Mechanisms of clathrin-mediated endocytosis

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Abstract | Clathrin-mediated endocytosis is a key process in vesicular trafficking that transports a wide range of cargo molecules from the cell surface to the interior. Clathrin-mediated endocytosis was first described over 5 decades ago. Since its discovery, over 50 proteins have been shown to be part of the molecular machinery that generates the clathrin-coated endocytic vesicles. These proteins and the different steps of the endocytic process that they mediate have been studied in detail. However, we still lack a good understanding of how all these different components work together in a highly coordinated manner to drive vesicle formation. Nevertheless, studies in recent years have provided several important insights into how endocytic vesicles are built, starting from initiation, cargo loading and the mechanisms governing membrane bending to membrane scission and the release of the vesicle into the cytoplasm.

Endocytosis generates small (60–120 nm) membrane vesicles that transport various cargo molecules from the plasma membrane of eukaryotic cells into the cytoplasm. The cargo consists mainly of transmembrane proteins and their extracellular ligands. These cargoes are involved in a broad range of physiological processes, including nutrient uptake, cell signalling, developmental regulation through morphogens, cell adhesion, etc. Multiple different endocytic pathways have been described in eukaryotic cells, but the major endocytic route for internalization of many cargoes is clathrin-mediated endocytosis[1]. For simplicity, throughout this Review, we will use the term endocytosis to specifically refer to clathrin-mediated endocytosis.

Conceptually, endocytosis is a fairly simple process that consists of a few sequential and partially overlapping steps (FIG. 1a). The process is initiated when endocytic coat proteins from the cytosol start to cluster on the inner leaflet of the plasma membrane. The protein coat assembly then continues by further recruitment of other coat proteins from the cytosolic pool. Cargo recruitment concentrates cargo molecules to the coated region of the plasma membrane. The assembling coat promotes membrane bending, which transforms the flat plasma membrane into a 'clathrin-coated pit'. The scission process constricts and cuts the neck of the membrane invagination to separate the clathrin-coated vesicle from the plasma membrane. Actin polymerization cooperates with the coat and scission proteins to promote membrane shaping. Finally, uncoating disassembles the endocytic protein machinery, releasing the nascent cargo-filled vesicle and allowing it to be trafficked further within the cell. This process is highly evolutionarily conserved and was likely present in the last common ancestor of eukaryotes possibly more than a billion years ago[2]. Nevertheless, certain important differences have evolved in different lineages (BOX 1).

Clathrin-mediated endocytosis is named after one key component of the endocytic machinery — the clathrin protein (BOX 2). However, over 50 other cytosolic proteins are involved in the formation of clathrin-coated endocytic vesicles[3,4]. All these proteins assemble from the cytosol to the endocytic site in a highly ordered manner[5–8]. Many of these proteins have been characterized in detail through the use of biochemical, structural and biophysical approaches and have known activities that can contribute to the different steps of endocytosis. However, it remains a major question how these proteins cooperate in the cell to form the endocytic machinery that functions in a robust and precise manner to package a wide variety of different cargoes into vesicles in a way that can be regulated and that responds to changes in the cell's physiology and its environment. Here, we review the current knowledge of the molecular mechanisms of endocytosis and highlight key unanswered questions.

Modular endocytic machinery

A striking feature of the endocytic protein machinery is that it assembles at the plasma membrane through recruitment of endocytic proteins from the cytosol in a highly regular sequence in which different proteins arrive and leave in a specific order (FIG. 1b). On the basis of their assembly dynamics, the endocytic proteins can be grouped into functional modules. This modular organization is largely conserved between different organisms[3,5,7,9].

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The endocytic proteins interact extensively within and between modules to coordinate the assembly and functions of the different parts of the endocytic machinery[3,10].

The first module to assemble is the clathrin coat. In addition to clathrin, the coat is composed of many other proteins, including clathrin-adaptor proteins and scaffold proteins. The clathrin-adaptor proteins, including the heterotetrameric adaptor protein AP2 complex, and monomeric adaptors, such as proteins of the clathrin assembly lymphoid myeloid leukaemia protein (CALM; also known as PICALM) family and epsins, bind to the lipids in the plasma membrane (see BOX 3 for details on the role of lipids, and specifically phosphoinositides (PIPs), in clathrin-mediated endocytosis) and to cargo molecules[11–16]. The scaffolds, such as clathrin, epidermal growth factor receptor substrate 15 (EPS15), epidermal growth factor receptor substrate 15-like 1 (EPS15R) and intersectins, interact with the clathrin adaptors and with themselves to cluster the coat components together[17–20]. The earliest assembling coat

