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Review

Molecular requirements for the internalisation step of endocytosis: insights from yeast

Alan L. Munn *

Laboratory of Yeast Cell Biology, Institute of Molecular Agrobiology, 1 Research Link, National University of Singapore, Singapore 117604, Singapore

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Abstract

Molecular genetic studies of endocytosis using the unicellular eukaryote *Saccharomyces cerevisiae* (budding yeast) have led to the identification of many cellular components, both proteins and lipids, required for this process. While initially, many of these requirements (e.g. for actin, various actin-associated proteins, the ubiquitin conjugation system, and for ergosterol and sphingolipids) appeared to differ from known requirements for endocytosis in higher eukaryotes (e.g. clathrin, AP-2, dynamin), it now seems that endocytosis in higher and lower eukaryotes share many requirements. Often, what were initially identified as actin cytoskeleton-associated proteins in *S. cerevisiae*, are now revealing themselves as clathrin-coated pit- and vesicle-associated proteins in higher eukaryotes. So rather than delineating two endocytic pathways, one actin-based and one clathrin-based, the combined studies on higher and lower eukaryotes are revealing interesting interplay in both systems between the actin cytoskeleton, clathrin coats, and lipids in the formation of endocytic vesicles at the plasma membrane. Recent results from the yeast system show that the Arp2/3p complex, Wiskott–Aldrich syndrome protein (WASP), and WASP-interacting protein (WIP), proteins involved in the nucleation step of actin filament assembly, play a major role in the formation of endocytic vesicles. This discovery suggests models whereby endocytic vesicles may be actively pushed from the plasma membrane and into the cell by newly forming and rapidly extending actin filaments.

Keywords: Actin; Amphiphysin; Arp2/3p; Ubiquitin; Wiskott–Aldrich syndrome protein; WASP-interacting protein

1. Yeast as a model eukaryote for molecular genetic studies

The budding yeast *Saccharomyces cerevisiae* has become a widely used eukaryotic model organism for the study of vesicular transport [1–8]. *S. cerevisiae* is unicellular, grows well in culture, is stable as either a diploid or haploid cell type, and is amenable to both classical genetic as well as molecular genetic manipulations. Haploid cell types (of which there are two: **a** and α) can be mated to form diploids (**a**/α) and diploids can be induced to undergo meiosis to yield recombinant **a** and α haploid spores. As in some other fungi, in *S. cerevisiae* it is possible to recover all four haploid products of a single meiosis and to phenotypically examine the progeny even when some of them are inviable [9]. A vast collection of well-characterised mutants affected in almost every aspect of cellular physiology is available [9]. Furthermore, the sequencing of the approximately

* Fax: +65-872-7007; E-mail: alan@ima.org.sg

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6000 genes which comprise the *S. cerevisiae* genome is now complete [10].

2. Endocytosis

Endocytosis is a term used to describe vesicular transport pathways used by eukaryotic cells to internalise extracellular fluid and particles as well as plasma membrane molecules. Endocytosis occurs in all eukaryotic cells which have been examined. It has been estimated that in some cultured mammalian cells, membrane material equivalent to 50% of the entire cell surface can be internalised every hour. Endocytosis, as referred to in this article, will specifically refer to internalisation of small particles, as distinct from phagocytosis, which involves internalisation of much larger particles (>500 nm diameter) and which is a more specialised endocytic process found only in certain types of cells [11].

Endocytosis of extracellular particles or molecules can be classified as fluid-phase or receptor-mediated, based on whether the particles or molecules are internalised in a free state or while bound to a specific cell-surface receptor. Fluid-phase endocytosis is non-saturable and particles or molecules are internalised at rates dependent on their extracellular concentration. In receptor-mediated endocytosis, uptake of the particle or molecule (ligand in this case) is saturable because it is dependent on binding to a specific cell-surface receptor and the ligand and receptor are internalised together. Endocytosis is an energy- and temperature-dependent process and can therefore be blocked when cells are treated with energy poisons or when incubated at low temperature (e.g. <4°C). It is also time-dependent, the half-time for internalisation of many plasma membrane proteins being 2–5 min under optimal conditions [11,12]. In some cases, internalisation of a cell-surface receptor is dependent on ligand binding (regulated endocytosis), while in other cases the receptor is internalised equally efficiently with or without ligand binding (constitutive endocytosis) [11].

3. Endocytosis in higher eukaryotic cells

Since the original report of endocytosis in mosquito oocytes in 1964 [13], most research on endocytosis has used higher eukaryotic cells, and in particular cultured mammalian cells. The characterisation of many endocytosed cell-surface receptors, e.g. the human low density lipoprotein, transferrin, and mannose 6-phosphate receptors, led to the discovery of short sequence motifs in the cytoplasmic domains which act as endocytic internalisation signals, e.g. the tyrosine-based NPXY and YXXΦ motifs (in the single letter amino acid code and where X is any amino acid and Φ is a bulky hydrophobic amino acid) and the dileucine (LL) motif [14–16]. Characterisation of the internalisation process using electron microscopy led to the discovery of clathrin, AP-2 adaptor complexes, and dynamin as major components of the coats of endocytic pits at the plasma membrane. The AP-2 adaptor complex has a role in recognition of internalisation motifs in cell-surface receptors and in clathrin coat assembly. Dynamin is involved in scission of endocytic vesicles from the plasma membrane. These topics have recently been reviewed [16–18].

