Harnessing actin dynamics for clathrin-mediated endocytosis

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Actin polymerization often occurs at the plasma membrane to drive the protrusion of lamellipodia and filopodia at the leading edge of migrating cells. A role for actin polymerization in another cellular process that involves the reshaping of the plasma membrane — namely endocytosis — has recently been established. Live-cell imaging studies are shedding light on the order and timing of the molecular events and mechanisms of actin function during endocytosis.

Lamellipodia
Sheet-like plasma-membrane protrusions at the leading edge of motile cells that are formed by actin polymerization.

Filopodia
Plasma-membrane spikes that are formed by actin polymerization.

Phagocytosis
A plasma-membrane-associated process in which a eukaryotic cell engulfs large particles, such as bacteria.

Macropinocytosis
A form of endocytosis in which extracellular fluid is taken up through the formation of large membrane vesicles.

Clathrin-mediated endocytosis
The uptake of receptors, membrane and cargo at the cell surface through a process that specifically involves the coat protein clathrin.

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The dynamic polymerization of actin has a central role in several processes that reshape the plasma membrane. These processes include the protrusion of lamellipodia and filopodia during cell migration, and different forms of endocytic internalization — for example, phagocytosis, macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis (FIG. 1). In this review, we focus on the role of actin in clathrin-mediated endocytosis, which is the main pathway for receptor-mediated endocytosis in most eukaryotic cells[1] (for other reviews on actin in clathrin-mediated endocytosis, see REFS 2–5). We present recent findings on this topic with an emphasis on live-cell imaging studies in *Saccharomyces cerevisiae*, in which the analysis of actin dynamics and function has been most comprehensive. We also attempt to synthesize all of the findings into a mechanistic model of how actin functions during endocytic internalization.

A basic scheme for the key events of clathrin-mediated endocytosis was established decades ago[6–8]. The first step in this pathway is the binding of extracellular cargo molecules to specific cell-surface receptors. These receptors and other membrane proteins that are destined for endocytosis are then sequestered by intracellular adaptor proteins to sites of endocytosis. The adaptors, together with clathrin, form an endocytic coat at the plasma membrane. The coated membrane bends to form an invagination that subsequently pinches off to form a cargo-filled vesicle. The protein coat that surrounds the newly formed endocytic vesicle is rapidly disassembled. The vesicles then deliver their cargoes to early endosomes by fusing with the endosomal membrane. From early endosomes, the cargo molecules can be recycled back to the plasma membrane or trafficked further to late endosomes and, finally, to lysosomal organelles for degradation. This classic model was based mainly on the results of electron-microscopy studies on fixed cells and on numerous biochemical assays.

During recent years, live-cell fluorescence microscopy has provided several new mechanistic insights into clathrin-mediated endocytosis. Although the resolution of light microscopy cannot compete with that of electron microscopy, light microscopy has one important advantage — microscopy at visual wavelengths can be carried out on living cells, so cellular processes can be followed in real time. Owing to the transient and localized nature of endocytic actin structures, live-cell imaging has been crucial in showing that a dynamic actin cytoskeleton participates directly in membrane dynamics during clathrin-mediated endocytosis and in understanding the role of actin in endocytosis. The dynamic connection between the actin cytoskeleton and sites of clathrin-mediated endocytosis in living cells was first shown in cultured mammalian cells[9]. As described below, the polymerization of actin seems to provide the force for the deformation and movement of a membrane at different steps along the endocytic pathway.

Linking the endocytic and actin machineries

One of the first hints that the actin cytoskeleton is involved in endocytosis came from the use of pharmacological agents to interfere with actin turnover. In mammalian cells, actin poisons inhibit endocytic uptake and the formation of coated vesicles[10–13]. However, the block in endocytosis in mammalian cells seems to be partial[10,12,13] or restricted to only the apical surface of epithelial cells[11]. In *S. cerevisiae*, both latrunculin A, which binds to actin monomers and prevents their assembly into filaments, and jasplakinolide, which stabilizes actin filaments (and prevents their depolymerization), block endocytosis completely[14,15].

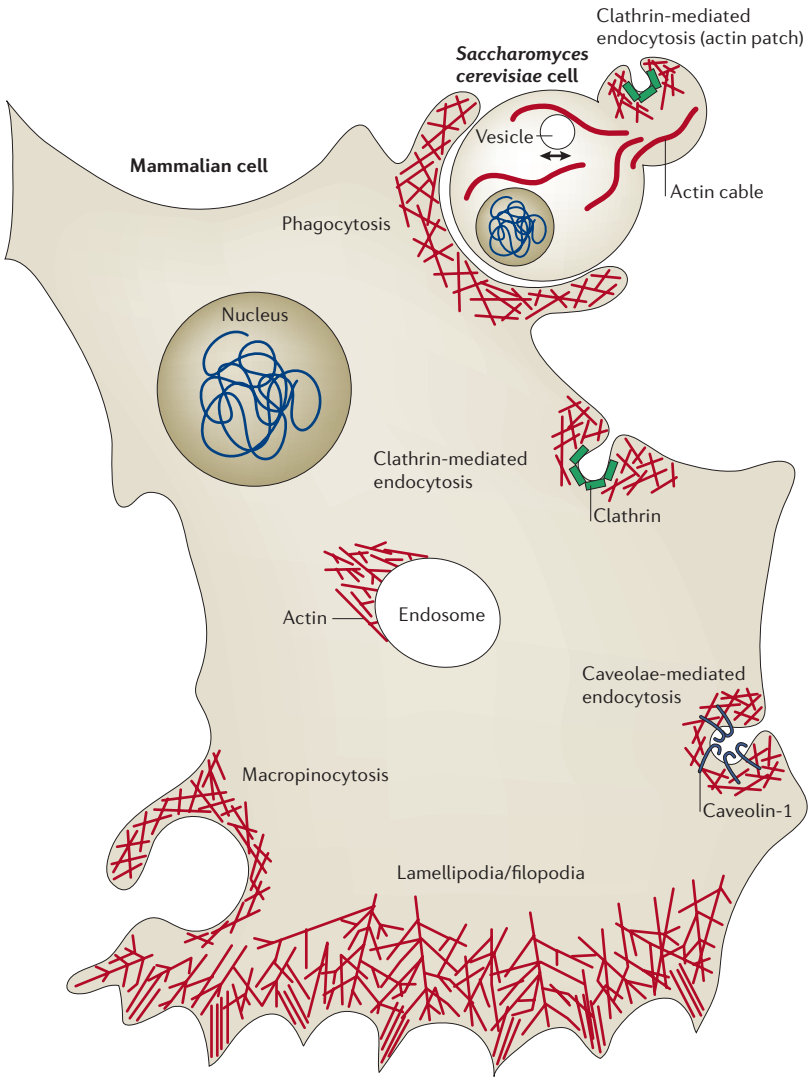


Figure 1 | **Actin-polymerization-driven processes in eukaryotic cells.** The dynamic polymerization of actin filaments (red) is involved in different processes that reshape or move cellular membranes. These processes include different forms of endocytic uptake at the plasma membrane — that is, clathrin-mediated endocytosis in *Saccharomyces cerevisiae* and mammalian cells, as well as caveolae-mediated endocytosis, macropinocytosis and phagocytosis in mammalian cells. In addition, actin assembly has a role in the movement of endosomes and/or endocytic vesicles. In mammalian cells, endosomes move by actin 'rocketing', whereas in *S. cerevisiae*, endocytic vesicles move together with actin cables as they are being assembled by formin proteins. Finally, the protrusion of lamellipodia and filopodia in migrating mammalian cells is dependent on actin polymerization.