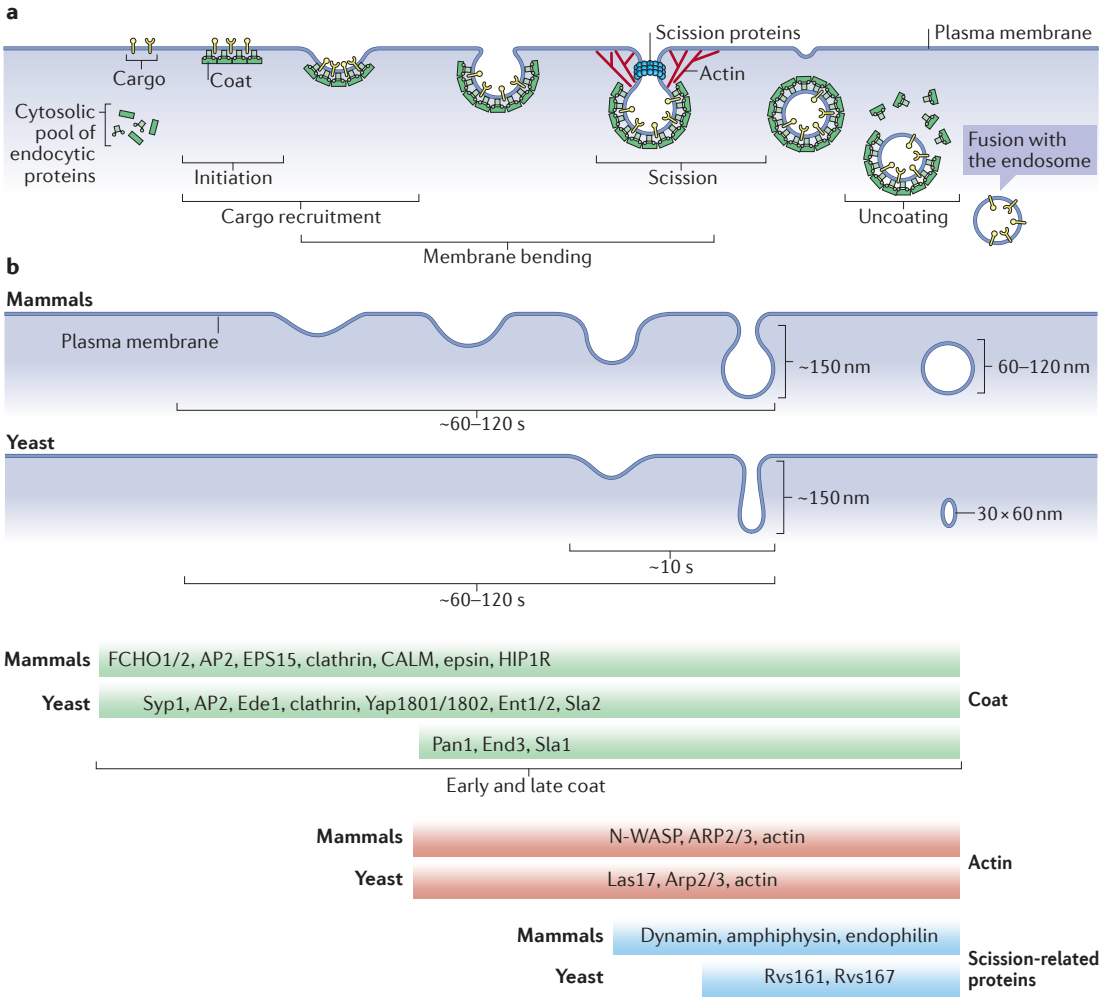


Figure 1 | **Stages of clathrin-mediated endocytosis and the associated modular machinery. a** | Protein modules driving the different steps of the endocytic process are assembled sequentially from soluble cytosolic protein pools. After initiation of the endocytic site, cargo is recruited to this site and the membrane is shaped into an invagination, which is finally separated from the plasma membrane by scission. Uncoating releases the proteins of the endocytic machinery back to the cytosolic pool and releases a vesicle that can participate in intracellular membrane trafficking events. **b** | Membrane shape changes during endocytosis are tightly coupled with the assembly of the endocytic protein machinery. The membrane shapes and the dynamics of membrane shaping differ between mammals and yeast. Clathrin-coated vesicles are wider in mammalian cells than in yeast, where they are pinched off from a narrow tubular invagination. The key protein components of the endocytic machinery involved in membrane shaping are largely conserved between mammals and yeast. AP2, adaptor protein 2; ARP2/3, actin-related protein 2/3 complex; CALM, clathrin assembly lymphoid myeloid leukaemia protein; Ede1, EH domain-containing and endocytosis protein 1; End3, actin cytoskeleton-regulatory complex protein End3; Ent1/2, epsin 1/2 complex; EPS15, epidermal growth factor receptor substrate 15; FCHO1/2, F-BAR domain only protein 1 and 2 complex; HIP1R, Huntingtin-interacting protein 1-related protein; Las17, protein-rich protein Las17; N-WASP, neural Wiskott–Aldrich syndrome protein; Pan1, actin cytoskeleton-regulatory complex protein Pan1; Rvs161, reduced viability upon starvation proteins 161; Rvs167, reduced viability upon starvation protein 167; Syp1, suppressor of yeast profilin deletion; Vps1/2, vacuolar protein sorting-associated protein 1 and 2 complex; Yap1801/1802, clathrin coat assembly protein AP180A and AP180B complex.

Box 1 | **Evolutionary differences in clathrin-mediated endocytosis**

The endocytic protein machinery is largely homologous between mammals and yeast, and many of the molecular mechanisms are clearly similar. However, there are some striking differences. Understanding these differences will be important for interpreting data from different organisms and will likely help us to better understand the mechanistic principles of endocytosis and how it has adapted to different physiological contexts.

Electron microscopy has revealed that the endocytic membrane shapes clearly differ (see also FIG. 1b). The endocytic membrane invaginations have comparable length (about 150 nm) but are much wider in mammalian cells than the narrow tubular invaginations in yeast. Accordingly, the endocytic vesicles in mammals are round and typically about 100 nm in diameter[34], whereas in yeast they are much smaller and ellipsoid (about 60 × 30 nm in diameter), corresponding to the dimensions of the tubular endocytic invaginations formed during membrane budding[59]. The difference in shape may be due to the high turgor pressure in yeast cells[91]. The turgor pressure pushes the plasma membrane outwards in yeast, making it energetically costly to generate the large balloon-like vesicles seen in mammalian cells. In addition, the temporal dynamics of membrane shaping differs. The overall lifetime of an endocytic event is in the same range in both mammals and yeast. However, in mammalian cells membrane bending initiates soon after the coat has started assembling and gradually progresses, whereas in yeast cells membrane bending occurs abruptly (within less than 10 seconds), just before scission.

In mammals, the clathrin coat seems to be the central driver of membrane bending. However, in yeasts, membrane bending is strongly actin-dependent and the contribution from the coat seems much smaller and may be limited to endocytic site selection and cargo recruitment. The early-arriving coat proteins, which in mammals are critical for the progression of endocytic vesicle budding[11,17], in yeast contribute to site selection similar to the process in mammals but are not essential for vesicle budding[20,21]. Clathrin itself can be deleted in yeast and endocytic vesicles are still formed, albeit at reduced efficiency[159,160]. It has been suggested that actin is essential in yeast cells because it is needed to produce the force necessary to overcome their high turgor pressure[161]. Accordingly, increasing membrane tension experimentally in mammalian cells makes endocytosis dependent on actin polymerization[52,162]. Under such high membrane tension, the clathrin coat may be insufficient to generate the force required to bend the membrane. Membrane tension in cells is generated by cytoskeletal forces and cell adhesion; thus, this parameter can be variable, depending on the cell type, differentiation stage, external stimulation, etc., possibly leading to variable requirements for actin during clathrin-mediated endocytosis in different cellular contexts[163].

Most of the key endocytic proteins have clear homologues between mammals and yeasts. However, the late coat module of yeast does not have a counterpart in mammalian cells and proteins of this module (actin cytoskeleton-regulatory complex proteins Pan1 and End3 as well as protein Sla1) have no direct homologues in mammals (although they contain domains that are found in epidermal growth factor receptor substrate 15 (EPS15) and intersectins). These yeast proteins form a complex that is essential for localizing the actin filament nucleation machinery to the endocytic site[85]. This late coat complex may be an evolutionary innovation in the lineage leading to yeast to decouple the early coat components, which primarily drive selection of the endocytic sites, from the membrane bending function to help separate these processes in time and to optimize the actin-based membrane bending process to drive endocytosis against high turgor pressure, which counteracts membrane shaping.

proteins form a 'pioneer module' that is responsible for initiating the endocytic process[7,11,17–21] (FIG. 2). In yeast, the coat module has been subdivided further into early, intermediate and late coat modules on the basis of the timing of their assembly[9].

After the coat has assembled, a network of actin filaments polymerizes at the endocytic site, forming the actin module. This module can be divided into regulatory components and the actin filament network. The regulatory components include proteins of the Wiskott–Aldrich syndrome protein (WASP) family, which are key activators of actin filament nucleation, myosin motor proteins and dynamin[7,22]. The actin filament network is composed of actin filaments nucleated by the complex of actin-related protein 2 (ARP2), ARP3 and a number of other actin-binding proteins[23].

The constriction and scission of the invagination neck is mediated by BAR domain (Bin, amphiphysin and Rvs) proteins such as endophilins and amphiphysins, and their yeast homologues Rvs161 and Rvs167. The BAR domain proteins cooperate with dynamin to mediate scission but have distinct assembly dynamics and define the scission module.

Finally, the proteins of the uncoating module localize to the endocytic site to drive disassembly of the endocytic machinery. These proteins include chaperones, protein kinases and lipid phosphatases[7,24]. These processes will be discussed in more detail in the following sections.

Initiation of endocytic events

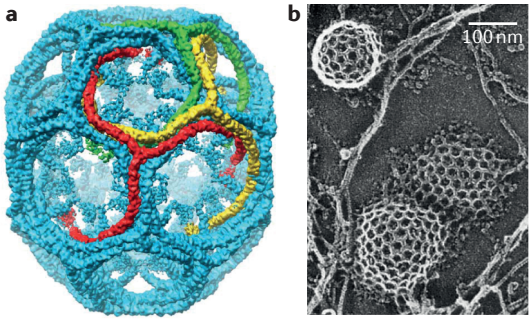
An endocytic event is initiated by clustering of the first endocytic proteins at the plasma membrane, forming the nucleus for the assembly of the vesicle coat. The initiation defines the site where the endocytic vesicle will be formed and is likely the key stage for regulating the frequency of endocytic events[17,25]. The endocytic frequency can also be controlled at a later stage after initiation by regulated disassembly of partially formed endocytic coats, so-called abortive coated pits[26,27].

Under some conditions, endocytic events appear to be initiated at random sites on the plasma membrane[26]. Random initiation could be explained simply by stochastic collision of endocytic adaptor proteins at the plasma membrane to form a seed for coat assembly[11]. However, in many cases the initiation is clearly spatially non-random. Endocytic events can occur repeatedly at some sites at the plasma membrane[25,28] or they can be concentrated to a certain region of a cell, such as the synapse in a neuron or the budding daughter cell in yeast.

Turgor pressure
Osmotic pressure within plant and fungal cells confined within a cell wall.

BAR domain
A crescent-shaped dimeric protein domain that binds membranes with its curved surface and thereby either senses membrane curvature or bends the membrane.

Box 2 | **Clathrin and the clathrin lattice**



Clathrin coats were first observed in the 1960s by electron microscopy of mosquito oocytes as a regular protein coating on the membrane of endocytic pits and vesicles[152]. The polygonal 'basketwork' of the clathrin coat was visualized a few years later on purified vesicles[164]. In 1975, Barbara Pearse purified the main protein component of these vesicles and named it clathrin[48].

