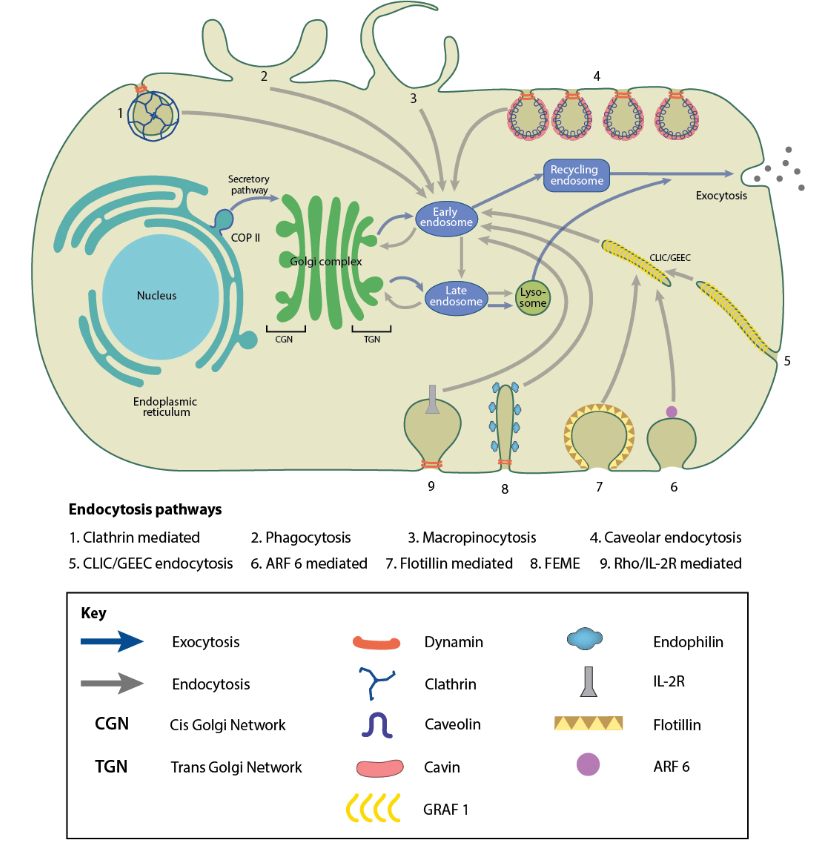
**Cellular context of endocytosis :**

The plasma membrane serves as the defining barrier between the interior and exterior of the 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 refers to the uptake of molecules too big to pass through the plasma membrane. It involves the invagination of the plasma membrane into a cargo-filled invagination, 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.



**Fig1: Endocytosis in the context of cellular trafficking, and some key proteins involved**

Apart from internalizing cargo, endocytosis allows regulation of the plasma membrane itself: its lipid and protein composition, and therefore many of its 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.

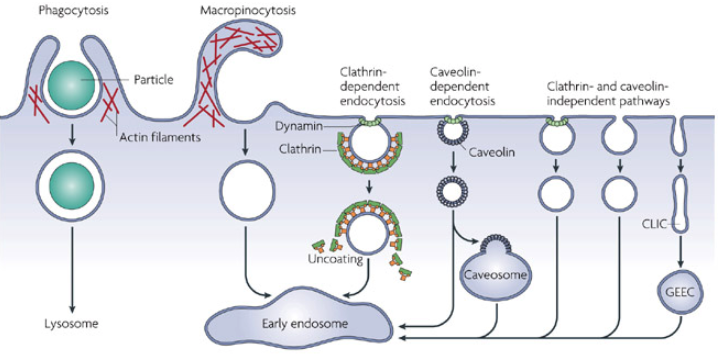
Endocytosis essentially forms the basis of all cellular responses, from incorporating external stimulus, to communication between different cellular compartments. This process arguably drove the development of organisms from single cell to multicellular eukaryotes.

Plasma membrane regulation and internalization of signalling molecules are critical for the function of the cell. Among the vast array of important cargo that are taken up via endocytosis are cholesterol(Goldstein and Brown, 1973; Anderson, Goldstein and Brown, 1976), insulin(Fan *et al.*, 1982), and other hormones. Not surprisingly, many human diseases have been linked to defects in the endocytic pathway like familial hypercholesterolemia(Goldstein and Brown, 1973; Anderson, Goldstein and Brown, 1976) -the study of which established the field of endocytosis-, Alzheimer’s(Maxfield, 2014), and some types of cancer(Mosesson, Mills and Yarden, 2008). 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 cells(Mercer, Schelhaas and Helenius, 2010). Other components of the cellular signalling 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(McMahon and Mills, 2004; Traub, 2005).

