

The scission phase

1.3.4 Membrane scission in mammalian cells

In mammalian cells, membrane scission is effected by the combined action of BAR domain proteins like Endophilin and Amphiphysin and the GTPase dynamin21. BAR proteins have been shown to tubulate membranes in-vitro, and can form helical scaffolds on invaginated membrane tubes22. Apart from the membrane-interacting BAR domain, these proteins have an Src homology 3 (SH3) region that acts as a scaffold for the proline-rich domains of dynamin23. Dynamin arrives at endocytic sites and undergoes a conformation change upon GTP hydrolysis24 that collapses the inner membrane leaflets, leading to membrane scission25. In dynamin 1, 2 double knock-out cells, clathrin-coated pits are formed, but vesicle formation is disrupted, resulting in accumulation of a large number of long tubes22. These experiments show that dynamin acts on invaginated pits to sever the membrane and form vesicles.

1.3.5 Membrane scission in yeast

In yeast, none of the three dynamin-like proteins have proline-rich domains that could interact with the SH3 domain of BAR proteins, suggesting that scission is not driven by the mammalian mechanism. Here, the Amphiphysin/ Endophilin homologue is the heterodimeric complex Rvs161/16726 (Rvs), of which Rvs167 has an SH3 domain. Rvs arrives at endocytic sites in the late stage of the process, and disassembles rapidly at the time of membrane scission27. Deletion of Rvs results in failure of membrane scission in nearly 30% of endocytic events16. This unique profile suggests that although Rvs is not necessary for scission, localization of the complex makes scission more efficient. The rapid disassembly of Rvs27, in the context of mammalian BAR protein data, has suggested that Rvs may form a similar scaffold on membrane tubes in yeast, whose disassembly is coupled to the scission step. What may regulate timing of scission in the absence of dynamin-BAR protein interaction however, has not been determined. Yeast cells are under high turgor pressure that makes forces from actin polymerization necessary for invagination28. There is therefore likely to be some interplay between scission-stage proteins and the actin network that could modulate the final shape transitions.

1.4 BAR domain proteins

1.4.1 Mammalian vs yeast BAR proteins

1.4.2 The Rvs complex

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:

- Is localization microscopy suitable to study endocytosis in budding yeast?

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 are proteins arranged within the endocytic machinery?

Using the methods I established, I studied the structural organization of endocytic proteins. First, I focused on their radial distribution, which had not been possible to study with live-cell fluorescence microscopy or EM. To increase the statistical power of my approach, I set up a high-throughput imaging and quantitative data analysis pipeline together with Joran Deschamps, another PhD student in the lab, which allowed me to image the organization of proteins at thousands of endocytosis sites. I found that endocytic proteins have an intricate radial organization, which mirrors their functional context and modularity. I also discovered that a set of proteins pre-pattern where actin polymerization will start, which likely supports the mechanistic robustness of the mobile phase. Results of these experiments are described in section **??**.

- How is the endocytic machinery organized during the highly dynamic mobile phase of endocytosis?

Visualizing the endocytic machinery during the most dynamic mobile phase with high spatial resolution is methodologically challenging, and has not been possible so far. Here, I proposed that localization microscopy, despite being a comparably slow technique, can overcome this gap. For this, I developed an approach to infer the time point of many fixed, unsynchronized endocytic sites by quantitatively analyzing their structures, and integrating the superresolution images with centroid trajectories determined in living cells (**Picco:2015iv**), and time-resolved membrane shapes measured by CLEM (**Kukulski:2012jl**). This has allowed me to visualize the highly dynamic formation of the actin network, which provides the force to invaginate the membrane, in relation to the zone where new filaments are nucleated. Results of this work are presented in section **??**.

3 results

This is where the results go. And then some more results. And more.