The clathrin molecule is composed of a clathrin heavy chain (~190 kDa) and a smaller clathrin light chain (~25 kDa). Three clathrin heavy and light chains form a trimeric clathrin 'triskelion'. In a triskelion, the heavy chains interact with each other in the central hub and protrude outwards as long legs. These legs interact with other triskelia to form polygonal lattices, the structural backbone of the clathrin coat. Clathrin can form lattices of different curvatures, from flat hexagonal lattices to tightly curved cages composed of hexagons and pentagons. Because regular pentagons cannot cover a flat surface, a pentagon creates a local curvature defect in a hexagonal lattice, which can be seen as a local bump. If many of these defects are clustered together, one can obtain a closed geometry that approximately fits in a sphere. At least 12 pentagons are needed to form such a closed shape, and the smallest cage that can be obtained is a dodecahedron, with only 12 pentagons. All determined molecular structures of clathrin cages have 12 pentagons. However, these highly curved structures always include hexagons. In particular, the smallest clathrin cage that it is physically possible to achieve (a so-called mini-coat) contains 12 pentagons and 4 hexagons[165]. The most common clathrin lattice observed in *in vitro* reconstitution assays — which corresponds to the size of synaptic vesicles — is called a hexagonal barrel and contains 12 pentagons and 8 hexagons. However, the prototypical clathrin cage found in fibroblastic cells is a truncated icosahedron that contains 12 pentagons and 20 hexagons.

In the figure (part **a**), the structure of a clathrin cage obtained by cryo-electron microscopy is shown; individual clathrin triskelia are highlighted in red, green and yellow. The figure (part **b**) also shows clathrin lattices of different curvatures, which were visualized by electron microscopy on the surface of a fibroblast plasma membrane. From the cryo-electron microscopy structures, one can see that three triskelia arms are tightly bound together to form the edge of a hexagon or a pentagon. Large-scale topological changes to the clathrin lattice have to be made to transform hexagons into pentagons, which represents an enormous energy cost that could be fuelled only by direct or indirect ATP consumption. Of note, clathrin does not directly interact with the membrane but is connected to it via various adaptor proteins. Part **a** of the figure is from REF. 165, Macmillan Publishers Limited. The image in part **b** of the figure is republished with permission of *The Journal of Cell Biology*, from Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation, Heuser, J. E. & Anderson, R. G. **108** (2), 389–400 (1989)[166]; permission conveyed through Copyright Clearance Center, Inc.

The nonrandom distribution of endocytic events suggests that some plasma membrane domains or regions have endocytosis-promoting properties, such as the ability to concentrate specific lipids or endocytic cargo proteins, which vary locally. Many endocytic adaptor proteins are recruited to the plasma membrane by binding to phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), and this interaction is necessary for the assembly and function of the endocytic coat[29,30] (see also BOX 3). In addition, local differences in the concentration of endocytosis-competent cargo could recruit and cluster adaptor proteins to promote the formation of a nucleus. High local cargo concentration could arise from localized exocytic delivery of cargo[9,31] or from cargo oligomerization induced by ligand binding[32]. However, it is currently unknown whether cargo molecules are an essential component for the initiation of an endocytic event or whether they are more passive passengers that can be recruited after the initiation. Studies on lipids and cargo are challenging owing to the difficulty of visualizing and manipulating these molecules *in vivo* and because there are possibly hundreds of different cargoes undergoing endocytosis at the same time. Therefore, the exact role of membrane domains and cargo molecules in regulating the initiation location or frequency remains an open question.

The molecular mechanisms by which the endocytic site is initiated are not completely understood. Early studies suggested that either the BAR domain proteins F-BAR domain only protein 1 (FCHO1) and FCHO2 (REF. 17) or the AP2 complex[11] function as a primary initiator in mammalian cells. Other studies have painted a more complex picture by suggesting that several endocytic proteins form a pioneer module that initiates endocytosis[18–21] (FIG. 2). The pioneer module in mammals is composed of, at minimum, the adaptor proteins FCHO1/2 and AP2 and the scaffold proteins EPS15, EPS15R and intersectins 1 and 2 (REFS 7,17,19). In yeast, homologous pioneer proteins act similarly to promote initiation[20,21]. These pioneer components interact with each other, and they have been proposed to act cooperatively to initiate the endocytic event. The adaptors FCHO1/2 and AP2 bind to the plasma membrane and recruit scaffold proteins, which then cluster the membrane-bound adaptors to form the endocytic site. This is followed by the recruitment of clathrin and other coat-associated proteins, thereby expanding the coat. In yeast, casein kinase I homolog Hrr25 (Hrr25) phosphorylates two pioneer proteins: EH domain-containing and endocytosis protein 1 (Ede1), which is an EPS15-like protein, and suppressor of yeast profilin deletion (Syp1), which is an FCHO1/2 homologue. Phosphorylation of Ede1 by Hrr25 promotes the initiation of endocytic

sites[33]. Similarly, mammalian casein kinases homologous to Hrr25 promote endocytic initiation[33]. Exactly how this complex network of interactions in the pioneer module triggers the initiation of endocytosis remains unknown.

Cargo loading

Clathrin-mediated endocytosis transports a large variety of different cargoes from the plasma membrane into the cell. This transport is important for a multitude of physiological processes in eukaryotic cells, such as the maintenance of membrane homeostasis and the regulation of intercellular signalling, including synaptic signalling and developmental signalling[34,35]. Also, many viruses act as endocytic cargoes to gain entry into the cell[36]. In addition to being passive passengers, the cargo molecules may directly affect the vesicle formation process itself via different mechanisms.

The basic principle in cargo recruitment is that the protein components of the clathrin coat bind to specific binding sites in the cytosolic parts of different transmembrane cargo molecules to recruit them to the region of the plasma membrane that will form the vesicle. This leads to specific cargoes being enriched in the forming vesicles and thereby being selectively endocytosed. A large number of coat-associated clathrin-adaptor proteins and scaffold proteins have been shown to directly interact with specific cargoes and function as cargo adaptors for them[37]. This list of cargo adaptors includes many of the early-arriving coat components, including the pioneer module proteins AP2, FCHO1 and EPS15 and the CALM family[37]. Thus, the cargo molecules may recruit the pioneer proteins to the plasma membrane and increase the likelihood of initiation of an endocytic event[32]. The key cargo–adaptor interactions are mediated by short linear sequence motifs or by covalent modifications, such as phosphorylation or ubiquitylation in the cargo proteins[38,39].

In addition to binding to cargoes, most cargo adaptors also interact directly with lipids and with other coat proteins, thereby forming a complex network of interactions that can mediate initiation of clathrin coat assembly and its further expansion in a highly complex and cooperative manner[10].

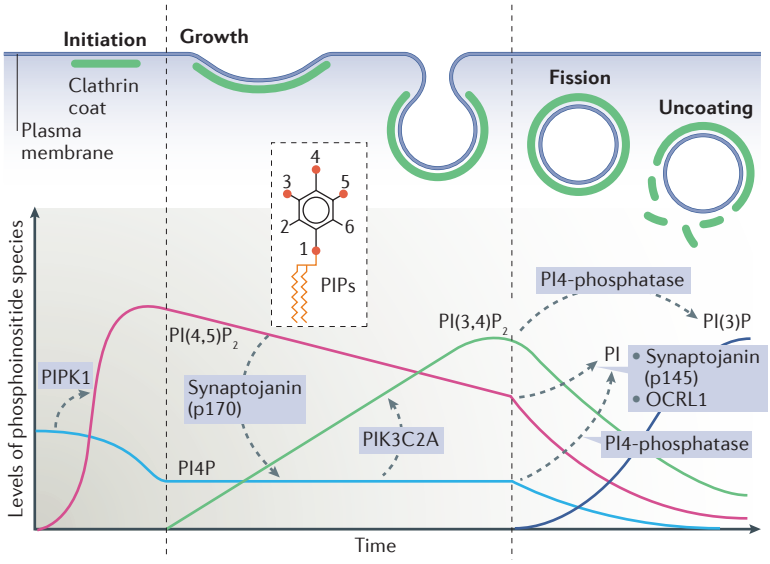
Several studies have suggested that there is a mechanism, a 'cargo checkpoint', that couples cargo loading and vesicle budding processes[9,26,27,40,41]. If the number of cargo molecules recruited does not exceed a certain threshold, the cargo checkpoint mechanism could either abort the endocytic site, leading to coat disassembly[26,27], or delay the completion of vesicle budding, potentially enabling recruitment of further cargo molecules[9,42]. These mechanisms would guarantee that the vesicles that are eventually formed are filled with cargo molecules.

Box 3 | **Phosphoinositides in clathrin-mediated endocytosis**

Many of the proteins involved in clathrin-mediated endocytosis have specific domains that bind to phosphoinositides (PIPs), the phosphorylated isoforms of the glycerolipid phosphoinositol (see the inset in the figure for the overview of PIP structure and inositol phosphorylation sites (red circles)). Among the seven possible isoforms of PIPs, at least phosphatidylinositol 3-phosphate (PI(3)P), phosphoinositol 4-phosphate (PI(4)P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P_2) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) have been suggested to have roles in endocytosis. PI(4,5)P_2 is the most important phosphoinositide for endocytosis, as many of the endocytic proteins, such as adaptors and dynamin, bind specifically to PI(4,5)P_2 (REF. 167). A critical role for PI(3,4)P_2 has been recently discovered in the late stages of endocytosis (see the main text)[118,119].