Although many early discoveries relating to endocytic pathways were identified in mammalian cell types(Hemmaplardh and Morgan, 1976; Karin and Mintz, 1981), description of endocytosis in *Saccharomyces.cerevisiae*(Riezman, 1985) 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(Boettner, Chi and Lemmon, 2012; Payne, 2013).

**Clathrin-mediated endocytosis (CME)**

Many different endocytic pathways that facilitate the internalization of cargo at the plasma membrane exist, as depicted in Fig.2, 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 cell(Bitsikas, Corrêa and Nichols, 2014). First identified by studying yolk uptake in mosquitos, ultrastructural studies of their oocytes (where frequency 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 oocyte(ROTH and PORTER, 1964). The bristle was noted in several cell types, and was later identified as a lattice of a single highly conserved protein(Pearse, 1976). This protein was named Clathrin, derived from the latin word for lattice. Clathrin is formed of light and heavy chains incorporated into a triskelion(Ungewickell and Branton, 1981) 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 membrane(Heuser and Reese, 1973) to rat vas deferens(Friend and Farquhar, 1967). Clathrin 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 cargo and targeting to different cellular compartments is achieved by Clathrin interaction with specialized adaptor proteins like the adaptor protein complexs (AP), which specify Golgi-to-early endosome traffic, while Golgi-localized gamma-adaptin (GGA) complexes specify Golgi-to-late endosome traffic(Payne, 2013).

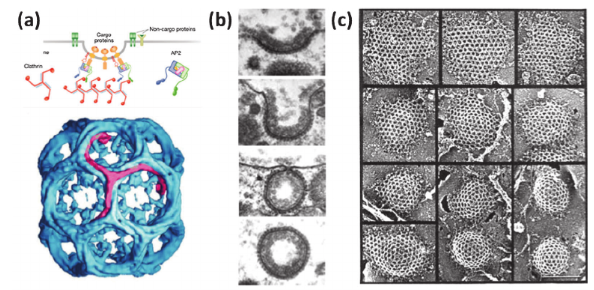


**Fig2: Pathways of entry into cells.**

**CME in mammalian and yeast cells**

**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(ROTH and PORTER, 1964; Kanaseki and Kadota, 1969). In multicellular organisms like *Caenorhabditis.elegans*, clathrin depleted by RNAi results in decreased endocytic uptake in oocytes and dead progeny(Grant and Hirsh, 1999), in *D.melanogaster*, deletion of clathrin heavy chain results in embryonic lethality(Bazinet *et al.*, 1993). In Hela cells, knock-down of the heavy chain by RNAi results in decrease in endocytosis by 80%(Huang *et al.*, 2004); 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 endocytosis(Payne and Schekman, 1985). Loss of clathrin changes the size of the vesicles formed at scission, and leads to decrease in the number of established endocytic sites(Kaksonen, Toret and Drubin, 2005; Kukulski *et al.*, 2016): 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 homologues(Weinberg and Drubin, 2012), there are some significant differences.



**Fig3: a) Mechanism of clathrin-mediated endocytosis and structure of clathrin cage that forms over a clathrin coat. b) Different stages of Clathrin coated pit progressing. Gallery of coated pit proﬁles on the inner surface of chicken ﬁbroblasts, illustrating the range of lattice curvature seen normally. Scale bar: 200 nm.**

**Actin forces are required for yeast CME**

Cortical actin patches were first seen in S.cerevisae, and they were later established as endocytic sites from the colocolization of other endocytic proteins. While the mammalian CME is heavily dependent on clathrin, the yeast system relies on actin and its proper organization for endocytosis(Kübler *et al.*, 1993). Serge et al., estimate that the forces required to pull the membrane are in the order of 1000-5000pN. This force requirement arises from high turgor pressure inside the yeast cells. Actin is required to pull the membrane inwards coupling the endocytic coat to actin is necessary for internalization(Raths *et al.*, 1993; Skruzny *et al.*, 2012).