4. Endocytosis in yeast

4.1. Fluid-phase endocytosis in yeast

Yeast cells carry out endocytosis [3–6,8,19]. This can be assayed in a variety of ways. The initial method used the membrane-impermeant fluorescent dye lucifer yellow carbohydrazide (LY). LY does not enter the cytoplasm, but is taken up in the fluid phase via endocytic vesicles. Uptake of LY is time-, temperature-, and energy-dependent and leads to accumulation of LY in the vacuole. LY accumulation can be easily visualised by fluorescence microscopy, because the vacuole is a relatively large organelle occupying about 30% of the total yeast cell volume. Defects anywhere in the endocytic pathway from the cell surface to the vacuole lead to a block in vacuolar LY staining. One limitation of LY assays is that it is not possible to determine which step in endocytic vesicular traffic is blocked, e.g. in a mutant [19,20].

4.2. Endocytosis of the mating-factor receptors

Yeast cells also have the ability to carry out recep-

tor-mediated endocytosis. Haploid cells of the **a** mating type express a cell-surface receptor (α-factor receptor, encoded by sterile 2, (*STE2*) gene) which specifically binds α-factor peptide secreted by cells of the α mating type. Similarly, cells of the α mating type express a cell-surface receptor (**a**-factor receptor, encoded by *STE3* gene) which binds **a**-factor peptide secreted by haploids of the **a** mating type. Both mating-factor receptors are polytopic G-protein-coupled membrane proteins with seven transmembrane domains similar to mammalian β-adrenergic receptors. Binding of ligand to these receptors activates signal transduction pathways that arrest cell division and induce morphological changes required for cell–cell fusion during mating [21].

Metabolically radiolabelled mating peptides (e.g. α-factor) can be used as markers for receptor-mediated endocytosis. As is the case for many ligands internalised by mammalian cells, labelled α-factor can be released from uninternalised receptors by washing cells at low pH. Once internalised, α-factor can no longer be removed from cells by low pH washes. Endocytosis of radiolabelled α-factor can therefore be measured as a time-dependent increase in low pH resistant radioactivity [20,22,23]. The use of radiolabelled α-factor as an endocytic marker allows for the specific assay of the internalisation step [20].

Internalisation of the α-factor receptor (Ste2p) can also be assayed. Ste2p internalisation can be measured by the progressive loss of α-factor binding sites at the cell surface [23] or by Ste2p degradation in the vacuole [24] after treatment with α-factor. Internalisation and degradation of Ste2p occurs in the absence of α-factor, but the rate increases 5–10 fold upon α-factor binding, indicating that Ste2p is subject to regulated endocytosis [23]. The **a**-factor receptor (Ste3p) is also internalised by endocytosis and degraded in the vacuole. As for Ste2p, Ste3p endocytosis can be followed by measuring Ste3p degradation in the vacuole [25]. In the absence of ligand, endocytosis and vacuolar degradation of Ste3p is rapid compared to that of Ste2p, indicating that Ste3p has a higher rate of constitutive endocytosis. Interestingly, although α-factor stimulates internalisation of Ste2p, this stimulation has been shown to be independent of G-protein signalling [26].

4.3. Endocytosis of other plasma membrane proteins

Early work focused on the mating-factor receptors. More recently, however, endocytosis of other cell-surface proteins has been investigated. Nutrient permeases, such as those specific for uracil (Fur4p) [27], galactose (Gal2p) [28], maltose (Mal61p) [29], general hexoses (Hxt6p and Hxt7p) [30], inositol (Itr1p) [31], general amino acids (Gap1p) [32], tryptophan (Scm2p/Tat2p) [33], and zinc (Zrt1p) [34] have been shown to undergo endocytic internalisation followed by vacuolar degradation. In these cases endocytosis is demonstrated by loss of permease activity from the cells and vacuolar-protease-dependent loss of the permease protein. Cell-surface proteins of the multidrug resistance family, e.g. Ste6p (which is involved in export of **a**-factor) [35] and Pdr5p (which is involved in export of low molecular weight cytotoxic compounds) [36] have also been shown to undergo endocytosis and vacuolar degradation. Other endocytosed surface proteins include the chitin synthases Chs1p and Chs3p, which are involved in synthesis of chitin (a cell wall component) at the neck region between the mother cell and bud at defined stages of the cell division cycle [37,38], and Snc1p, a v-SNARE involved in Golgi to plasma membrane vesicular transport [39]. Not all surface proteins are internalised, however. The plasma membrane H^+ ATPase, Pma1p, is stably maintained at the plasma membrane. Pma1p does not undergo endocytic internalisation and is not found in endosomes [35,40]. Internalisation of these proteins has been shown to require some common gene products, but it will be interesting to see if there are also differences in the internalisation requirements of distinct cell-surface proteins. It is perhaps surprising that most of these internalised yeast proteins are polytopic (the sole exception being Snc1p). In contrast, the 'classical' mammalian cell-surface receptors whose endocytosis was first studied have a single membrane-spanning domain [14]. Until quite recently it was not even clear if polytopic surface proteins are subject to endocytosis in higher eukaryotes.

4.4. Endocytic signals in yeast

While the sequence motifs and/or other structural features which regulate internalisation of most *S. ce-*

revisiae surface proteins are not known, two internalisation motifs in the mating-factor receptors have been characterised in detail. Important information for internalisation of Ste2p and Ste3p lies in the cytoplasmic carboxy-terminal tail domains [25,41]. The sequence motif SINNDAKSS in the cytoplasmic tail of Ste2p [41] and NPFXD (where X is any amino acid) [42] in the cytoplasmic tail of Ste3p have been shown to be sufficient to confer endocytic internalisation. Neither sequence is necessary for internalisation of otherwise full-length receptor molecules, indicating that Ste2p and Ste3p have multiple independent internalisation motifs. In the case of receptors whose cytoplasmic tails have been truncated experimentally to remove other internalisation motifs, the SINNDAKSS and NPFXD sequences have been shown to be required for internalisation [41,42].