Another line of evidence for the involvement of actin in endocytosis came from genetic experiments that were carried out in *S. cerevisiae*. Using actin mutants, the Riezman laboratory showed that normal actin function is required for the internalization step of the endocytosis of mating pheromone α-factor[16]. The later trafficking steps along the endocytic pathway were not affected[16]. Similarly, mutations in several actin-binding proteins resulted in a block in endocytic internalization in *S. cerevisiae*. In cells in which the gene that encodes the actin-filament-crosslinking protein Sac6 (also known as fimbrin) was deleted, the internalization of α-factor was blocked[16] (see TABLE 1 for descriptions and homologies of the *S. cerevisiae* and mammalian proteins that are discussed in this article). Deletions of the genes that encode the *S. cerevisiae* type-I myosins Myo3 and Myo5, which are actin-dependent motor proteins, also caused a strong defect in receptor-mediated endocytosis[17]. In addition, cofilin mutations and the actin Val159Asn mutation, which both cause defects in actin-filament turnover, blocked the endocytic uptake of Lucifer yellow. These results indicate that active actin-filament assembly and disassembly are required for endocytosis.

The localization of actin also indicates that it might function in endocytosis. In *S. cerevisiae* cells, punctate cortical actin structures called actin patches were shown to colocalize, albeit only partially, with many endocytic proteins[2]. Also, electron-microscopy studies on mammalian cells showed that actin filaments were often associated with coated pits[13]. In neuronal synapses, actin was also found to colocalize with sites of clathrin-mediated endocytosis[18]. Similarly, an immunoelectron-microscopy study found that *S. cerevisiae* actin patches localized to membrane invaginations that could be intermediates of endocytic vesicle formation[19]. However, a follow-up study, which used double immunoelectron microscopy, failed to show the colocalization of endocytic cargo molecules with actin patches[20].

Finally, an abundance of studies have uncovered a large number of protein–protein interactions that are indicative of links between the endocytic machinery and the actin cytoskeleton. These studies of biochemical interactions show that many endocytic proteins can potentially be linked to the actin cytoskeleton, either directly or indirectly[2,5,21]. Taken together, these experiments clearly indicate a close functional link between the actin cytoskeleton and the internalization step of endocytosis. However, an understanding of how actin functions in endocytosis remained elusive.

Glimpses of actin in living cells

Live-cell imaging studies of both mammalian and *S. cerevisiae* cells have recently revealed a highly regular timeline of events at endocytic sites. Different endocytic proteins are recruited to and disassemble from endocytic sites in an ordered sequence[9,22–31]. Many features of this sequence are conserved between mammalian and *S. cerevisiae* cells, as are the proteins themselves (FIG. 2; TABLE 1).

In mammalian cells, actin appears at clathrin-coated structures on the plasma membrane in transient bursts that occur near the end of the lifetime of the clathrin-coated pit and overlap with the internalization of clathrin-coated vesicles[9,32] (FIG. 2). The actin structures that are involved in these bursts have been visualized in mammalian cells by expressing green fluorescent protein (GFP) fusions of actin or by microinjecting fluorescently labelled actin, and using total internal reflection fluorescence (TIRF) microscopy (BOX 1). Internalization is marked by the movement of fluorescently labelled clathrin away from the cell surface and can be followed by comparing the signal intensities in TIRF and epifluorescence images[9]. This ratio

Caveolae-mediated endocytosis
A form of uptake at the plasma membrane that involves the protein caveolin.

Cofilin
A conserved actin-binding protein that is thought to be involved in actin-filament severing and disassembly.

Table 1 | ***Saccharomyces cerevisiae* and mammalian protein homologies***

Saccharomyces cerevisiae proteins	Mammalian proteins	Functions
Abp1	ABP1	Arp2/3-complex activator in *S. cerevisiae*, actin-binding protein, scaffold protein
Act1	Actin	Cytoskeletal filaments
Ark1, Prk1	AAK1, GAK	Protein phosphorylation
Bbc1	–	WASP regulator
Bzz1	Syndapin	WASP-interacting protein
Capping proteins (Cap1/2)	Capping proteins (CAPZ-α, CAPZ-β)	Barbed-end actin-filament capping proteins
Chc1, Clc1	Clathrin	Vesicle coat component
Inp52	Synaptojanin	PtdIns(4,5)P_2 5-phosphatase
Las17	WASP, N-WASP	Arp2/3-complex activator
Myo3, Myo5	Type-I myosins	Arp2/3-complex activator in *S. cerevisiae*, motor protein
Pan1 complex (Pan1, Sla1, End3)	Intersectin, EPS15	Adaptor protein, Arp2/3-complex activator (Pan1 in *S. cerevisiae*), WASP regulator (Sla1, Intersectin)
Rvs161, Rvs167	Amphiphysin	Membrane bending or curvature sensing
Sac6	Fimbrin	Actin-filament-crosslinking protein
Sla2	HIP1R	Actin-binding protein, PtdIns(4,5)P_2-binding protein
Vrp1	WIP	WASP-interacting protein
–	Cortactin	Arp2/3-complex activator
–	Dynamin-1, -2	GTPase, vesicle scission

*This table gives descriptions and homologies of the *S. cerevisiae* and mammalian proteins that are discussed in this article. The '–' symbol indicates that there is no known homologue. AAK1, adaptor-protein-complex-2-associated kinase-1; Abp1/ABP1, actin-binding protein-1; Ark1, actin-regulating kinase-1; Arp2/3, actin-related protein-2/3; Cap 1/2, barbed-end capping proteins; CAPZ, capping protein muscle Z-line; Chc1, clathrin heavy chain-1; Clc1, clathrin light chain-1; EPS15, epidermal-growth-factor-receptor-pathway substrate-15; GAK, cyclin-G-associated kinase; HIP1R, Huntingtin-interacting protein-1 related; Myo, myosin; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; Rvs, reduced viabililty upon starvation; (N-)WASP, (neuronal) Wiskott–Aldrich syndrome protein; WIP, WASP-interacting protein.