**CME in yeast is highly regular**

In yeast, over fifty proteins are recruited, interact, and disassemble during endocytic 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 the 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 vesicles(Kaksonen, Toret and Drubin, 2005). Coat proteins arrive upon initiation of endocytic sites. The actin and WASP modules arrive next, and include 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(Kaksonen, Toret and Drubin, 2005; Picco *et al.*, 2015; Mund *et al.*, 2017). The multiple stages of endocytosis are discussed below.

Early initiation phase:

A variable initiation phase establishes endocytic sites and selects cargo(Brach *et al.*, 2014). The earliest proteins to arrive at sites, Ede1 and Syp1 are not required for the formation 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 factors(Brach *et al.*, 2014), 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 loaded(Weinberg and Drubin, 2012) before later (energy consuming) phases are triggered.

Coat module:

Coat proteins serve to template later proteins(Mund *et al.*, 2017), as well as form the link between the actin module(Skruzny *et al.*, 2012), ingressing membrane, and cargo associated with it. Unlike in mammalian cells, as mentioned earlier, clathrin adaptors and the clathrin triskelion are not necessary for the progression of sites, although deletion of clathrin introduces a high variability in the timing of scission(Kukulski *et al.*, 2016). 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(Kaksonen, Sun and Drubin, 2003; Skruzny *et al.*, 2012). The complex between proteins Sla1, Pan1 and End3, which 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(Wendland and Emr, 1998; Sun *et al.*, 2015; Toshima *et al.*, 2016). The arrival of Sla1 is a strong predictor of successful endocytosis(Kaksonen, Toret and Drubin, 2005; Kishimoto *et al.*, 2011). These coat proteins are pulled upwards into the cytoplasm, and follow the moving membrane.

Actin module

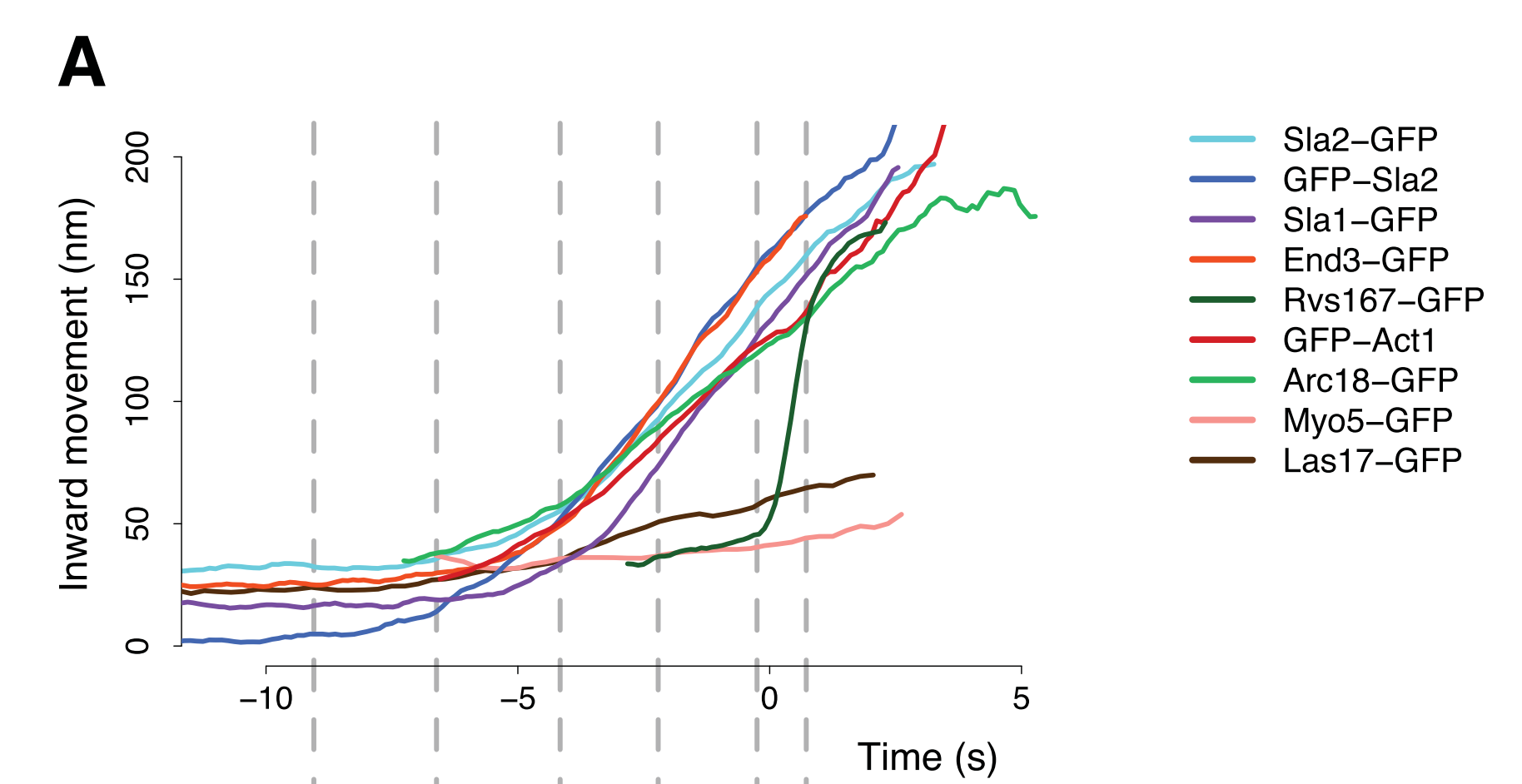
Once the coat proteins are assembled, proteins that nucleate and organize the actin machinery are recruited. Actin filaments are nucleated by the Arp2/3 complex, and act in concert with actin nucleation promoting factors (NPFs), such as the yeast WASP homologue Las17, type 1 myosins Myo3 and Myo5, Pan1, 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 membrane(Picco *et al.*, 2015). Las17 is a potent actin nucleator, without which endocytosis essentially fails(Yidi Sun, 2006). Myo3/5 are non-processive motors that interact with and can translocate actin filaments, but whose mechanistic contribution to endocytosis is unknown. Deletion of either Myo5 or Myo3 has subtle phenotypes, but deletion of both effectively blocked endocytosis(Yidi Sun, 2006). Abp1 binds actin filaments and activates the Arp2/3 complex.