An important clue to the function of the Ste2p SINNDAKSS sequence came from the discovery that lysine 337 (K337) within this motif is ubiquitinated in a ligand-dependent process [43]. Ubiquitination is necessary for rapid internalisation of Ste2p, since *ubc* (ubiquitin conjugation enzyme) mutants which are defective in ubiquitination of Ste2p (*ubc4 ubc5* double mutants) exhibit a defect in Ste2p internalisation [43]. In addition, mutation of K337 to arginine (which cannot be ubiquitinated) (K337R) abolishes both ligand-dependent ubiquitination and internalisation of a truncated Ste2p, even in cells with a normal ubiquitin conjugation system [41,43]. The Ste2p cytoplasmic tail contains seven other lysine residues apart from K337, of which six are probably subject to ubiquitination and one is clearly not ubiquitinated. One of the other six ubiquitinated lysines (K374) is, like K337, quite strongly ubiquitinated. The existence in the Ste2p cytoplasmic tail of other lysine residues which can act as ubiquitin acceptors explains why SINNDAKSS itself is not absolutely necessary for internalisation of full-length Ste2p. Replacement of all seven ubiquitinated lysines in the Ste2p cytoplasmic tail with arginine leads to lack of ubiquitination and a severe defect in Ste2p internalisation, even in the context of a full-length cytoplasmic tail [44].

Ubiquitination of cellular proteins is often used to target such proteins for degradation by the cytosolic 26S proteasome complex [45]. The 26S proteasome complex has a well-characterised role in degradation of polyubiquitinated cytosolic (and some membrane-spanning) proteins in *S. cerevisiae* and in higher eukaryotes. Unlike most ubiquitinated proteins, however, Ste2p is degraded in the vacuole. 26S proteasome-deficient strains of yeast are not defective in degradation of ubiquitinated Ste2p [43]. Proteins which are targeted to the 26S proteasome and degraded in the cytoplasm are polyubiquitinated, i.e. each lysine is modified by a chain composed of many ubiquitin molecules in covalent linkage. Unlike these proteins, each modified lysine in Ste2p is ubiquitinated with a single ubiquitin molecule, i.e. Ste2p is monoubiquitinated. Monoubiquitination of Ste2p is sufficient for internalisation, since in-frame addition of the amino acid sequence of ubiquitin (even a mutated ubiquitin lacking all lysine residues) to a truncated Ste2p lacking the remaining cytoplasmic tail lysines restores Ste2p internalisation [44]. Sequences within ubiquitin that contain information specifying endocytic internalisation have recently been characterised [46].

The ubiquitination of K337 is specifically induced by ligand binding and recent work has shown that this is due to phosphorylation of the neighbouring three serine residues in the SINNDAKSS sequence. While the Ste2p tail is constitutively phosphorylated, ligand binding induces hyperphosphorylation of Ste2p [47]. Exchange of all three serines for alanine in the SINNDAKSS motif (S331A, S338A, and S339A) in a truncated (but endocytically functional) Ste2p leads to a defective Ste2p species which cannot be phosphorylated, either constitutively or upon α-factor binding, and which cannot be ubiquitinated or internalised [43,48]. Double mutants defective in the yeast casein kinase I homologue 1, *YCK1*, and *YCK2* genes, have defects in phosphorylation, ubiquitination, and internalisation of wild-type Ste2p. Yck1p and Yck2p may therefore represent the kinases responsible for Ste2p phosphorylation [48].

Ubiquitination has also been shown to be important for internalisation of other plasma membrane proteins in *S. cerevisiae*, e.g. Fur4p [49], Ste3p [50], Pdr5p [51], and several other proteins (Gal2p [28], Ste6p [52], Gap1p [32]). In contrast to Ste2p, in the case of the uracil permease, Fur4p, polyubiquitin chains are required for normal kinetics of internalisation. In most polyubiquitinated proteins the ubiquitin chains are crosslinked via the carboxy-terminal

glycine of ubiquitin covalently bonding to the ε amino group of K48 in ubiquitin. In contrast, crosslinking of ubiquitin attached to Fur4p is mainly via the ε amino group of K63 of the ubiquitin molecules. Perhaps this unusual ubiquitin linkage is used to specifically target Fur4p to the vacuole instead of the cytosolic 26S proteasome for degradation [53]. A PEST-like sequence containing several serine residues in the cytoplasmic amino-terminal domain of Fur4p is important for phosphorylation, ubiquitination, and endocytosis of this protein. However, it is not known if this PEST-like sequence, like the SINNDAKSS sequence, is sufficient on its own to act as an endocytosis signal or if phosphorylation of the PEST-like sequence induces conformational changes in Fur4p which are required to expose an endocytosis signal elsewhere in the protein [54]. Ubiquitination of Fur4p is different from that of Ste2p in a second way because it does not require the ubiquitin conjugating proteins Ubc4p or Ubc5p, but instead requires the ubiquitin protein ligase nitrogen permease inactivator 1, Npi1p (also called Rsp5p) [49,55,56]. Npi1p is also required for inactivation of other permeases, e.g. the general amino acid permease, Gap1p [56]. Gap1p has a sequence EEKAI in its cytoplasmic tail which is important for permease turnover and which may be ubiquitinated [32,57].

The motifs used in higher eukaryotes to direct internalisation by endocytosis (the NPXY and YXXΦ tyrosine-based motifs and the dileucine motif), while present in some yeast membrane proteins, are not commonly used as internalisation motifs. Tyrosine-containing motifs are used by resident proteins of the *trans* Golgi network (TGN) to specify retention or retrieval to this compartment of the secretory pathway [58] and dileucine motifs have been found to direct newly-synthesised vacuolar resident proteins from the TGN to the vacuole [59]. Interestingly, Gap1p is unique in that it also has a dileucine-like motif in its cytoplasmic domain which is necessary, together with the EEKAI motif described above, for normal kinetics of internalisation and degradation [32,57].