(intensity in TIRF/ intensity in epifluorescence) decreases when the fluorescent structures move into the cell and out of the zone of TIRF excitation[9]. The increase in the intensity of actin fluorescence correlates with the translocation of the clathrin signal away from the plasma membrane[9]. Merrifield and colleagues reported that bursts in actin polymerization occurred at 80% of the internalizing clathrin-coated pits[9]. Internalization also coincides with the transient recruitment of dynamin-1, a GTPase that is involved in vesicle scission[9]. Actin polymerization is therefore tightly coupled both spatially and temporally to the vesicle-budding step of endocytosis.

A similar sequence of events takes place at endocytic sites in *S. cerevisiae* (FIG. 2). The two main actin cytoskeletal structures in *S. cerevisiae* are actin cables and actin patches (FIG. 1). The cables are orientated along the growth axis of the cell and are involved in the trafficking of many organelles to growing daughter cells[33]. Actin patches are punctate dynamic structures that are at or close to the plasma membrane[34,35]. The dynamics of actin patches have been followed using different fluorescently tagged actin-cytoskeleton proteins[23–25,34–39]. For a long time the role of actin patches remained unknown. However, it has recently become evident that they mark endocytic sites at the plasma membrane and newly formed primary endocytic vesicles[24,25,28,37].

Another long-standing issue in the studies of endocytosis in *S. cerevisiae* has been the unresolved role of clathrin. The question of whether clathrin localizes to endocytic sites at the plasma membrane in *S. cerevisiae* has been difficult to answer owing to technical challenges. However, using TIRF microscopy, it has recently been shown that clathrin does localize to *S. cerevisiae* endocytic sites, as was expected from work in other species, and that actin polymerization bursts occur at the clathrin-containing endocytic sites during the vesicle-budding phase[25,28]. Actin patches are highly transient, with lifetimes of only about 15 seconds[24,39]. Similar to the case in mammalian cells, these transient actin structures form at the plasma membrane, where they colocalize with endocytic sites that are marked with clathrin and other endocytic proteins[25,28]. The short lifetime of the actin patches is divided into an initial phase of restricted motility followed by a phase of rapid motility during which the patch disassembles[24]. The initial phase probably coincides with the internalization movement of clathrin-coated endocytic structures[25] and the rapid motility phase probably corresponds to the movement of clathrin-uncoated, actin-filament-covered endocytic vesicles[37,40].

The localization and timing of the actin polymerization bursts are strikingly similar in both *S. cerevisiae* and mammalian cells (FIG. 2). Furthermore, the spatial and

Lucifer yellow
A fluorescent dye that enters cells by endocytosis and is often used as a marker for bulk endocytic uptake.

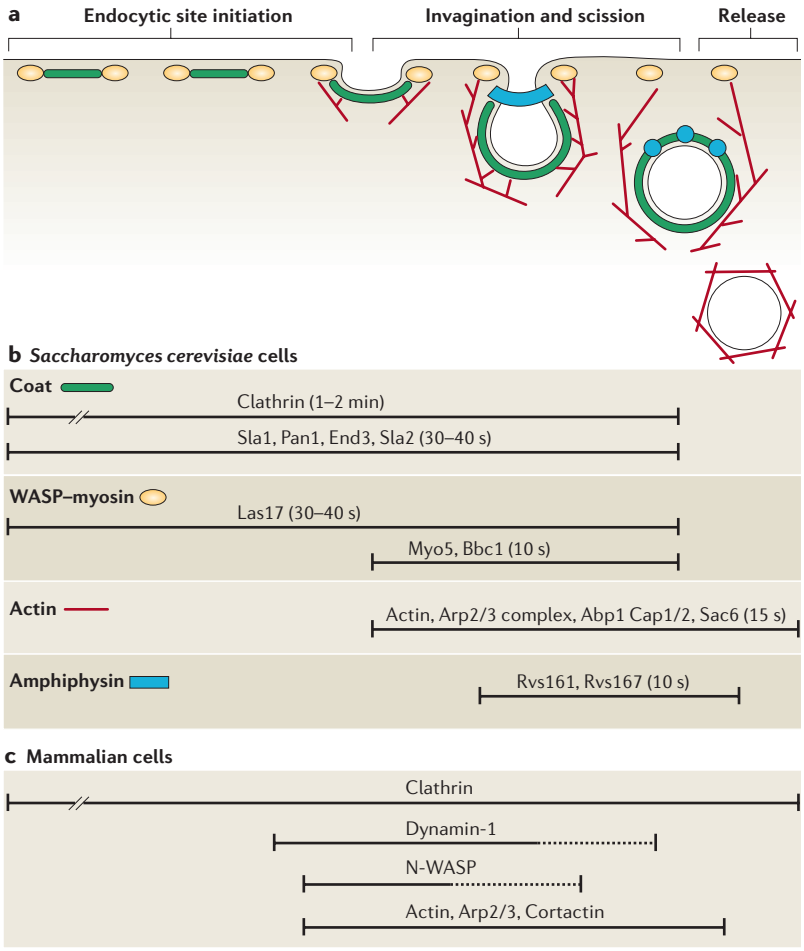


Figure 2 | **The sequential assembly of proteins at endocytic sites. a** | The different steps of endocytic internalization: endocytic site initiation, membrane invagination and scission, and vesicle release. The four protein modules that are involved in endocytic internalization in *Saccharomyces cerevisiae* are shown schematically — that is, the coat (green), the Wiskott–Aldrich syndrome protein (WASP)–myosin complex (yellow), the actin network (red) and the amphiphysin complex (blue). Components of these different protein modules are assembled and disassembled dynamically. **b** | The temporal localization of the constituent proteins for each module in *S. cerevisiae*[23–25]. **c** | The approximate temporal localization of proteins during endocytic internalization in mammalian cells[9,27]. Dashed lines indicate ambiguity in the time frame of the protein dynamics. Endocytic protein modules have not been defined for mammalian cells. Abp1, actin-binding protein-1; Arp2/3, actin-related protein-2/3; Cap1/2, barbed-end capping proteins; Myo, myosin; N-WASP, neuronal WASP ; Rvs, reduced viability upon starvation. For further information on the proteins involved, see TABLE 1.

temporal localization of several endocytic and actin-cytoskeleton proteins has been determined in both *S. cerevisiae* and mammalian cells, and it is apparent that the endocytic internalization processes in these different organisms are variations on the same ancestral theme (FIG. 2). However, there are also striking differences, such as the importance of dynamin. In mammalian cells, dynamin is essential for the scission of clathrin-coated vesicles[41,42]. By contrast, dynamins do not seem to have a direct role in endocytic internalization in *S. cerevisiae*[43,44]. Also, the relative importance of clathrin and adaptor protein complex-2 (AP2) seems greater in mammalian than in *S. cerevisiae* cells.