Bbc1, 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 sites(Picco *et al.*, no date). Bzz1, an F-BAR protein, relieves Las17 of NPF activity inhibition by Sla1(Yidi Sun, 2006). Vrp1 stimulates the Arp2/3 complex, recruits myosins, and interacts with Las17(Anderson *et al.*, 1998; Wong *et al.*, 2010).

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 inwards, taking the membrane along with it(Picco *et al.*, 2015).

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 (Kukulski *et al.*, 2012; Picco *et al.*, 2015). 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 /ref{yeast\_scission})



**Fig4: Sterotypicity of yeast endocytosis allows averaging of centroids of GFP-tagged endocytic proteins**

**that are tracked as they move away from the plasma membrane.**

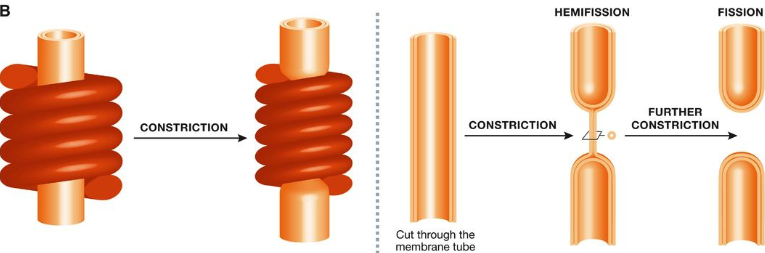
**Membrane scission in mammalian cells.**

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 (Shpetner and Vallee, 1989), 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 (Grigliatti *et al.*, 1973; Poodry and Edgar, 1979; van der Bliek and Meyerowrtz, 1991). Shibire codes multiple isoforms of dynamin that are differentially expressed across the organism (Chen *et al.*, 1991). 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 (Ferguson *et al.*, 2009).

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(Antonny *et al.*, 2016). *In-vitro*, dynamin oligomerizes into helical structures with the PH domain apposed against the membrane, and the GTPase domain facing away from the membrane (Sweitzer and Hinshaw, 1998; Zhang and Hinshaw, 2001). 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(Zhao *et al.*, 2016). Disruption of its GTPase activity results in membrane tubes that accumulate dynamin, as well as the BAR domain proteins endophilin and amphiphysin(Takei *et al.*, 1995; David *et al.*, 1996; Ringstad, Nemoto and De Camilli, 1997).