4.5. Some differences between endocytosis in higher eukaryotes and in yeast

Most of the proteins important for endocytic internalisation in mammalian cells, e.g. clathrin heavy and light chain, are present in *S. cerevisiae*. Initial characterisation of the requirements for endocytic internalisation in yeast, however, led to the remarkable observation that mutant strains in which the single gene for clathrin heavy chain, *CHC1*, is deleted (*chc1Δ*) are still able to carry out endocytosis, although with slower kinetics [60]. The same is true for strains in which the clathrin light chain gene (*CLC1*) is deleted (*clc1Δ*) [61]. Temperature-sensitive mutations affecting clathrin heavy chain (*chc1-ts*) do, however, have strong defects in endocytic internalisation immediately after shift to the restrictive temperature [62]. The differences observed between *chc1-ts* immediately after shift and *chc1Δ* mutant strains may indicate the existence of a cellular adaptive response that compensates for loss of clathrin and restores endocytosis in clathrin-deficient cells after some time.

S. cerevisiae has possible homologues of the AP-2 adaptor complex subunits, but these proteins are not only dispensable for endocytosis, but may not even associate with clathrin in yeast cells [63,64]. Yeast has three dynamin-like proteins. One, mitochondrial genome maintenance 1, Mgm1p, is localised on mitochondria [65], and the other two are cytoplasmic isoforms (dynamin-related protein 1, or Dnm1p and vacuolar protein sorting 1, or Vps1p) [66,67]. Dnm1p and Vps1p have been tested and are not required for endocytic internalisation [66,67]. Mutants carrying a complete deletion of the *DNM1* gene are weakly defective in transport between endosomes and the vacuole and have defects in mitochondrial morphology [66,68]. In contrast, Vps1p is required for the transport of newly-synthesised proteins from the TGN to the vacuole [67].

4.6. Screens for endocytosis-deficient mutants of yeast

A variety of mutant screens have been developed to isolate yeast mutants with general defects in the internalisation step of endocytosis. The initial approach was to create banks of random mutants showing temperature-sensitive defects in viability and then assaying each mutant for a defect in α-factor internalisation at the restrictive temperature. This approach was based on the prediction that, like mutants defective in the secretory pathway, mu-

tants defective in endocytosis (*end*) would have severe viability defects and that only conditional mutations (e.g. temperature-sensitive, where the defect is only manifest at elevated temperatures) would be viable [1]. This approach led to the isolation of the *end3* and *end4* mutants [69] and subsequently to the isolation of the *end5*, *end6*, and *end7* mutants and a second *end4* allele [70]. While these *end* mutants are temperature-sensitive for viability, as expected, most are defective for endocytic internalisation at all temperatures. This shows that endocytosis, unlike secretory vesicular traffic, is not essential for cell viability under all environmental conditions (see [8]).

Another approach used to isolate endocytic mutants was based on an interesting discovery. The vacuolar membrane ATPase 2, or *VMA2* (also known as *VAT2*), gene encodes the 60 kDa subunit of vacuolar H^+-ATPase, an enzyme responsible for maintaining the acidity of this compartment [71]. Mutants in which *VMA2* is deleted (*vma2Δ*) have defects in vacuolar acidification, but, perhaps surprisingly, remain viable. Interestingly, the viability of these mutants is pH-sensitive: they are only viable when cultured in acidified (pH <6) medium. One explanation for the pH-dependent viability of these strains was that vacuolar acidification is indeed required for viability but *vma2Δ* mutants can acidify their endosomal pathway and vacuole by internalising acidic medium via fluid-phase endocytosis. Based on this, the combination of a mutation blocking fluid-phase endocytosis with *vma2Δ* should lead to a lethal inability to acidify the vacuole, even on acidic medium [71]. Consistent with this, haploid *end3 vma2Δ* or *end4 vma2Δ* double mutant spores arising from *end3*$\times$*vma2Δ* and *end4*$\times$*vma2Δ* genetic crosses were inviable [72]. This result showed that *end* mutants can be identified by simply screening for lethality in combination with *vma2Δ*. Using such an approach, the *end8*, *end9*, *end10*, and *end11* [72,73] and *end14* [74] internalisation mutants were isolated.

Although this screening strategy has proven very successful for isolation of novel *end* mutants, the original explanation for the lethality may not be correct. A recent study has shown that while acidic medium does lead to the predicted increase in vacuolar acidity in *vma2Δ* mutant cells, this acidification occurs even when endocytosis is blocked [75]. This acidification is dependent on the presence of ammonium ions in the medium, but the actual mechanism is not clear yet. One possibility is that ammonium may be taken up by a plasma membrane permease and then delivered to the vacuole via a vacuolar permease. In the vacuole it would then dissociate to yield protons and ammonia (which can diffuse out of the vacuole). Since acidification occurs even in the absence of endocytosis, why are *end vma2Δ* double mutants inviable? One possible explanation is that an increased physiological requirement for vacuolar acidity exists in *end* mutants. If this were the case, vacuolar acidification would still occur in *end* mutants but would not be sufficient to maintain cell viability.