Arp2/3 complex
A protein complex that promotes the nucleation of actin filaments and creates a branched actin meshwork.

Formin-family proteins
A family of proteins that contain a formin homology-2 domain and can promote actin-filament assembly.

Dynamic actin filaments have also been observed to colocalize with endosomes in cells from various vertebrate species[45–49] (FIG. 1). Actin 'comet tails' form on one side of the endosomes and actin-filament assembly in the comet tails seems to push the endosomes along. The biological role of the actin rocketing of endocytic organelles is not clear. It might have a role in moving the endosomes so that they can make contact with microtubules, which are used as tracks for long-range trafficking, or it could aid in the fusion of endocytic organelles. A similar actin-comet-tail-propelled movement has also been proposed as a mechanism for endosome motility in *S. cerevisiae* cells[50]. However, no actin comet tails or actin-related protein-2/3 (Arp2/3)-complex activators have been observed to associate with *S. cerevisiae* endosomes, so whether this mechanism exists in *S. cerevisiae* remains an open question.

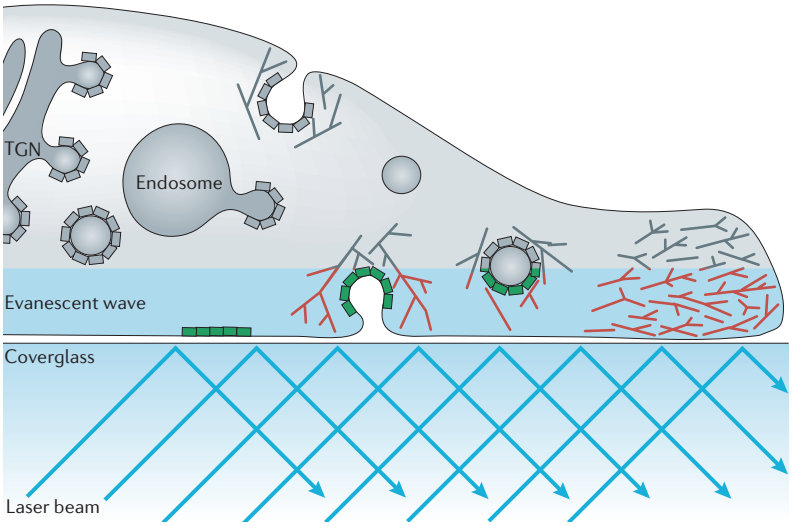
S. cerevisiae cells use a different actin-polymerization-dependent mechanism for endocytic vesicle motility. Actin cables are bundles of actin filaments that grow continuously by polymerization that is nucleated at the plasma membrane by formin-family proteins[51]. Endocytic vesicles become attached to the cables and move together with the growing cables[37,52] (FIG. 1). This process seems to improve the the efficiency of the trafficking of internalized cargo[52].

In summary, actin polymerization seems to have a direct role during at least two steps in the endocytic pathway. First, actin appears at the plasma membrane during the formation of the primary endocytic vesicles. Subsequently, in some vertebrate cells, dynamic actin structures associate with motile endosomes.

The roles of actin in endocytic internalization

Dynamic actin structures seem to associate with different endocytic intermediates. But what are the functional roles of actin during endocytosis? Actin could function during several different stages of clathrin-mediated endocytosis (FIG. 2). In both mammalian and *S. cerevisiae* cells, the first step of endocytosis in which dynamic actin structures has been visualized is just prior to the internalization of the endocytic coat[9,24]. This timing indicates possible roles for actin in membrane invagination, vesicle constriction, vesicle scission and vesicle movement. Actin might also have earlier, possibly indirect, roles in the spatial organization and lateral movement of clathrin-coated endocytic sites[32,53,54]. It was shown recently in mammalian cells that vesicle scission takes place when the actin polymerization burst reaches its peak level[26]. Merrifield and colleagues used mouse fibroblast cells that were expressing a pH-sensitive form of GFP that was fused to the extracellular domain of the transferrin receptor. The cells were perfused with rapidly alternating media of neutral and low pH, and the surface-exposed receptors changed their fluorescence intensity depending on the pH of the medium. However, when the receptors were internalized they became protected from the pH changes. This method allowed vesicle scission events to be observed in relation to actin and clathrin dynamics at a time resolution of 2 seconds[26]. The exact timing of

Box 1 | **Total internal reflection fluorescence microscopy**



One of the common problems in imaging studies of membrane-trafficking events is that it is often difficult to resolve different structures. Typical mammalian cells contain many different kinds of actin structures that are used at various locations in a cell for structural support, motility and trafficking. Similarly, clathrin localizes to the plasma membrane and to internal organelles such as endosomes and the Golgi complex. Total internal reflection fluorescence (TIRF) microscopy has been useful in studies of endocytosis because it allows researchers to excite fluorescent molecules specifically at the interface between the sample and the coverglass. In TIRF microscopy, the fluorescent molecules are excited with a beam of light (usually from a laser) that is directed at the sample at an angle that is greater than the critical angle such that all of the light is reflected at the coverglass–sample interface. Although no light gets into the sample, an evanescent wave is created on the sample side. This wave dissipates rapidly as the distance from the surface of the coverglass increases. The evanescent wave excites fluorophores that are within ~200 nm of the coverglass surface (the structures highlighted in red (actin) and green (clathrin) in the figure, but not those that are further away (the structures with the same shape, but that are coloured grey)). TIRF has been used to reveal the actin polymerization bursts at endocytic sites in mammalian cells[9,26,32] and the localization of clathrin at endocytic sites in *Saccharomyces cerevisiae* cells[25,28]. TGN, *trans*-Golgi network.

vesicle scission has not been determined in *S. cerevisiae*. However, the fact that the *S. cerevisiae* actin patches enter their phase of rapid motility briefly after maximal actin accumulation occurs indicates that the timing of vesicle scission in relation to actin-filament assembly is similar in both mammalian and *S. cerevisiae* cells[24]. Actin polymerization therefore seems to begin when the coat and the plasma membrane start to invaginate to form a coated pit, and net actin polymerization seems to stop when vesicle scission occurs. This timing indicates that actin polymerization could have roles in invagination, constriction and scission.