**Fig5: Schematic of membrane constriction by dynamin assembled into a helical scaffold**

Dynamin interacts with BAR proteins to cause scission

Dynamin arrives at clathrin-coated pits via interaction with BAR proteins endophilin and amphiphysin(Ferguson *et al.*, 2009). 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, which direct BAR proteins to specific lipids within membranes, or Src homology 3 (SH3) domains that mediate protein-protein interaction. These SH3 regions act as a scaffold for the proline-rich domains of dynamin (Grabs *et al.*, 1997).

Dynamin and BAR proteins interact via PRD and SH3 regions

Dynamin’s PRD interacts with the SH3 domains of BAR proteins endophilin and amphiphysin(Grabs *et al.*, 1997; Cestra *et al.*, 1999; Farsad *et al.*, 2001; Meinecke *et al.*, 2013). Endophilin recruitment is reduced in the absence of dynamin, and appears to inhibit the GTPase action of dynamin(Farsad *et al.*, 2001; Meinecke *et al.*, 2013; Hohendahl *et al.*, 2017), 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 scission(Meinecke *et al.*, 2013). 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(Neumann and Schmid, 2013; Hohendahl *et al.*, 2017). 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(Shupliakov *et al.*, 1997; Meinecke *et al.*, 2013).

**Membrane scission in yeast**

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 fusion(Cerveny *et al.*, 2007). Vps1 is essential for vacuolar protein sorting(Rothman *et al.*, 1990), is involved in fission and fusion of vacuoles(Peters *et al.*, 2004) and peroxisomes(Hoepfner *et al.*, 2001), is required for regulation of golgi to endosomal trafficking(Gurunathan, David and Gerst, 2002), and may arrive at early endocytic events(Nannapaneni *et al.*, 2010). None of the three yeast dynamins have the typical PH domain(Bui *et al.*, 2012; Moustaq *et al.*, 2016) that in mammals 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 clathrin and other endocytic proteins (Yu, 2004; Nannapaneni *et al.*, 2010; Goud Gadila *et al.*, 2017), though other work has failed to observe localization of Vps1 at endocytic sites(Kishimoto *et al.*, 2011; Goud Gadila *et al.*, 2017). The role of Vps1 in endocytosis is not clear, but it is a candidate for the role of the canonical dynamin in CME.

Yeast BAR domain proteins Rvs161/167 regulate scission timing

In yeast, the Amphiphysin/ Endophilin homologue is the heterodimeric complex Rvs161/167(Friesen *et al.*, 2006) (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 scission(Picco *et al.*, 2015). Deletion of Rvs results in failure of membrane scission in nearly 30% of endocytic events(Kaksonen, Toret and Drubin, 2005). Scission failure is identified by the movement inwards of the endocytic protein coat 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 phenotype(Kishimoto *et al.*, 2011). 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.

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(Kübler and Riezman, 1993; Aghamohammadazadeh and Ayscough, 2009). There is therefore likely to be some interplay between scission-stage proteins and the actin network that could modulate the final shape transitions.

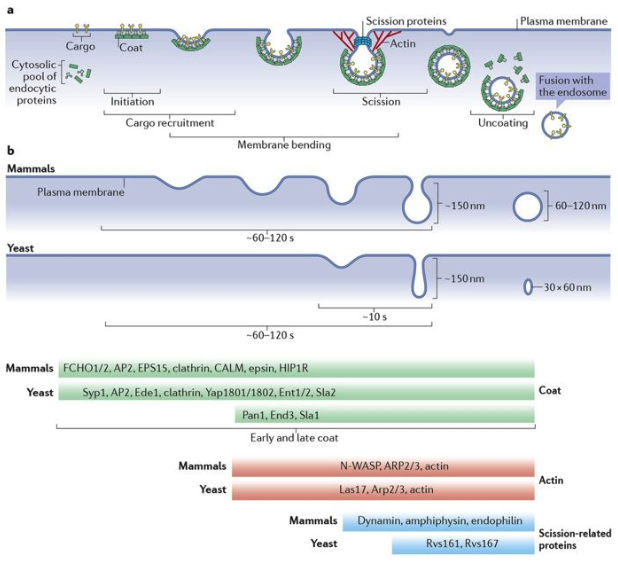
Proposed scission mechanisms:

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 endocytosis(Nannapaneni *et al.*, 2010). *vps1Δ rvs167Δ* double mutant has been shown to increase membrane retraction rates after invagination(Rooij *et al.*, 2010), an indication of scission failure. Another hypothesis has proposed that lipid hydrolysis by yeast synaptojanin-like proteins can cause vesicle scission(Liu *et al.*, 2006). 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 occur(Simunovic *et al.*, 2017). 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 scission(Snead *et al.*, 2018). 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 regions, and these regions 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 domains scaffold membrane tubes and stabilize them, preventing scission(Boucrot *et al.*, 2012; Dmitrieff and Nédélec, 2015). For a more detailed discussion on these models, see /ref{results}

Many of these theories are contradictory, based on *in-vitro* data, using mammalian BAR proteins, at protein concentrations 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. Therefore, the mechanism for membrane scission in yeast is yet to be determined.



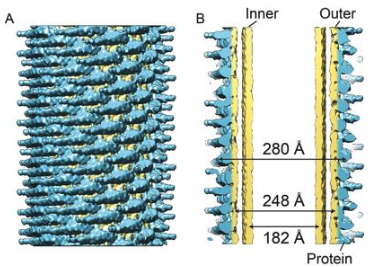
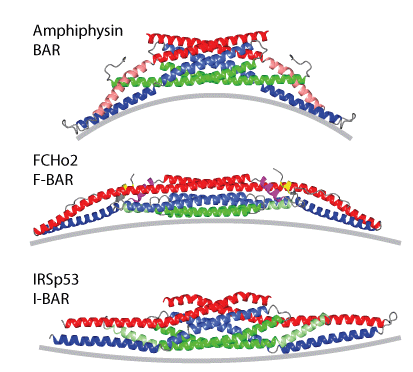
**Fig6: Schematic of endocytosis in mammalian cells compared to yeast**

BAR domain proteins

The proteins in 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(Sakamuro *et al.*, 1996; Ren *et al.*, 2006).

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, and have an intrinsic curvature defined by the dimerization angle. This curvature categorizes BAR proteins to classical BAR (high curvature), 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(Farsad *et al.*, 2001; Peter *et al.*, 2004). 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.



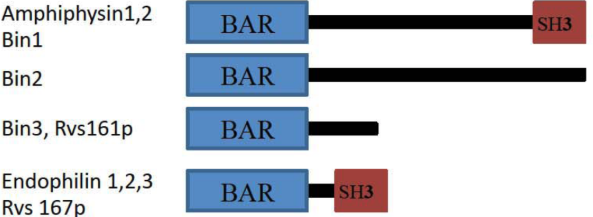
**Fig7: Left: Schematic of structures of BAR dimers with different curvature. Right: 3D reconstruction of an amphiphysin/BIN1-mediated tube with a diameter of 280 Å. The density corresponding to the protein is colored in blue and the lipid corresponding parts are coloured in in yellow.**

**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 bilayer (Peter *et al.*, 2004). 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 (Kozlovsky and Kozlov, 2003; Boucrot *et al.*, 2012). BAR domains lacking this helix are not able to efficiently tubulate vesicles(Jennifer L Gallop, 2006). The N-helix also increases efficiency of binding to liposomes(Farsad *et al.*, 2001) in a curvature sensitive manner, and confers salt sensitivity(Jennifer L Gallop, 2006).

High resolution structural data has shown that NBAR proteins can form helical scaffolds on tubular membranes (Peter *et al.*, 2004; Shimada *et al.*, 2007; Mim *et al.*, 2012). 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 tube(Boucrot *et al.*, 2012). 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 stability (Boucrot *et al.*, 2012).

Both BAR proteins implicated in CME , Amphiphysin and Endophilin are shown to tubulate membranes *in-vitro*(Peter *et al.*, 2004; Jennifer L Gallop, 2006; Mim *et al.*, 2012) and form a helical scaffold. The tubule diameter resembles the diameter of the proteins themselves, and involve lateral interactions of the neighboring BAR domains(Sorre *et al.*, 2012). Both BAR domain proteins are able to form mixed helices in the presence of dynamin(Takei *et al.*, 1999; Farsad *et al.*, 2001).