The discovery of many PIP-binding domains in molecules driving clathrin-mediated endocytosis suggests a central role for these lipids in recruiting and organizing the sequence of events during endocytosis. For example, adaptors such as adaptor protein AP2, epsin and proteins of the clathrin assembly lymphoid myeloid leukaemia protein (CALM) family each have a PI(4,5)P_2 binding domain. Dynamin interacts strongly with PI(4,5)P_2 through its pleckstrin-homology domain. Other endocytic proteins bind to other PIPs: sorting nexin 9 (SNX9), which has a role during membrane curvature build-up, has an FYVE domain that binds to PI(3,4)P_2, whereas auxilin has a PTEN domain that binds to PI(3)P and to PI(3,4)P_2.

Most of the knowledge we have on the role of PIPs in recruiting and organizing the proteins of the clathrin machinery comes from studies of the kinases and phosphatases involved in the conversions of PIPs[139]. These proteins localize to endocytic sites at specific times (see the figure). The sequence starts with phosphatidylinositol 4-phosphate 5-kinase 1 (PIPK1), which interacts with AP2 and catalyses the rapid production of PI(4,5)P_2 from PI4P to recruit many of the players involved in endocytic initiation[140]. During the assembly of the clathrin coat, the presence of the synaptojanin isoform p170 (REF. 142) is thought to degrade PI(4,5)P_2 and to enable the formation of PI(3,4)P_2 by phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit α (PIK3C2A) from the PI4P generated by synaptojanin or from the plasma membrane pool. PI(3,4)P_2 is proposed to further recruit SNX9, which is involved in curvature build-up and dynamin recruitment[118,119]. The second isoform of synaptojanin, p145, is recruited after vesicle fission to dephosphorylate the remaining PI(4,5)P_2 to phosphatidylinositol (PI)[142]. At the same time, PI(3,4)P_2, which has accumulated during vesicle budding, recruits the auxilin–HSC70 (heat shock cognate 71 kDa protein) complex to complete uncoating (see also main text). Finally, phosphoinositol 4-phosphatase (PI4)[151] and inositol polyphosphate 5-phosphatase OCRL1 (REF. 150) participate, respectively, in converting PI(3,4)P_2 to PI(3)P and degrading the remaining PI(4,5)P_2 to give an endosomal identity to the vesicle membrane[151].



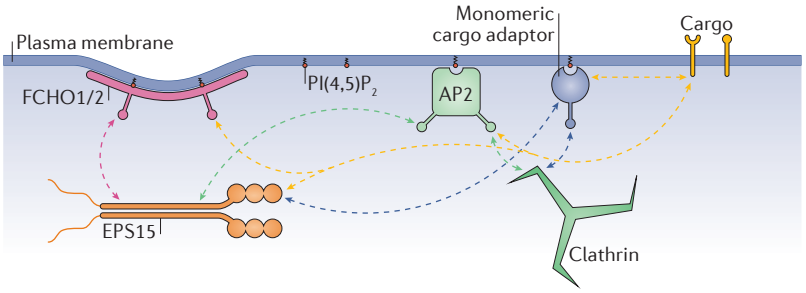


Figure 2 | **Initiation of membrane budding during endocytosis is mediated by the pioneer module.** Proteins of the pioneer module, which comprise the F-BAR domain only protein 1 and 2 complex (FCHO1/2), the adaptor protein AP2 complex, epidermal growth factor receptor substrate 15 (EPS15) and monomeric cargo adaptors, initiate endocytic events and start the recruitment of clathrin. The pioneer proteins form a network of interactions between themselves, clathrin, membrane phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and cargo molecules.

We are just beginning to understand the molecular mechanisms by which cargoes may control the progression of endocytic events. Interestingly, the AP2 complex can act to coordinate cargo recruitment with coat assembly, thus possibly regulating the initiation and/or maturation of the endocytic coat. Binding of $PI(4,5)P_2$ and cargo to the AP2 complex is associated with a large conformational change in AP2, which reveals the clathrin-binding site of AP2 and thereby enables it to recruit clathrin to the assembling coat[13,14,43]. Specifically, cargoes with tyrosine-based recognition motifs such as the transferrin receptor can activate the AP2 complex in this way and promote coat assembly[13]. The function of the AP2 complex can be regulated by other proteins such as adaptin ear-binding coat-associated protein 1 (NECAP1), which modulates the interactions of the AP2 complex with clathrin and other endocytic proteins[44]. In addition, cargo ubiquitylation status, possibly recognized by epsins, can regulate the progression of endocytic events[42].

Cargoes can also have direct physical effects on the progression of endocytic vesicle budding. Recruited cargo molecules occupy space, and invagination of the plasma membrane to form the endocytic vesicle will reduce the space available for the extracellular domains of these cargoes; thus, accumulation of cargoes would make the membrane bending harder to accomplish[45,46]. This highlights the importance of coordination between cargo loading and endocytic vesicle formation.

Mechanisms of membrane bending

Lipid bilayers made with cellular phospholipids are usually flat (that is, they lack spontaneous curvature at their energy minimum), owing to the cylindrical shape of most of the lipids. Thus, endocytosis needs to curve the membrane, which is opposed by a certain resistance of the membrane to bending. The energy associated with bending of the membrane at a given point of its surface is expressed as κc^2, where κ is the bending rigidity and c its local curvature, which is the inverse of the radius for a sphere. Several endocytic modules contribute to membrane bending during vesicle formation: the coat, the actin filaments and, finally, the scission proteins. Many different molecular mechanisms have been described that can bend membranes and are likely cooperating during endocytosis.

Membrane bending by the coat. The first function proposed for clathrin (and other protein coats operating in the endocytic and secretory systems) was membrane bending. Because clathrin was very soon found to assemble into icosahedral cages (BOX 2), it was postulated that polymerization of clathrin could induce membrane curvature by imposing the shape of the assembled clathrin lattice onto the membrane[47,48]. This mechanism, called scaffolding, was extended to the many polymeric structures involved in membrane trafficking, in particular for the coat proteins I and II (COPI and COPII, respectively) involved in Golgi transport.

There have been two opposing hypotheses of how clathrin could scaffold the membrane: early electron microscopy studies of clathrin structures found, at the plasma membrane of cells, clathrin lattices of varying curvature, from flat to the highly curved buds[49] (BOX 2). These observations led to the hypothesis that clathrin would polymerize initially flat, and then through a gradual reorganization of its lattice while keeping its surface constant would curve into a spherical coat, forcing the membrane to bud (see FIG. 3a, Model 1). Solid geometrical and biochemical arguments arose in opposition to this model: the number of clathrin–clathrin bonds to be modified to form a single pentagon from a hexagon in order to create curvature (see BOX 2) and the affinity of such bonds would make this flat-to-spherical transition extremely energetically costly[50,51]. Thus, it was instead proposed that clathrin would directly polymerize into a curved lattice on the membrane (FIG. 3a, Model 2). In this model, the budding would occur by progressive increase of the surface area covered by clathrin.

The lack of quantitative data on the dynamics of curvature build-up in cells and the robustness of *in vitro* and biochemical data on the polymerization of clathrin have favoured Model 2. However, recent *in vivo* and *in vitro* data have challenged this view, putting the two models under discussion again.

Surprisingly, endocytic rates have been shown to depend on membrane tension, suggesting that increased tension competes with clathrin polymerization[52]. Indeed, the force generated by clathrin polymerization onto the membrane is in the same range as cellular membrane tensions (typically ~10^{-4} N m^{-1}), and the recruitment of clathrin to the membrane and its polymerization can be efficiently inhibited by increasing membrane tension *in vitro*[53]. Strikingly, similar values of polymerization energy have been measured for the COPI coat[54], which may indicate that even very structurally different coats may have evolved to converge to a given force of polymerization, which is well balanced with typical values of membrane tension.

However, the main conclusion of these biophysical studies is that the membrane is not a passive element subject to the action of clathrin but that it actively contributes to the efficiency of membrane shaping and that the elastic energy required for membrane budding can

Membrane tension
In-plane force counteracting membrane surface expansion.