Other genetic screens have involved looking for wild-type genes which when present at high copy-number can suppress an *end* mutation of interest. The rationale is that if a critical protein–protein interaction has been affected in the mutant, overexpression of the partner protein may reverse the phenotype. An example of this approach was the isolation of the local anaesthetic sensitive 17, *LAS17* (also known as *BEE1*), gene as a high-copy-number suppressor of the *end5* mutant. Using the yeast two-hybrid system the interaction between the protein defective in *end5* (Vrp1p/End5p) and the protein encoded by *LAS17* (Las17p) was confirmed. Subsequent testing of a strain in which the *LAS17* gene was deleted (*las17Δ*) showed that Las17p is also required for endocytosis [76,77].

In addition to screens for novel *end* mutants, some gene products important for endocytosis were discovered by taking known mutants affected in specific proteins or processes and testing them for defects in endocytosis. In this way actin (*act1*), the actin filament bundling protein fimbrin (suppressor of actin 6, *sac6*) [78], the Ca^{2+} regulatory protein calmodulin (*cmd1*) [79], unconventional type I myosins (*myo3*, *myo5*) [80], the actin filament severing protein cofilin (*cof1*) [81], and actin-related protein 2 (*arp2*) [82,83] were implicated in the endocytic process. A screen for novel mutations that block endocytosis and thus suppress the viability defect of a *pma1* mutant in which the plasma membrane ATPase Pma1p is mistargeted to the vacuole (*sop*) as well as the testing of known mutants for endocytic defects both led to the discovery that yeast homologues of synaptojanin are required for internalisation. Mammalian

synaptojanin is a neuronal inositol polyphosphate 5-phosphatase. The yeast homologues are known as inositol polyphosphate 5-phosphatases (Inp51p, Inp52p and Inp53p) or, alternatively, as synaptojanin-like proteins (Sjl1p, Sjl2p, and Sjl3p). Inp53p/Sjl3p is also known as suppressor of *pma1* 2 (Sop2p) [84,85]. All these proteins are required for both fluid-phase and receptor-mediated internalisation.

4.7. The importance of cytoskeletal proteins

One of the most remarkable findings from molecular genetic studies of endocytosis in yeast was the correlation between mutations affecting internalisation and those affecting the distribution of the cortical actin cytoskeleton. Yeast cells have two types of filamentous actin (F-actin) structure that are visible by immunofluorescence staining: actin patches and actin cables. Actin patches are predominantly at the cortex just underlying the plasma membrane and actin cables are present in the middle of the cell. Both of these structures undergo characteristic changes in distribution during the cell division cycle, e.g. in cells about to form a daughter cell (bud) the actin patches concentrate in the region where the bud will form and actin cables align so that they point towards this site. Many mutants defective in actin itself or actin-associated proteins cause a loss of this polarised distribution and both patches and cables are randomly distributed throughout the cell division cycle [86]. The majority of *end* mutants isolated in the various mutant screens described above exhibit a more random distribution of cortical actin patches than is seen in wild-type cells [70,74,76,87]. Some of the *END* gene products are *S. cerevisiae* orthologues of well-known cytoskeleton-associated proteins in higher eukaryotes (e.g. actin, fimbrin, cofilin, myosins), while others are regulators or components of the actin cytoskeleton, e.g. End3p, Pan1p, Sla2p/End4p, Vrp1p/End5p, Rvs161p/End6p, and Rvs167p, which originally had no higher eukaryotic counterparts.

Not all mutations that perturb the distribution of cortical actin patches cause defects in endocytic internalisation. Mutations affecting an unconventional type V myosin (*myo2*), the actin-filament-binding protein tropomyosin 1 (*tpm1*), or actin-monomer-binding protein profilin of yeast 1 (*pfy1*) do not have strong effects on endocytic internalisation, even though the cortical actin patches are severely delocalised [3,8,70]. This suggests that if actin-containing cytoskeletal structures are directly involved in endocytic internalisation, they may be distinct from cortical actin patches. These putative endocytosis-specific cytoskeletal structures may not be as easily visualised by immunofluorescence as cortical actin patches and may therefore have been overlooked in the initial characterisations of the yeast cortical actin cytoskeleton.

What types of proteins are affected in endocytosis-defective mutants?

4.7.1. Actin/Act1p/End7p

The *end7* mutation affects actin (Act1p) itself and causes loss of cortical actin patch polarisation. The *end7* mutation is a substitution of glycine 48 with aspartic acid in the DNase I-binding loop of subdomain 2 of actin. Subdomain 2 is implicated in contacts between actin monomers within filaments [3,70].

4.7.2. Fimbrin/Sac6p

The actin filament bundling protein fimbrin is encoded by the suppressor of actin mutations 6, or *SAC6*, gene. Fimbrin was found to be necessary for endocytosis through testing a *sac6* deletion mutant [78].

4.7.3. Calmodulin/Cmd1p

The essential calcium regulatory protein calmodulin is necessary for endocytosis: temperature-conditional *cmd1* mutants have defects in internalisation. Many *cmd1* mutants defective in endocytosis have delocalised cortical actin patches and one pool of Cmd1p partially colocalises with cortical actin patches [88,89]. Interestingly, a mutant in which Cmd1p lacks all high-affinity calcium-binding sites is still able to internalise normally [79].

4.7.4. End3p

end3 mutants display delocalised cortical actin patches and abnormal clumps of F-actin in the cytoplasm. End3p contains a consensus site for interaction with phosphatidylinositol 4,5 bisphosphate (PtdInsP2), but this binding site is not essential for End3p function. End3p also has two copies of a short amino acid sequence motif at its carboxy-ter-

minus and the presence of at least one of these repeat sequences is required for function [87]. Finally, End3p has a domain homologous to mammalian epidermal growth factor (EGF) receptor kinase phosphorylation substrate 15 (Eps15) [90] ('Eps15 homology' or 'EH', domain) [91,92] at its amino-terminus. The EH domain contains a conserved EF hand motif which is essential for End3p stability and function [87].