**Fig8: Schematic of domains of endocytic NBAR proteins**

**NBAR protein in endocytosis: Amphiphysin**

Two mammalian isoforms of Amphiphysins (Amph) exist. AmphI is enriched in neurons in mammals, while AmphII (Bin1) is expressed in other tissue types, with one isoform enriched in muscle T-tubule junctions(Lee *et al.*, 2002). The only Amphiphysin (d-Amph) in flies is expressed in various tissues, and enriched at muscle T-tubule junctions. The d-Amph dimer forms a coiled coil, with each BAR domain made of three long, kinked alpha-helices(Peter *et al.*, 2004). *In-vitro*, liposome tubulation activity of Amphiphysin is concentration dependent. At very high concentrations, Amphiphysin is also able to sever tubular membrane to form vesicles(Peter *et al.*, 2004).

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 adaptors(Razzaq *et al.*, 2001) and can polymerize clathrin into invaginated lattices in a BAR domain dependent manner(Peter *et al.*, 2004), while both bind dynamin, and the lipid phosphatase Synaptojanin(Cestra *et al.*, 1999).

**NBAR protein in endocytosis: Endophilin**

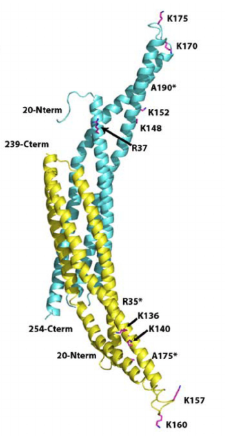
Endophilins A1-A3 (EndoA) were discovered as SH3 domain containing proteins (Giachino *et al.*, 1997) that co-localized with dynamin, and interacted with Synaptojanin(Ringstad, Nemoto and De Camilli, 1997) and amphiphysin(Micheva *et al.*, 1997): all already identified as important regulators of synaptic vesicle recycling by endocytosis. A second mammalian protein was later discovered as related to endophilin, and termed EndophilinB (EndoB). Other sequenced eukaryotes have a single isoform of EndoA and B.

EndoA1 isoform is found in neurons, EndoA2 is expressed ubiquitously, and EndoA3 is enriched in the brain and testes. All three are found at presynaptic membranes. Crystal structure of EndoA1 shows the same overall structure as that of amphiphysin, with an additional amphiphatic helix similar to the N-helix, located at the centre of the crescent-shaped dimer(Weissenhorn, 2005; Jennifer L Gallop, 2006). 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(Huttner and Schmidt, 2000; Gallop, Butler and McMahon, 2005), suggesting different roles for the two BAR domain proteins in membrane interaction. Endophilin interacts with dynamin, NWASP and Synaptojanin proteins via its SH3 domain(Cestra *et al.*, 1999; Otsuki, Itoh and Takenawa, 2003; Hohendahl *et al.*, 2017)

**NBAR protein in yeast endocytosis: Rvs167/161 (Rvs)**

RVS167 and RVS161 (reduced viability upon starvation) genes were discovered in a screen that tested for survival under starvation conditions(Crouzet *et al.*, 1991). Rvs167 and Rvs161 are both NBAR domain proteins that are thought to form obligate heterodimeric complexes (Rvs) *in-vivo*(Pierre Sivadon, Crouzet and Aigle, 1997; Lombardi and Riezman, 2001). 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 (Lombardi and Riezman, 2001; Kaksonen, Toret and Drubin, 2005; Boeke *et al.*, 2014), 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 not(Brizzio, Gammie and Rose, 1998). It is consistent however, that at endocytic sites the Rvs proteins function 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 interchangeable(P Sivadon, Crouzet and Aigle, 1997). 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 alone(Pierre Sivadon, Crouzet and Aigle, 1997), suggesting that the BAR domains are the main functional unit of the complex



**Fig9: Homology model of Rvs complex based on Amphiphysin structure.**

**Rvs167 in yellow, Rvs161 in cyan**

Deletion of the genes show abnormal actin morphology, confer salt sensitivity, as well as amino-acid and lipid sensitivity, and have abnormal budding pattern(Bauer *et al.*, 1993; Sivadon *et al.*, 1995; Lombardi and Riezman, 2001; Toume and Tani, 2016). 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-vitro*(Youn *et al.*, 2010).

Averaged centroid tracking of the Rvs complex has shown that Rvs arrives in the scission stage of endocytosis (ref). 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 (ref). 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 vesicle(Picco *et al.*, 2015). 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.

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