Elastic energy
Energy required to deform an elastic material. For lipid membranes, it contains a term for bending and a term for stretching, both taking the form of the energy associated with a harmonic spring: a constant called the modulus or rigidity, multiplied by the shape change to the square. Thus, bending energy is the bending rigidity multiplied by membrane curvature to the square, whereas stretching energy is the compressibility modulus multiplied by the area difference to the square. The elastic energy of the membrane is the sum of these two terms.

Amphipathic helix
A short polypeptide, typically between 10 and 20 amino acids in length, that contains hydrophobic and hydrophilic residues. This polypeptide spontaneously folds into an α-helix when binding to a lipid membrane. In this configuration, all hydrophobic residues are aligned on the cylindrical face of the helix that is buried in the bilayer whereas the hydrophilic moieties are aligned on the hydrated face.

compete with the clathrin polymerization energy. These phenomena would have different consequences in the two models described above. The effect of increasing elastic energy is to limit membrane curvature. Thus, in the case in which clathrin polymerizes at a constant curvature, as proposed in Model 2 (FIG. 3a), increased elastic energy may fully block clathrin assembly onto the membrane or stall clathrin-mediated endocytosis at various steps of assembly. By contrast, if clathrin can polymerize on surfaces of different curvature (FIG. 3a, Model 1), elastic energy cannot block polymerization. It can, however, participate in regulating the shape of the vesicle, as the final curvature will be a balance between membrane elastic energy and clathrin polymerization energy, with high elastic energy promoting larger vesicles and low elastic energy promoting formation of vesicles close to the spontaneous clathrin cage size.

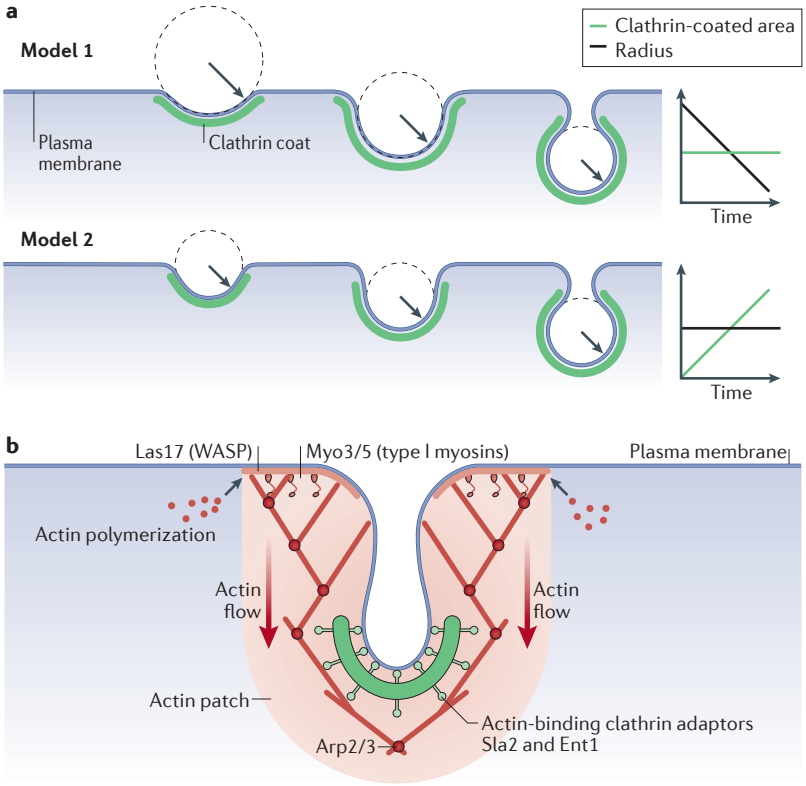


Figure 3 | **Mechanism of clathrin coat-mediated and actin-mediated membrane bending. a** | Models of membrane bending by clathrin coat. Model 1: The clathrin coat first assembles into a flat patch of a given area (not shown). Without changing its area, the clathrin lattice is then remodelled by introducing pentagon units of clathrin to increase curvature (see BOX 2 for details). Model 2: The clathrin coat assembles directly into a bud of constant curvature. The area coated by clathrin increases with time from a shallow pit, eventually reaching the full spherical shape. The graphs show representation of area (green) and curvature (black) evolution during the budding. Arrows are radii at different steps of clathrin pit formation — they progressively reduce in Model 1, whereas they stay constant and small in Model 2. **b** | The model for actin-polymerization-driven membrane invagination in yeast. Actin filaments are nucleated around the base of the invagination by actin-related protein 2/3 complex (Arp2/3), which is activated by Las17 (protein-rich protein Las17; homologue of mammalian Wiskott–Aldrich syndrome protein (WASP)) and type I myosins (Myo3/5). Continued polymerization pushes the actin network into the cell (actin flow). The clathrin-coated membrane is coupled to the actin network by protein Sla2 (homologue of mammalian Huntingtin-interacting protein 1-related protein (HIP1R)) and epsin 1 (Ent1) and is pulled inwards with the actin network.

As described above, many *in vitro* findings are consistent with Model 2, as clathrin polymerization is blocked by increased tension and bending rigidity[53,55]. These findings may be true for conditions used in these studies (for example, the choice of clathrin adaptors — namely, AP180 and epsin) but could be very different for other conditions. Notably, *in vivo*, well-ordered, flat clathrin patches, containing AP2 and other adaptors, have been observed repeatedly[40,49,56]. Moreover, *in vitro*, clathrin can make very large, perfectly hexagonal lattices on hard substrate, showing that clathrin alone can polymerize into completely flat lattices[57].

A recent study measured the curvature of clathrin-coated pits and the membrane area covered by clathrin in mammalian cells through the use of correlative light and electron microscopy (CLEM) at different stages of progression through budding[58]. The authors found that while the membrane area covered by clathrin does not change, the average curvature of the pits increases with progression of budding, supporting Model 1. Similar results were also obtained in yeast, where curvature build-up is clearly temporally disconnected from clathrin accumulation[59].

At the moment, both models have gained substantial support from various experimental studies. Two questions remain. First, are these two models really exclusive? Second, because Model 1 is supported mostly by experimental data in the cell environment and Model 2 by *in vitro* experiments, what could be the reason for the different behaviour of clathrin in those two contexts?

First, clathrin is binding to biological membranes through adaptors, which have been shown to participate in membrane deformation. For example, the amphipathic helix of the epsin N-terminal homology (ENTH) domain of epsin or the AP180 N-terminal homology (ANTH) domain of CALM can promote curvature induction[12,16,53] (whereas the equivalent ANTH domain of AP180, which does not have an amphipathic helix, does not promote membrane bending)[53,60]. These amphipathic helices act as wedges, creating local curved defects that propagate the curvature on a larger area once they are clustered together through polymerization of clathrin. Thus, the sequence of adaptor recruitment to the pit could favour curvature build-up by integrating a higher fraction of membrane bending adaptors with time[11].

Second, it could be that integration of accessory factors rigidifies the clathrin lattice, participating in the curvature build-up through elastic relaxation of a curved clathrin lattice, which when assembled flat would be initially stretched. This possibility is supported by the observation that binding of the clathrin light chain to the clathrin heavy chain rigidifies the clathrin lattice, which shows that rigidity of the clathrin coat can be dynamically modulated[57]. This idea was initially proposed for COPII vesicle budding[61], again suggesting an evolutionarily conserved functional convergence of COP and clathrin coats despite their structural differences.

Third, a striking feature of the clathrin lattice *in vivo* is its high turnover rate evidenced by fluorescence recovery after photobleaching (FRAP) experiments[58,62,63]. These findings suggest that, *in vivo*, the clathrin lattice

is rapidly reorganized, which drives membrane deformation by providing flexibility to the lattice to accommodate changes in membrane curvature associated with budding. What cellular machinery could drive such high turnover? The complex between auxilin (also known as cyclin-G-associated kinase (GAK)) and the constitutively active heat shock cognate 71 kDa protein (HSC70; also known as HSPA8) is essential to membrane uncoating after scission of the bud (see below). It is mostly recruited to the clathrin coat after scission[24], but some earlier functions during clathrin coat assembly have also been proposed[64]: HSC70, as a chaperone protein, could function to provide the necessary energy source to remodel the clathrin lattice, transforming hexagons into pentagons to enable the change of curvature of the assembling polymeric coat (see BOX 2). HSC70 is clearly involved in coat disassembly (see below). Nevertheless, in *in vitro* experiments in which clathrin cages are disassembled with ATP and HSC70, a dynamic steady state in which assembly and disassembly occur simultaneously is obtained after long incubation times[65]. These findings support the possibility that HSC70 promotes the required turnover of triskelia for curvature change.