4.7.5. Pan1p

Pan1p has two long sequence repeats at the amino-terminal end and each repeat contains one EH domain. Pan1p is required for polarised cortical actin patch distribution and also localises to cortical actin patches [93]. Pan1p binds to End3p and the complex is necessary for the internalisation step of endocytosis [92,94]. The Pan1p–End3p interaction is mediated through the essential repeated carboxy-terminal motif in End3p binding to the second long amino-terminal repeat sequence in Pan1p. The interaction does not require the EH domain in the second long repeat of Pan1p, but requires neighbouring sequences within the repeat [92]. Pan1p is phosphorylated by a serine, threonine specific protein kinase known as Prk1p, and this phosphorylation negatively regulates Pan1p function. At least one role of End3p binding is to protect Pan1p from phosphorylation by Prk1p [95].

4.7.6. Sla2p/End4p

end4 mutants, like *end3* and *pan1*, have delocalised cortical actin patches. Other alleles of *end4* were isolated based on their lethality in combination with mutations affecting actin-binding protein 1 (Abp1p), another actin-associated protein. Hence, the protein affected by the *end4* mutation is known as Sla2p (synthetic lethal with abp1 2) [96]. Sla2p partially colocalises with cortical actin patches and has a carboxy-terminal domain which shows homology to the mammalian actin-binding protein talin. Surprisingly, the talin homology domain is not required for Sla2p localisation or function. The essential domain of Sla2p lies in the amino-terminal region and this region does not exhibit strong homology to other yeast proteins [74]. In vitro reconstitution of actin patch assembly using permeabilised yeast cells and fluorochrome-labelled actin monomers requires Sla2p [97]. This protein therefore has a role in actin filament assembly.

4.7.7. Las17p/Bee1p

las17 mutants also have delocalised cortical actin patches [98]. Las17p is also known as Bee1p because it is homologous to higher eukaryotic Wiskott–Aldrich syndrome protein (WASP). Las17p is a proline-rich protein that has a carboxy-terminal actin-binding motif. Las17p partially colocalises with cortical actin patches and is implicated in actin filament assembly (see Section 7) [99].

4.7.8. Vrp1p/End5p

The *END5* gene is also known as very rich in proline 1, *VRP1*, because End5p (also known as verprolin), like Las17p, is proline-rich. *end5* mutants have cytoskeletal defects similar to the other *end* mutants described above. End5p has an amino-terminal actin-binding motif and also partially colocalises with cortical actin patches [76,100,101].

4.7.9. Rvs161p/End6p and Rvs167p

The protein affected in the *end6* mutant is known as reduced viability upon starvation 161, or Rvs161p. Rvs161p, and a second yeast protein, Rvs167p, are highly homologous over the entire sequence of Rvs161p. Rvs167p has, however, two additional carboxy-terminal domains not present in Rvs161p: a GPA domain rich in glycine, proline, and alanine residues, followed by a src homology 3, or SH3, domain. SH3 domains are important mediators of protein–protein interactions and bind to specific proline-rich amino acid sequence motifs [102–104]. Amphiphysin, a mammalian neuronal protein, shows sequence similarity to Rvs161p and Rvs167p and has a similar domain structure to Rvs167p [104,105]. The common amino-terminal domain within Rvs161p, Rvs167p, and amphiphysin is now known as the Bar domain because it is found in bin1 (a mammalian amphiphysin-like protein), amphiphysin, and Rvs proteins [106]. Mutations in *RVS161/END6* and *RVS167* lead to a loss of cortical actin patch polarity. Rvs161p interacts with Rvs167p in the yeast two-hybrid system [107] and because of the similar phenotypes associated with deletion of either gene it has been suggested that they function together [103]. The two proteins may not always function together,

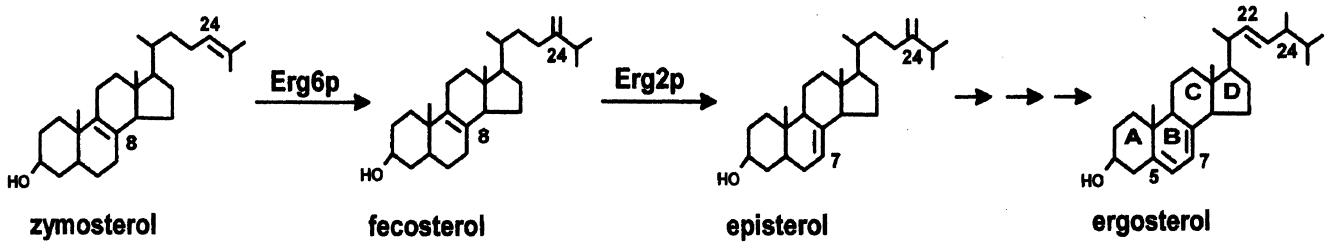

Fig. 1. The biosynthetic pathway for ergosterol showing the steps catalysed by Erg2p (C-8 sterol isomerase) and Erg6p (C-24 sterol methyltransferase) and the structures of the various intermediates. Mutants in which both Erg2p and Erg6p are missing accumulate mainly zymosterol which lacks not only the C-24 methyl group and the C-7,8 desaturation, but also the C-5,6 desaturation. Zymosterol is unable to support normal kinetics of endocytic internalisation due to the presence of only a single desaturation in the B-ring. Reprinted from [73] with permission by the American Society for Cell Biology.

however, because Rvs167p interacts with actin in the two-hybrid system [108] while Rvs161p has not been reported to, and the localisations of the two proteins seem to be different. Rvs167p localises to cortical actin patches [106] while Rvs161p has a more diffuse distribution at the cell cortex and in the cytoplasm [109].