Experimental perturbations of actin function provide important insights into the endocytic roles of actin. In *S. cerevisiae*, when actin polymerization is blocked by latrunculin A, the clathrin coats and coat-associated proteins are stabilized at the plasma membrane and the internalization movement is completely blocked[24,25,28]. Similarly, coat internalization is blocked by certain Arp2/3-complex mutants[55]. These results indicate that actin polymerization in *S. cerevisiae* is needed for the initial formation of invaginated coated pits.

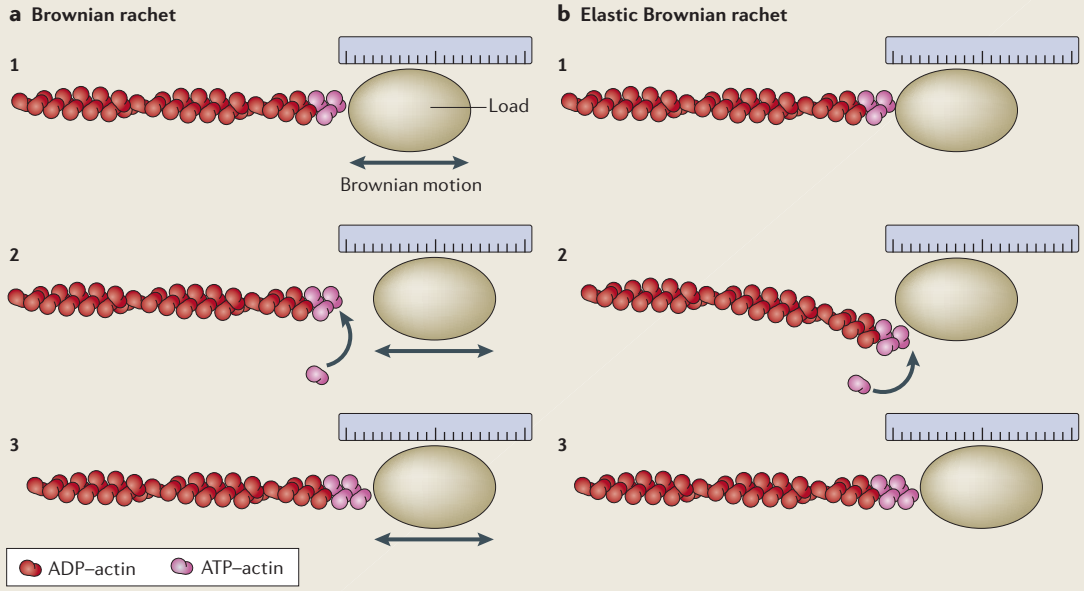
In mammalian cells, the scission of clathrin-coated vesicles from the plasma membrane was reduced by 82% in cells that were treated with the inhibitor of actin polymerization latrunculin B (REF. 26). However, whether endocytic invaginations were formed was not determined in this study. Using electron microscopy, Yarar and colleagues showed that although endocytosis was inhibited in cells that were treated with latrunculin A or jasplakinolide, the number of invaginated coated pits was actually higher in treated cells than in control cells[32]. This indicates that invaginations form without actin function in mammalian cells but that efficient vesicle scission requires actin. Also, constriction of the vesicle neck was impaired when actin function was perturbed[32]. By contrast, *S. cerevisiae* cells seem to need actin for membrane invagination preceding vesicle scission[24]. This apparent difference between *S. cerevisiae* and mammalian cells could be due to differences in membrane tension. *S. cerevisiae* might need more force to induce plasma-membrane invagination owing to the high osmotic pressure in these cells that results in high plasma-membrane tension. By contrast, in mammalian cells, vesicle scission might be the step that is most dependent on the forces that are provided by actin-filament assembly. One fact that might complicate the interpretation of results is that mammalian cells use the actin cytoskeleton to maintain plasma-membrane tension[56]. When the actin cytoskeleton is perturbed globally in the cell, reduced membrane tension might facilitate the formation of invaginations and explain the reduced need for actin during this step.

Mechanics of actin-driven internalization

Certain intracellular bacterial and viral pathogens such as *Listeria*, *Shigella* and *Vaccinia* spp. use an actin-rocketing mechanism for their motility inside mammalian host cells[57,58]. Brownian-ratchet models show that actin polymerization alone can generate force to move objects[59] (BOX 2). This kind of polymerization-driven mechanism has been proposed to be responsible for the motility of pathogens, such as those mentioned above, in the cytoplasm of infected mammalian cells (FIG. 3a) and for the protrusion of the leading edge of migrating cells (FIG. 3b). The actin-polymerization-driven motility of bacteria, coated plastic microbeads and lipid vesicles has also been observed in cytoplasmic extracts[47,60–63], and has been reconstituted using purified proteins[64]. Only actin, the Arp2/3 complex, cofilin and capping protein are needed for this type of motility *in vitro*[64]. Proteins at the surface of the propelled object (for example, the *Listeria monocytogenes* surface protein ActA) activate the Arp2/3 complex. Activated Arp2/3 complexes bind to the sides of actin filaments and activate the nucleation of new filaments. This leads to the formation of comet-tail-like actin structures that are composed of branched actin-filament networks. The comet tail grows at the surface of the object and 'rockets' it forward.

This actin-rocketing model can explain the motility of endosomes that have actin tails[45–49]. The requirements are simply that the organelle nucleates actin-filament assembly at its surface and that polymerization takes

Box 2 | **Brownian-ratchet models**



Brownian-ratchet models provide mechanisms by which actin polymerization can drive the motility of 'loads' such as the leading edge of migrating cells, endosomes or pathogenic bacteria that have invaded mammalian cells[59,102,103]. In the original Brownian-ratchet model (see figure, part **a**), the actin filament (red) grows through the addition of ATP–actin monomers towards the load, until it is so close to the load that there is no more space for the addition of further monomers. However, if the load undergoes Brownian motion, it will occasionally move far enough away from the filament end to allow a new monomer to be added to the filament. The continuing growth of the actin filament biases the Brownian movement of the load in the direction of the filament growth.

The elastic Brownian-ratchet model (see figure, part **b**) is a modification of the original model. In the elastic model, the random bending of the actin filament provides the space for monomer addition. The elastic energy that is stored in the bent filament then pushes the load forward.

place asymmetrically on the organelle (FIG. 3a). This asymmetry has been shown to arise spontaneously in an *in vitro* system that used plastic microbeads that were coated with an Arp2/3-complex activator[65].