Membrane bending by actin filaments. In addition to the clathrin coat, the actin cytoskeleton can contribute to membrane bending during the formation of clathrin-coated vesicles. The early evidence for a role for actin in endocytosis came from studies that showed inhibition of endocytosis when the actin cytoskeleton was perturbed with inhibitors or mutations of factors involved in actin dynamics[66–70]. Live imaging studies later revealed that actin assembles locally and transiently at the endocytic sites in both mammalian[6,7,28,71,72] and yeast cells[73,74]. Actin polymerization is associated with later phases of endocytosis when considerable membrane bending is present. After vesicle scission, the actin filaments depolymerize in seconds. In yeast cells this association lasts only for about 15 seconds[5,74]; in mammals it lasts about twice as long[7,71].

Inhibiting actin polymerization in yeast cells blocks endocytosis very strongly at the beginning of membrane invagination[59,75]. In mammalian cells, the inhibition of actin polymerization has produced various effects on the progression of endocytosis[28,52,67,76]. Endocytic membrane shaping is blocked in mammals at a later stage than in yeast when the plasma membrane has already formed U-shaped invaginations[52].

In yeast cells, the studies of endocytic actin structures (actin patches) are facilitated by the fact that they are very distinct and easily visualized owing to the lack of a cortical actin cytoskeleton, which obscures actin at endocytic sites in mammalian cells. Therefore, most of the our mechanistic understanding of the role of actin in endocytosis comes from yeast studies. The yeast actin patch starts forming at the endocytic sites at about the time when membrane bending is initiated[59,75]. The patch then grows to a maximum diameter of about 200 nm, surrounding the forming membrane invagination and finally the nascent vesicle[59,75,77,78]. Similarly, in mammalian cells, polymerizing actin has been shown to form a network of filaments around the rim of the clathrin coat[79]. Light and electron microscopy data suggest that in both mammals and yeast, actin filaments polymerize preferentially in a region surrounding the coat and the base of the growing membrane invagination[79,80].

The key components regulating the formation of the actin filaments at the endocytic sites are the WASP family proteins that recruit and activate the ARP2/3 complex to nucleate actin filaments[22,74,81]. In yeast cells, the nucleation by the Arp2/3 complex at the endocytic sites has been shown to be further controlled by a complex network of regulatory proteins[22,74,82,83]. The polymerized actin filaments recruit a large number of actin-binding proteins, including filament crosslinkers, cappers, myosin motors and depolymerization factors, many of which have been shown by mutant studies in yeast to be important for endocytosis to occur[23].

The mechanisms that couple the polymerizing actin filaments to vesicle budding are not well understood. However, in yeast, two EPS15-like coat-associated proteins, actin cytoskeleton-regulatory complex proteins Pan1 and End3, are critical for recruiting the actin filament nucleation machinery to endocytic sites[84,85]. To harness the polymerizing actin filaments for membrane shaping in concert with the forming coat, they need to be coupled to the clathrin coat. The cooperative action of two families of clathrin-adaptor proteins is essential for this coupling: the epsins and proteins belonging to the Huntingtin-interacting protein 1-related protein (HIP1R; Sla2 in yeast) family. These proteins bind to $PI(4,5)P_2$ in the membrane, to clathrin and to actin filaments, and their deletions result in arrested endocytosis and continuous unproductive actin polymerization at the endocytic sites in both yeast and mammalian cells[9,15,52,73,86–88].

Filament polymerization is likely the main mechanism by which the actin machinery produces force for bending the endocytic membrane. Localized polymerization at the plasma membrane would generate a flow of actin filaments directed from the base of the forming invagination towards the tip. A coupling between the actin filaments and the clathrin coat would transmit the force to bend the membrane (FIG. 3b). The mechanisms would thus be analogous to actin-driven protrusion of lamellipodia, where actin filaments are nucleated by the ARP2/3 complex and polymerize at the plasma membrane, generating a flow of the actin network, which is coupled to cell–extracellular matrix adhesions[89]. Theoretical studies support this model of actin-polymerization-driven endocytosis[90,91]. In addition to actin polymerization, myosin motor proteins could contribute to actin-dependent force generation. In yeast, type I myosins localize to the endocytic sites together with actin and are essential for vesicle budding[22,74,77,92]. Similarly, in mammalian cells, myosins localize to endocytic sites and facilitate the endocytic process[7,93]. The mechanism by which the myosins work is not well understood, but it has been suggested that they act as dynamic anchors linking actin filaments to the membrane at the base of the invagination so that the force produced by actin polymerization can be efficiently harnessed for membrane shaping[94].

Fluorescence recovery after photobleaching
(FRAP). Microscopy method for measuring local exchange of fluorescently labelled molecules.

Type I myosins
A highly conserved subfamily of monomeric myosin motors involved in cell motility and membrane traffic.

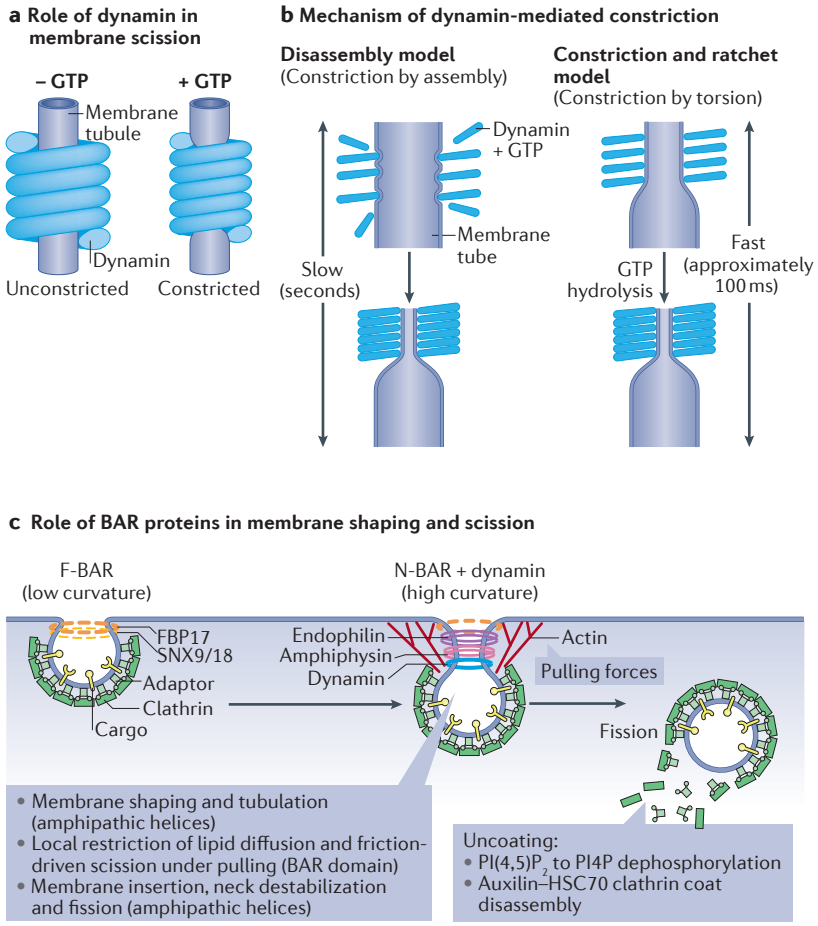


Figure 4 | **Membrane fission by dynamin and BAR proteins followed by vesicle uncoating. a** | Dynamin forms helical collars around membrane tubules *in vitro*. Two states of dynamin are observed: unconstricted in the absence of nucleotides and constricted in the presence of GTP. Whether dynamin assembles directly into the constricted state in the GTP-bound form or whether GTP hydrolysis is required to fuel active constriction of the helix is still debated. **b** | Models of membrane constriction by dynamin. In the disassembly model (left), when bound to GTP, dynamin adopts a very tight helical conformation, which constricts the membrane by assembly around the neck, and reaches hemi-fission. In the constriction and ratchet model (right), the helix of dynamin assembles around the membrane in a partially constricted form, and GTP hydrolysis energy promotes active constriction of the polymer to reach hemi-fission. **c** | Bin/Amphiphysin/Rvs (BAR) proteins participate in the curvature build-up at the neck of clathrin-coated pits to recruit dynamin. First, F-BAR proteins, including formin-binding protein 17 (FBP17; also known as FNBP1) and sorting nexins 9 and 18 (SNX9/18), are recruited to the pit at low curvature (owing to the presence of shallow BAR domains), and then N-BAR proteins with highly curved BAR domains (endophilin and amphiphysin) are recruited to promote higher curvature and dynamin recruitment. Dynamin then further constricts the neck to allow fission. N-BAR proteins also further contribute to membrane fission. By forming a polymer-like coat over the constricted membrane, they limit the diffusion of lipids; this generates friction between the lipids and the N-BAR proteins, which results in a build-up of tension in the non-coated part of the vesicle, eventually leading to friction-driven scission. This mechanism depends on pulling forces generated by the actin cytoskeleton (see FIG. 3b) or microtubule motors (not shown), which extend the vesicle. In addition, amphipathic helices of N-BAR proteins directly contribute to membrane shaping during vesicle budding. After scission, the vesicle undergoes uncoating. This uncoating is driven by the activity of the heat shock cognate 71 kDa protein (HSC70) chaperone, which is recruited by auxilin and disassembles the clathrin polymer. Auxilin–HSC70 machinery is most likely downstream of lipid composition changes, most prominently, the dephosphorylation of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$ to phosphoinositol (4)-phosphate (PI4P) and concomitant accumulation of phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) (see also BOX 3).