4.7.10. Arp2p and Arc35p/End9p

The Arp2/3p complex comprises seven subunits, of which the two larger subunits are actin-related proteins encoded by the *ARP2* and *ARP3* genes [82,83,110]. The Arp2/3p complex plays a critical role in assembly of F-actin containing structures [110]. Both *arp2* and *end9* mutations have been shown to block endocytosis [72,83]. The *end9* mutation affects the 35 kDa subunit of the Arp2/3p complex (encoded by the *ARC35* gene) [110,111].

4.7.11. Myo3p and Myo5p

S. cerevisiae has two type I unconventional myosins, encoded by the *MYO3* and *MYO5* genes. These myosins have a conserved ATPase motor domain at the amino-terminus which can bind actin. They also have an SH3 domain and a carboxy-terminal acidic domain. *myo5* mutants have weak defects in endocytosis and *myo3 myo5* double mutants are blocked in endocytosis and exhibit delocalised cortical actin patches [80,112]. Type I myosins partially colocalise with cortical actin patches [112,113].

4.8. Specific lipid requirements for endocytosis

Specific sterols and sphingolipids, two classes of lipid abundant in the yeast plasma membrane, are also required for endocytic internalisation. The *end8* mutation affects the long chain base 1, *LCB1* gene, which encodes a subunit of serine palmitoyl transferase. This enzyme catalyses the condensation of palmitoyl-CoA and L-serine into 3-dehydro D-sphinganine and CoA, the first committed step in sphingolipid synthesis [72,114]. *end11* mutants, on the other hand, are blocked in ergosterol synthesis [72,73]. The *end11* mutation affects the ergosterol 2, *ERG2*, gene. *ERG2* encodes C-8 sterol isomerase and *end11/erg2* mutants are specifically defective in the isomerisation of the C-8,9 desaturation to a C-7,8 desaturation in the B-ring of the sterol molecule (see Fig. 1). *erg6* mutations, which affect methylation of C-24 in the sterol side-chain (Fig. 1), do not have severe effects on endocytosis. However, combination of *end11/erg2* and *erg6* mutations leads to loss of the C-5,6 desaturation in the B-ring as well as the expected lack of isomerisation of the C-8,9 to a C-7,8 desaturation and defects in side-chain methylation. This additional unexpected loss of the C-5,6 desaturation is presumably due to the C-5,6 desaturase being unable to use sterols lacking both *ERG2*- and *ERG6*-dependent modifications as a substrate. *end11/erg2 erg6* double mutants have severe defects in endocytic internalisation. It is likely that for sterols to support endocytic internalisation, two desaturations, either C-5,6 and C-7,8 or C-5,6 and C-8,9 are required in the B-ring. The role of sterols in endocytosis is specific because even the *end11/erg2 erg6* double mutation does not severely affect other membrane trafficking pathways [73].

5. Insights into endocytosis emerging from yeast

A major finding from work in *S. cerevisiae* has been that endocytosis is an actin-dependent process. That the involvement of actin in endocytosis is direct has been demonstrated using mutants with temperature-conditional mutations affecting actin. Thus, *act1-1* mutants that are temperature-sensitive for actin function can endocytose at 24°C, but lose this ability immediately upon shift to 37°C [78]. Another approach has been the use of drugs which cause F-actin depolymerisation. Depolymerisation of F-actin using the drug latrunculin A has been shown to rapidly abolish endocytic internalisation in *S. cerevisiae* cells [115]. In earlier studies using mammalian cells, actin inhibitors such as cytochalasins had given variable results in endocytosis inhibition experiments. In some cell types some types of endocytosis had been affected in some studies. For example, cytochalasin D blocks endocytosis from the apical surface of polarised epithelial cells, but has no effect on endocytosis from the basolateral surface even though F-actin at both surfaces is disrupted [116]. Cytochalasin D also blocks internalisation through some clathrin-independent internalisation pathways (see below) [117,118]. More recent studies with latrunculin A, which has stronger effects on actin polymerisation than cytochalasin D, indicate that endocytosis through clathrin-coated pits can also be blocked by actin depolymerisation [119].

The role of amphiphysin in endocytosis in mammalian cells was also revealed after earlier studies using yeast. Amphiphysin is a mammalian neuronal protein related to Rvs161p/End6p and Rvs167p. After the initial observation that Rvs161p/End6p and Rvs167p are required for endocytosis of both LY and α-factor in yeast [70], amphiphysin was shown to be important for endocytic internalisation in neurons [120]. While amphiphysin is mainly cytosolic, a fraction is tightly associated with synaptic coated vesicles [105]. Amphiphysin contains a carboxy-terminal SH3 domain and this binds to a proline-rich domain in the carboxy-terminus of dynamin [121,122]. Disruption of this interaction in vivo or in vitro leads to the accumulation of invaginated clathrin-coated pits unable to pinch off the plasma membrane, a defect similar to that observed when dynamin function is affected [120,123]. In vitro, purified amphiphysin can bind to artificial phospholipid liposomes and induce the formation of membranous tubules. Similar tubules can be formed when purified dynamin is added to liposomes and dynamin can also sever these tubules into vesicles in a GTP-dependent reaction. Simultaneous addition of amphiphysin and dynamin to liposomes leads to colocalisation of amphiphysin and dynamin in spiral-shaped structures surrounding the membranous tubules and stimulation of the GTP-dependent vesiculating activity of dynamin [124,125]. Amphiphysin also interacts with the AP-2 complex and may function as a linker between dynamin and AP-2 present in clathrin-coated pits [123]. While amphiphysin is expressed mainly in neurons, lower levels of amphiphysin are expressed in other cell types and amphiphysin has been shown to have general functions in endocytosis in non-neuronal cells [123]. In addition, in mammals another amphiphysin-like protein (amphiphysin II, of which Bin1 mentioned above is a splice variant) is encoded by a distinct gene. Amphiphysin II, like the original amphiphysin isoform (amphiphysin I), is mainly expressed in neurons, but is expressed at lower levels in other cell types. Interestingly, amphiphysin II binds directly to clathrin heavy chain and the two amino acid sequence motifs which mediate this interaction are conserved in the sequence of amphiphysin I [126]. There are also reports that while neuronal amphiphysin I localises to clathrin-coated pits, some isoforms of amphiphysin II (e.g. Bin1) may be associated with the cortical actin cytoskeleton in some mammalian cells [127].