However, the rocketing model that has been seen in *Listeria* spp. might not be applicable at sites of vesicle formation at the plasma membrane. If actin filaments are nucleated at an endocytic site and form an actin tail, the resulting force would actually oppose membrane invagination (FIG. 3c). The membrane would need to be significantly invaginated prior to the initiation of actin-filament assembly, and the actin filaments would need to be orientated so that the force would be directed into the cell (FIG. 3d), or orientated to constrict the neck of the invagination to help the vesicle scission event. Different kinds of schematic models have been proposed[2,5], but detailed information about the organization and polarity of the actin filaments at endocytic sites is lacking. Interestingly, an *S. cerevisiae* strain that carries deletion mutations in two genes that are linked to actin regulation has provided insights into actin organization. Cells that lack the genes *BBC1* and *SLA1* can still carry out endocytosis, but they have highly enlarged actin structures at endocytic sites[25]. The large size of the actin structures made it possible to use photobleaching studies to analyse the turnover of actin during endocytic internalization. These experiments showed that actin polymerizes at the plasma membrane, and that the clathrin coat and the invaginating membrane move into the cell at the same rate as the growing actin network[25].

On the basis of these findings and the dynamics of proteins in wild-type cells, a new model for actin-driven endocytosis has been proposed[24,25] (FIGS 3e,4). In this model, the endocytic coat is initially surrounded by a complex of proteins (including Myo5 and Las17, a Wiskott–Aldrich syndrome protein (WASP); TABLE 1) that remains on the plasma membrane at the rim of the invaginating endocytic pit and efficiently activates the Arp2/3 complex to nucleate actin filaments. These filaments then bind to the endocytic coat and continued nucleation leads to the formation of a cone of crosslinked actin filaments that pulls the attached coat inwards and invaginates the underlying membrane[19,66].

This model is supported by both localization data and deletion phenotypes for many endocytic proteins. The model assumes that the vesicle coat can bind to actin filaments. Candidate proteins for linking the coat to the actin meshwork include *S. cerevisiae* Sla2 and its mammalian homologue HIP1R (Huntingtin-interacting protein-1 related). These proteins can bind directly to both actin filaments and clathrin, and they colocalize with the clathrin coat[67–69]. Deletion of the *S. cerevisiae SLA2* gene and knockdown of *HIP1R* expression lead to a dramatic phenotype in which actin polymerization is uncoupled

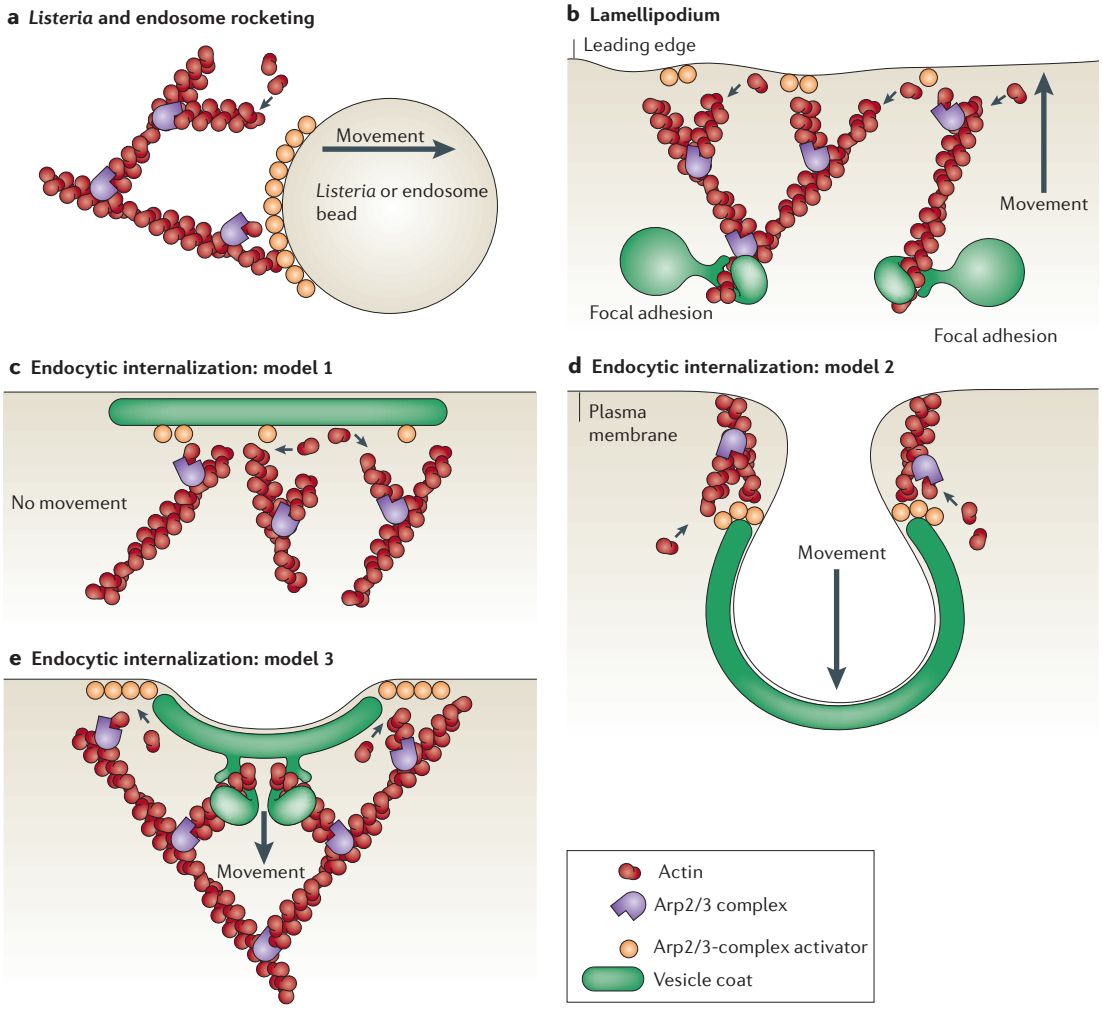


Figure 3 | **Modelling actin-driven endocytic internalization. a** | An actin-rocketing model can explain the motility of endosomes and intracellular pathogens such as *Listeria* species. Actin-related protein-2/3 (Arp2/3)-complex activators are asymmetrically associated with the surface of the object being moved. Arp2/3-complex-nucleated filaments form an actin 'comet tail' that is composed of branched filaments. **b** | The protrusion of a lamellipodium is also driven by actin polymerization. Arp2/3-complex activators , such as WAVE proteins (Wiskott–Aldrich syndrome protein-family verprolin-homologous proteins), localize to the leading edge and activate the Arp2/3 complexes there. The growing branched network flows away from the leading edge. However, when the growing actin network is bound to focal-adhesion complexes that are connected to the extracellular matrix, the leading edge is pushed outwards. **c** | An actin-rocketing model in which the filaments are nucleated by the vesicle coat cannot explain endocytic internalization. If the vesicle coat contained Arp2/3-complex activators, the forming actin comet tail would generate a force that would oppose internalization. **d** | An alternative model requires that the plasma membrane has been bent to form a coated pit before actin polymerization is initiated. Actin is polymerized at the rim of the coated pit to form a network of filaments that pushes the coat towards the cell centre. **e** | In a third model, actin polymerization is nucleated by Arp2/3-complex activators that surround the coated membrane, and actin filaments form a cone that surrounds the coat. The coat binds to the filaments. The coat and the underlying membrane are pulled towards the interior of the cell by the movement of the growing actin network.