Vesicle scission

Scission is the step in which the forming vesicle is separated from the donor membrane. In canonical clathrin-mediated endocytosis, this step is catalysed by the large GTPase dynamin, which assembles into a helical collar at the neck of the clathrin-coated pit and breaks it. There is general agreement in the community for the role of dynamin in vesicle fission in mammals based on three observations: first, dynamin assembles into fairly tight oligomers (internal radius of 10 nm), enabling initial constriction of the neck; second, membrane fission requires GTP hydrolysis; and third, dynamin oligomers further constrict in the presence of GTP[95] (FIG. 4a). Although there is still discussion concerning the details of the use of GTP-derived energy in this reaction (see FIG. 4b), there is strong agreement that GTP hydrolysis is required to reach a highly constricted state that spontaneously (through thermal fluctuations of the membrane) transitions to a hemi-fission and then fission state[95].

Although the dynamin-mediated membrane fission reaction has been reconstituted *in vitro*[96–99], it remains unknown how this reaction proceeds and how it is precisely regulated *in vivo*. In particular, dynamin interacts with many partners through its proline-rich domain, among which BAR proteins are of particular importance (see below)[100].

In yeast, the role of dynamin in clathrin-mediated endocytosis remains an open question. Some studies have shown that the yeast homologue of dynamin, vacuolar protein sorting-associated protein 1 (Vps1), does not contribute to clathrin-mediated endocytosis[5,101] whereas other studies have suggested a clear role for Vps1 in endocytic scission[102,103]. Therefore, Vps1 may have a regulatory, but not an essential, role in scission.

Two questions about the mechanism of fission of clathrin-coated pits have garnered the most attention from scientists in recent years. First, how are fission proteins, in particular dynamin partners such as BAR proteins, recruited in a timely manner to the neck of endocytic buds at the progression to vesicle scission? Second, how do these proteins contribute to fission; that is, do they regulate or actively participate in membrane constriction?

BAR domain proteins have been proposed to have several roles in the fission reaction, including the recruitment of other fission proteins, notably including dynamin itself, as well as an active role in membrane constriction and fission[104,105]. They have also been implicated in actin recruitment to the bud, which could be important for both membrane shaping (see previous subsection) and membrane scission (see below). However, it needs to be pointed out that the exact contribution of BAR-containing proteins to dynamin-mediated fission is thus far unclear, with some studies reporting an enhancement and some an inhibition of dynamin fission by BAR proteins[106–109]. The BAR domain is a dimeric membrane-binding module that has a crescent shape[110]. An impressive feature of the BAR domain is that it tubulates membranes upon binding at high density, and the radii of the tubules fit

the curvature of the BAR crescent[111–113]. At lower, probably more physiologically relevant densities, the BAR domain was shown to be a curvature sensor[110,114]. Thus, BAR domain proteins were initially proposed to recruit dynamin to the neck of endocytic buds because of the high curvature of these necks. The structure and the membrane-binding properties of BAR domain proteins are consistent with this function, and numerous studies *in vitro*[107,110,115] and *in vivo*[116,117] support this role. Various BAR domains differ in their curvature: F-BAR domains have a shallow curvature (radius of 60–80 nm) whereas N-BAR domains have a very high curvature (with typical radii of 10 nm). Data supporting a role of BAR domains in recruiting dynamin suggest that the recruitment sequence of various BAR proteins largely matches the changing membrane curvature at the endocytic cite: proteins with shallow F-BAR domains (for example FCHO1/2) are recruited earlier than proteins with N-BAR domains (such as endophilin and amphiphysin), and proteins with BAR domains with intermediate curvature, such as sorting nexin 9 (SNX9), are recruited in between[7,118–120] (FIG. 4c). Interestingly, the sequence of recruitment may be coordinated by both an increasing curvature of the membrane, which recruits the BAR domain when it matches their curvature, and specific species of PIPs that are sequentially produced during assembly of the clathrin coat (see BOX 3). For example, SNX9 is specifically recruited through the production of phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) by phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit α (PIK3C2A) at the assembled clathrin pit[118]. Although the PX domain of SNX9 binds PI(4,5)P2 and $PI(3,4)P_2$, simulations show that only $PI(3,4)P_2$ actively recruits SNX9 at the endocytic site, where many proteins compete for $PI(4,5)P_2$ binding.

The structure and the membrane properties of some of the BAR domain proteins is consistent with their direct role in membrane fission. In particular, N-BAR proteins contain an amphipathic helix located on the amino terminus from the BAR domain and use this helix to insert into the outer leaflet of the plasma membrane and induce membrane curvature[121]. This helix on its own was shown to induce sufficient bending to constrict and break membrane necks[116]. In the same study, it was also proposed that the BAR domain restricts the curvature of the membrane to the size imposed by its own curvature, thereby precluding further membrane bending and fission[116]. Moreover, the BAR domain of endophilin, which is an N-BAR protein that binds dynamin, was shown to insert in between turns of the dynamin helix when endophilin was in molar excess with respect to dynamin. This insertion was proposed to block the mechanoenzymatic constriction of dynamin and thus membrane fission[122]. Interestingly, at regions where the curvature of the neck of the vesicle is fixed by the curvature of the BAR domain, BAR domains have been shown to form large tubular polymers that coat the tubulated membrane[106,111,113,123,124] and to limit the diffusion of lipids, forming stable, phosphoinositide-rich membrane domains[125]. This limited lipid diffusion imposed by the BAR polymer was shown to lead to membrane fission under pulling forces *in vitro*[126,127]. The proposed mechanism of this fission is that limited diffusion of lipids below the BAR polymer coat generates friction between the lipids and the coating polymer, which results in a build-up of tension in the non-coated part of the tubule — driven by pulling forces — eventually resulting in pore formation and scission. The pulling forces may be generated either by microtubule motors such as dynein[123] or by actin polymerization[122]. In this way, BAR proteins could drive dynamin-independent fission, thereby providing one potential explanation for a nonessential role of dynamin for endocytosis in yeast. Indeed, Rvs proteins (BAR-containing yeast proteins) have been suggested to form a large scaffold over the neck of clathrin endocytic structures in yeast cells, and fission of the vesicles is concomitant with bud displacement, suggesting the importance of pulling forces during scission[59,80]. Moreover, the overall shape of the clathrin-coated endocytic structures is fairly tubular in yeast, which is consistent with the mechanism involving thinning of the neck under pulling forces. Thus, BAR proteins seem to be important for orchestrating membrane curvature changes as the endocytic vesicle is formed, but it appears that BAR domains themselves may have both an inhibitory and promoting effect on fission and that other mechanisms, such as the presence of amphipathic helices or additional pulling forces, are responsible for the direct scission-promoting effects.

Disassembly of the coat

The formation of the primary endocytic vesicle ends with the disassembly of the protein machinery that created it. Disassembly releases the endocytic machinery proteins, which can be reused for another endocytic event, and frees the new vesicle so that it can fuse with an early endosome to initiate an intracellular trafficking event. Two key mechanisms have been described that participate in uncoating: breakage of the clathrin–clathrin interactions in the lattice by the chaperone HSC70, and dephosphorylation of $PI(4,5)P_2$ (FIG. 4c). These two mechanisms probably work in a coordinated manner, but their exact contributions to uncoating and how they work together in this process are not known.

Direct role of HSC70 in vesicle uncoating. HSC70 has been shown to be essential for clathrin uncoating[64,128,129]. The recruitment of HSC70 to clathrin-coated vesicles requires the protein auxilin, which harbours a clathrin-binding domain that interacts with the globular carboxy-terminal domain of clathrin, and a J domain that binds ATP–HSC70. Importantly, auxilin also binds to dynamin[130–132] and is mostly recruited to endocytic vesicles after scission, which most likely makes an important contribution to regulating the sequence of fission and uncoating.