from the internalization of the vesicle coat[24,70]. Another protein that can potentially link actin filaments to the coat is *S. cerevisiae* Pan1, which shares homology with the mammalian endocytic proteins EPS15 (epidermal-growth-factor-receptor-pathway substrate-15) and intersectin. Pan1 binds to actin filaments and colocalizes with the clathrin coat[25,71]. Furthermore, Sac6 is essential for internalization, but not for actin polymerization[25], which indicates that the integrity of the actin-filament network is important for force transduction (FIG. 4).

In *S. cerevisiae*, the type-I myosins Myo3 and Myo5 are also important for endocytosis[17], and actin-patch internalization is severely defective in mutants of these myosins[23,39]. The myosins remain localized at the plasma membrane with Las17 while the coat proteins are internalized (FIG. 4), and the timing of their appearance at endocytic sites corresponds to the onset of actin polymerization[23]. A similar sequence of recruitment of a type-I myosin and a WASP homologue has also recently been described in the fission yeast

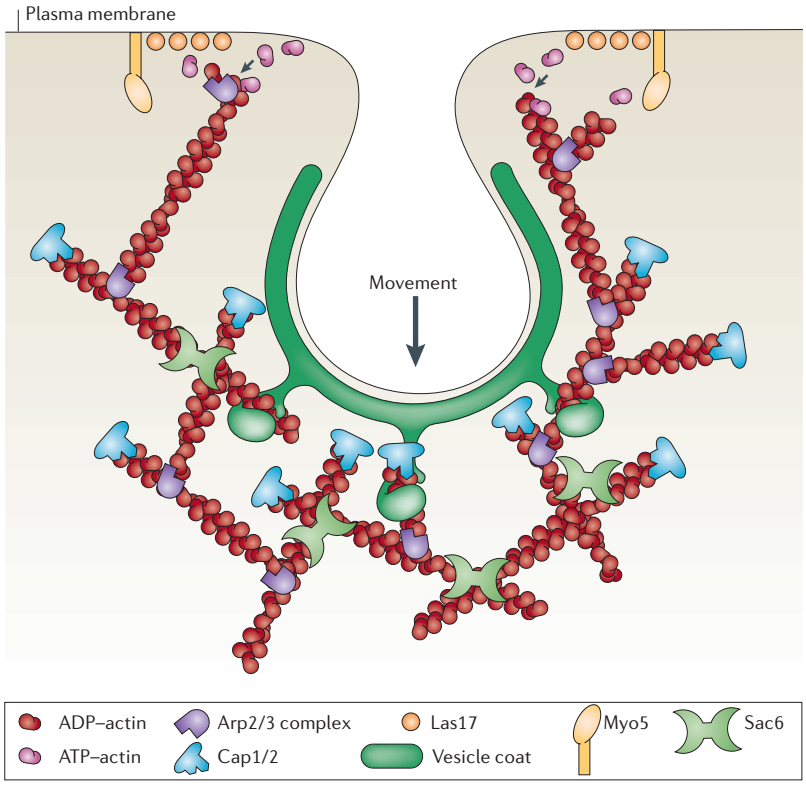


Figure 4 | **Current model for actin-driven endocytic internalization.** This schematic diagram illustrates putative functions of different actin-cytoskeleton proteins during endocytic internalization in *Saccharomyces cerevisiae*. Las17 (Wiskott–Aldrich syndrome protein (WASP) in mammals) together with the myosins Myo3 (not shown) and Myo5 activate the actin-related protein-2/3 (Arp2/3) complex at the cell surface. Myosins might also generate force on the actin network or anchor the actin filaments to the plasma membrane through their motor domains. The activated Arp2/3 complexes form branched actin filaments that grow through the addition of ATP–actin monomers near the plasma membrane. Older filaments are capped at their barbed ends by capping proteins (Cap1/2). The branched filaments are further crosslinked by Sac6. The crosslinked actin network is linked to the underlying vesicle coat by actin-binding proteins such as Sla2 and Pan1, which are represented by green hand-like structures . The growth of the actin network leads to the invagination of the coated membrane. For further information on the proteins involved, see TABLE 1.

Schizosaccharomyces pombe[30]. The myosin motor domains could push the growing actin filaments away from the membrane, or they could transiently anchor the growing barbed ends to the plasma membrane. However, *S. cerevisiae* type-I myosins can also activate the Arp2/3 complex[72,73]. Further work is needed to clarify the relative contributions of myosin motor activity and actin polymerization to endocytic internalization. There is currently no evidence of a role for type-I myosins in the vesicle-budding step in mammalian cells.

Controlling the actin engine

The actin polymerization that is associated with endocytic processes seems to be tightly regulated. This is especially evident at endocytic sites on the plasma membrane, where transient bursts of actin polymerization are precisely coordinated with the recruitment of other endocytic proteins[9,23–28,30]. Actin polymerization is initiated at preformed clathrin-coated structures and stops rapidly after vesicle scission[26]. How is this regulation achieved? The main control point for actin polymerization is the Arp2/3-complex-mediated nucleation event, which is the rate-limiting step in actin polymerization[74]. In mammalian cells, the Arp2/3-complex activators N-WASP and cortactin are known to localize to the sites of vesicle budding[27,75]. Interfering with N-WASP or cortactin function impairs receptor-mediated endocytosis[75–77]. In *S. cerevisiae,* there are up to five potential Arp2/3-complex activators that localize to endocytic sites — Las17, Pan1, Myo3, Myo5 and actin-binding protein-1 (Abp1)[23,24,30]. The dynamics of four of these proteins (Las17, Pan1, Myo5 and Abp1) have been studied in detail in living cells. Surprisingly, each shows a different motile behaviour, which indicates that they might harness the force of actin polymerization at different stages of vesicle formation. Mutants of Las17, Pan1, Myo3 and Myo5 cause severe defects in actin organization and endocytosis[17,78,79]. Abp1 does not have a significant role in initiating actin polymerization, but seems to have an inhibitory role in that it turns off actin polymerization[25,80].