It was shown *in vitro* that the formation of the HSC70–auxilin–clathrin complex stimulates the ATPase activity of HSC70 and promotes coat disassembly[65]. Interestingly, *in vitro*, after the initial disassembly of the clathrin coat, balanced polymerization–depolymerization of clathrin

is observed, reminiscent of the proposed role of HSC70 in clathrin coat remodelling that may promote membrane curvature during vesicle formation. This turnover has been clearly associated with the nucleotide load of HSC70: when associated with ADP, HSC70 still binds to clathrin through auxilin. However, once ATP replaces ADP in the nucleotide pocket of HSC70, the complex then dissociates.

Two models have been proposed for how auxilin–HSC70 could cause disassembly of the clathrin coat (see REF. 133 for review). One model, which is based on structural data of the auxilin bound to the clathrin cage[134], proposes that HSC70 and auxilin create a large steric hindrance, increasing the excluded volume below the vertices of the clathrin lattice. As a consequence, thermal fluctuations of molecules bumping onto this excluded volume would create a large force, loosening clathrin–clathrin interactions, leading to disassembly. This model is referred to as the 'steric wedge' model.

Another model, which is based on the ability of HSC70 to generate entropic forces along unstructured polypeptides connecting auxilin to clathrin[135], suggests that auxilin–HSC70 acts as a 'wrecking ball'. ATP consumption is proposed to enhance the Brownian motion of the globular fold of HSC70, which would then collide into the clathrin lattice vertices owing to its attachment to clathrin and auxilin through their unstructured regions. These collisions would apply a local pressure on the vertices of the clathrin lattice that could disrupt the coat[136].

PI(4,5)P_2 dephosphorylation in the regulation of scission and uncoating. Conversion of PI(4,5)P_2 to phosphoinositol (4)-phosphate (PI4P) is a key factor involved in endocytic vesicle uncoating. This reaction was initially identified in neuronal synapses and was shown to depend on the PI(4,5)P_2 phosphatase synaptojanin[137]. Neurons of mice, in which the brain-specific synaptojanin 1 isoform (encoded by *Synj1*) was knocked out, accumulate coated synaptic vesicles as well as PI(4,5)P_2 (REF. 138). These data suggested that PI(4,5)P_2 dephosphorylation is required for uncoating (at least in neuronal cells) and that mechanisms of PI(4,5)P_2 dephosphorylation act independently — probably through destabilizing membrane–coat interactions — to drive uncoating. These mechanisms most likely are upstream of the auxilin–HSC70 machinery, promoting its action on the clathrin coat.

Phosphoinositide metabolism is essential for many steps of clathrin-mediated endocytosis[139] (BOX 3). As discussed above, PI(4,5)P_2 synthesis and accumulation is important for the assembly of the clathrin-coated pit, whereas, as shown here, PI(4,5)P_2 dephosphorylation mediates coat disassembly. Further confirmation of the importance of PI(4,5)P_2 in endocytic processes came from findings that genetic disruption of PI(4,5)P_2 synthesis substantially impaired formation of synaptic vesicles and synaptic vesicle trafficking[140] and that chemically induced removal of synaptojanin from the plasma membrane completely stopped the dynamics of clathrin-coated pits in fibroblastic cells[30,141].

So, how is PI(4,5)P_2 metabolism regulated through the process of endocytosis? The long isoform of synaptojanin (p170 for 170 kDa), which contains a clathrin and AP2 binding cassette, is present throughout clathrin coat maturation and is probably required for the tight regulation of PI(4,5)P_2 levels during clathrin coat assembly[142]. Notably, however, the short synaptojanin isoform (p145, which stands for 145 kDa), which lacks the clathrin and AP2 binding cassette, is recruited together with dynamin by the BAR protein endophilin in fibroblast cells at later stages of endocytosis. This finding is consistent with previous findings that endophilin 1, the brain-specific isoform, recruits synaptojanin 1 to endocytic sites at synapses[143,144]. These data strongly suggested that dynamin-mediated fission is coupled to recruitment and action of synaptojanin through the common partner endophilin. Indeed, synaptojanin is also recruited by highly curved membranes and has an additional effect in stimulating dynamin-dependent fission[145]. Thus, it seems that PI(4,5)P_2 dephosphorylation by synaptojanin promotes both dynamin-mediated membrane fission and concomitant vesicle uncoating mediated by the auxilin–HSC70 complex. Of note, synaptojanin is recruited by endophilin probably slightly before dynamin[7,120], whereas auxilin harbours a phosphatidylinositol 3-phosphate (PI(3)P) and PI(3,4)P_2 binding module (the PTEN homology domain) and is recruited to clathrin-coated pits only if this PTEN domain is present and if sufficient levels of PI(3)P and PIP(3,4)P_2 are available in the membrane of the vesicle[24]; thus, it seems that auxilin–HSC70 recruitment could occur only when sufficient amounts of PI(4,5)P_2 have been dephosphorylated by synaptojanin and when PI(3,4)P_2 (and later PI(3)P) has accumulated (see also BOX 3). Overall, these data suggest that PI(4,5)P_2 dephosphorylation is upstream of both membrane fission and vesicle uncoating and serves to control these events and to synchronize them in time.

More recently, apart from synaptojanin, another PI(4,5)P_2 phosphatase, inositol polyphosphate 5-phosphatase OCRL1 (encoded by *OCRL*), was directly implicated in the final steps of clathrin-mediated endocytosis[146]. OCRL1 interacts with clathrin and AP2 (REFS 147,148) and seems to act temporally after dynamin-mediated scission to participate in dephosphorylation of PI(4,5)P_2 (REF. 149). The precise timing of OCRL1 recruitment after scission depends on marking of the endocytic vesicle with the endosomal small GTPase Ras-related protein Rab35 (RAB35)[150] and is thought to participate in converting the membrane identity of newly formed endocytic vesicles to endosomal membranes. This membrane identity is also endowed by the concomitant RAB35-mediated recruitment of a multivalent adaptor protein specific for endosomal membranes, DCC-interacting protein 13α (APPL1)[151], which also interacts with OCRL1. Thus, OCRL1 has an important role in the generation of an endosome-specific phosphoinositide composition through degradation of PI(4,5)P_2. However, the precise roles of OCRL1 in membrane trafficking, and in particular its phosphatase activity in clathrin-mediated endocytosis, remain obscure.

Entropic forces
Forces that arise while the system tries to maximize its entropy. These forces typically arise from frustrated thermal fluctuations, which will then counteract the constraints by applying forces onto them. The pressure of an ideal gas is an entropic force. In lipid membranes, repulsive forces between closely apposed bilayers (less than a few tens of nanometres) — known as Helfrich forces — are entropic forces. They arise from thermal undulations of the bilayer surface. In polymer physics, thermal fluctuations usually lead to the folding of the polymer molecule into globular conformations. If one pulls on both ends of the molecule, an entropic force is felt.

Conclusions and perspectives

The process of clathrin-mediated endocytosis has been studied for more than 5 decades[152]. We have gained a detailed understanding of most of the protein components involved in the process and of many of the molecular mechanisms driving the individual steps of endocytosis, such as clathrin coat assembly, membrane bending, scission and vesicle uncoating. However, we still have a rather poor understanding of how all the different proteins and their activities are precisely coordinated to produce endocytic vesicles in a highly regular and robust manner.

The key challenges are the molecular complexity of the endocytic machinery, its dynamic nature and small size. Overcoming these challenges will require application of analytical methods that have high temporal and spatial resolution and that at the same time are high-throughput methods that enable systematic studies. To achieve a solid mechanistic understanding of endocytic events, we need to obtain quantitative data about the activities and numbers of individual molecules functioning at endocytic sites at different stages of endocytosis. Recent advances in quantitative live-cell imaging[11,27,80,153], electron microscopy and super-resolution light microscopy[72,154] are paving the way towards this comprehensive mechanistic understanding.

Furthermore, our understanding of endocytosis comes almost exclusively from studies performed on single cells in culture. As a result, how endocytosis operates and how it is regulated in the multicellular context of tissues, how it contributes to various processes (such as developmental patterning, neural plasticity and cell signalling) and how its deregulation is implicated in pathological conditions remain largely unknown. Novel gene editing[155,156] combined with imaging approaches[157,158] will be critical for entering this almost uncharted territory of membrane trafficking.

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