The regulatory complexity of endocytosis does not end here. The Arp2/3-complex activators are themselves subject to regulation by many interacting proteins. Mammalian N-WASP might be regulated during the endocytic internalization process by ABI1 (Abl-interacting protein-1), syndapins, intersectin and CDC42 (REFS 77,81–83). In *S. cerevisiae*, Las17 can be regulated by Sla1, Bbc1 and Vrp1 (REFS 78,84). Vrp1 and Sla1 also interact with Myo5 and Pan1, respectively[85,86]. Deletions of the genes that encode Sla1, Bbc1 and Vrp1 cause different endocytic phenotypes[25]. First, the deletion of *SLA1* leads to a delay in the initiation of actin polymerization at endocytic sites[25]. Second, the deletion of *BBC1* causes an enhancement of the actin-driven internalization movement[25]. Third, the deletion of *VRP1* uncouples actin polymerization from the internalization movement[25]. Protein phosphorylation also has a role in regulating the actin polymerization bursts at the internalization sites. The *S. cerevisiae* actin-regulating kinases Ark1 and Prk1 are involved in turning off the actin-polymerization machinery at the newly formed endocytic vesicles. Inhibiting the kinases Ark1 and Prk1 leads to the accumulation of clusters of actin-filament-covered endocytic vesicles in *S. cerevisiae* cells[25,40,87]. These kinases function at least in part by phosphorylating Pan1 and inhibiting its function[71,88]. Interestingly, Abp1 recruits the kinases Ark1 and Prk1 to endocytic sites[87]. In addition, Abp1 recruits the *S. cerevisiae* synaptojanin Inp52, which is a phosphatidylinositol-4,5-bisphosphate 5-phosphatase that can also negatively regulate actin-filament assembly[89]. The localization of Abp1 depends on actin filaments[15], so Abp1 might function in a negative feedback loop that is triggered by nascent actin filaments and functions to turn off actin polymerization.

In mammalian cells, dynamin potentially has an important role in coordinating actin-filament assembly at endocytic sites. Dynamin is thought to oligomerize around the neck of an endocytic membrane invagination

and facilitate vesicle scission, and it has been proposed to function either by providing force for the constriction of the neck or by functioning as a regulatory GTPase[41,42]. The peak accumulation of dynamin-1 at endocytic sites precedes peak actin accumulation[9] (FIG. 2c). Dynamin can interact with the actin machinery through several linking proteins such as cortactin, intersectin and syndapin[90–92]. These proteins bind to dynamin, the Arp2/3 complex, actin filaments and N-WASP. Dynamin might regulate actin-filament assembly at endocytic sites, coordinating it with the vesicle scission event. Dynamin might also be linked to the actin cytoskeleton through a family of proteins that contain BAR or related F-BAR domains, which both bind to lipids and can tubulate membranes, potentially facilitating endocytosis[93,94].

In summary, actin polymerization by the Arp2/3 complex is regulated by several activator proteins and numerous proteins that control the activity of the activators. Unravelling the complex regulation that initiates and turns off actin polymerization at endocytic sites will be an important challenge for future research.

Many uses for the actin module

The molecular machinery that is based on actin and the Arp2/3 complex is well conserved throughout eukaryotes. Interestingly, the same protein machinery has a central role in many different cellular processes. Actin and the Arp2/3 complex are involved in other forms of endocytic internalization including macropinocytosis[46], phagocytosis[95] and caveolae-mediated endocytosis[96] (FIG. 1). Also, actin and the Arp2/3 complex are central components in the lamellipodia of motile cells[97].

The functional similarities between the actin meshwork in lamellipodia and at endocytic sites are intriguing (FIG. 3b,e). Each lamellipodium is composed of a dense Arp2/3-complex-nucleated branched actin-filament meshwork[97]. Actin filaments are nucleated by the Arp2/3 complex at the leading edge of the lamellipodium, and actin filaments continuously flow from the leading edge towards the centre of the cell[97]. This actin meshwork is thought to be anchored to the extracellular matrix through adhesion sites, so that the growing filament ends can push the leading edge of the cell forward (FIG. 3b). The proteins that regulate actin polymerization seem to have analogous localizations in both lamellipodia and at endocytic sites (FIG. 3b,e). Actin nucleation in lamellipodia is regulated by Arp2/3-complex activators, such as WAVE proteins (WASP-family verprolin-homologous proteins), that localize to the leading edge of lamellipodia[98,99]. Actin at endocytic sites seems to be regulated by WASP-family proteins, namely N-WASP in mammalian cells, Las17 in *S. cerevisiae* and Wsp1 in *S. pombe,* that are homologous to WAVE. Mammalian cortactin, which colocalizes with actin at clathrin-coated pits and endosomes[26,45,75], is also localized throughout the actin-filament meshwork of lamellipodia[100]. Furthermore, capping protein and cofilin, which both regulate actin dynamics in lamellipodia[74], also function at endocytic sites in *S. cerevisiae*[25,101]. The specific functions of these proteins are probably similar in these different processes, which indicates that they form a functional module that has retained its basic mechanism of forming a branched actin-filament meshwork. It also indicates that this module has been adapted for several different uses over the course of evolution. The regulation of the actin–Arp2/3-complex module comes mostly through the activation or inhibition of the Arp2/3 complex. The force produced by this module can be harnessed by proteins (such as endocytic coat proteins or adhesion proteins) that bind to the actin-filament meshwork.

Future directions

Although live-cell imaging has provided answers to many of the questions about endocytosis, it has raised even more new questions. The endocytic machinery seems to be much more dynamic than had been previously appreciated. The regularity with which different steps follow each other, and the precision with which numerous proteins assemble at endocytic sites signifies a remarkable level of molecular choreography. We can literally see order emerge from the apparent chaos of the random motions of soluble endocytic proteins as they assemble to form endocytic structures. How this ordered sequence of assembly and disassembly takes place is an important unanswered question. Many loops of positive and negative feedback are probably involved in creating the assembly–disassembly sequence.

Other key questions concern the mechanism by which actin drives endocytosis at the plasma membrane. Is the energy for vesicle budding derived from actin polymerization, the motor proteins, or both? How are actin filaments organized at an ultrastructural level at endocytic sites, and how is their nucleation regulated? Why is actin more crucial for endocytosis in *S. cerevisiae* than in mammalian cells? Does actin have a direct role in vesicle scission?

New quantitative live-cell imaging and image-analysis methods will have an important role in answering many of the remaining questions about the mechanisms of endocytosis. However, live-cell imaging will be most powerful when it is used in combination with electron microscopy and biochemical, pharmacological and genetics tools.

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Competing interests statement
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