Following the discovery that End3p is required for endocytic internalisation in yeast [69,87], the mammalian EH-domain-containing protein Eps15, was shown to be a component of clathrin-coated pits in mammalian cells [128]. Eps15 localises specifically to the edges of clathrin-coated pits through interaction with the AP-2 adaptor complex [129–131]. More recently, Eps15 interaction with AP-2 has been shown to be required for assembly of clathrin-coated pits and for endocytosis [132,133]. EH domains have been shown to bind to motifs in other proteins containing the amino acid sequence NPF (in single letter amino acid code) [134]. Epsin is a mammalian NPF-motif-containing clathrin-coated pit protein which interacts strongly with Eps15 as well as AP-2 and which is required for receptor-mediated internalisa-

tion of both Tf and EGF [135]. In yeast, the EH-domain-containing protein Pan1p is the closest relative of mammalian Eps15. The EH domains of Pan1p bind to two epsin homologues, Epsin-N-terminal-homology-domain-containing protein 1, or Ent1p, and Ent2p. Intriguingly, yeast Ent1p binds clathrin heavy chain in vitro through a carboxy-terminal sequence motif (Ent2p also has the motif, but was not tested for clathrin heavy chain binding). The yeast epsins have been shown to be required for both cortical actin patch localisation and endocytic internalisation [136]. It is not yet clear whether mammalian epsin is required for actin cytoskeletal organisation or function in mammalian cells.

A human homologue of yeast Sla2p/End4p has also been described more recently. This protein, Huntingtin-interacting protein 1 (HIP1)-related protein (HIP1R) is a member of a family of proteins that interact with Huntingtin. Mutations affecting Huntingtin cause Huntington's disease, a human genetic disorder associated with degeneration of the nervous system. HIP1R is associated with clathrin-coated pits as well as actin-rich membrane ruffles in mammalian cells [137].

Synaptojanin is a neuronal inositol polyphosphate 5-phosphatase [138]. Synaptojanin-like proteins have been shown to be required for internalisation in *S. cerevisiae* [84,85] and synaptojanin itself is implicated in internalisation in mammalian neurons [138]. Like dynamin, synaptojanin also contains a proline-rich domain and the SH3 domain of amphiphysin binds to synaptojanin through this proline-rich domain [139–141]. While the exact role of phosphatidylinositides in endocytosis is not yet known, PtdIns(4,5)P2 plays a key regulatory role in actin polymerisation.

WASP is a mammalian proline-rich protein related to *S. cerevisiae* Las17p. Wiskott–Aldrich syndrome is a human X-linked recessive genetic disorder characterised by low numbers of circulating platelets and lymphocytes. Patients with Wiskott–Aldrich syndrome have severe immunodeficiencies which arise from an inability of certain subtypes of T lymphocyte to proliferate upon T cell receptor activation [142]. The disease has been shown to be attributable to mutations in WASP, a protein expressed in various haematopoietic cells [142,143]. At least two other, more ubiquitously-expressed, WASP-like proteins are known in humans (neural-, or N-WASP, and another protein known variously as WASP-family verprolin-homologous protein (WAVE) or the human homologue of *Dictyostelium discoideum* suppressor of cAMP receptor 1 (SCAR1)). Diseases associated with defects in these proteins are not yet known [144–147]. After the discovery that Las17p is required for endocytosis in yeast, there have been reports linking WASP-family proteins to endocytic internalisation in mammalian cells. Wiskott–Aldrich syndrome T lymphocytes have endocytic defects, suggesting a requirement for the haematopoietic form of WASP in endocytosis [148]. In neuronal cells, N-WASP has been shown to interact with syndapin I, an SH3-domain-containing protein that also interacts with dynamin and synaptojanin [149]. Syndapin I, and a more ubiquitously-expressed isoform, syndapin II, have been shown to play an important role in endocytic clathrin-coated vesicle formation in mammalian cells [150,151].

WASP-interacting protein (WIP) is a ubiquitously expressed human protein. The carboxy-terminal domain of WIP interacts with the amino-terminal domain of WASP in the yeast two-hybrid system [152]. *S. cerevisiae* Vrp1p/End5p shows sequence similarity to WIP and in the yeast two-hybrid system, the carboxy-terminal domain of Vrp1p interacts with the amino-terminal domain of Las17p, analogous to the mammalian WIP–WASP interaction [76]. Interestingly, while it is not yet known if WIP is required for internalisation in mammalian cells, expression of WIP in *end5* mutant yeast cells rescues some of the *end5* phenotypes including endocytosis [153]. This result shows that the function of this family of proteins has been conserved through evolution.

The investigation of lipid requirements for endocytic internalisation in *S. cerevisiae* cells is contributing to our understanding of the role of lipids in endocytosis in higher eukaryotes. In mammalian cells, sterols (the major form in mammals being cholesterol) are necessary for the formation of specialised detergent-insoluble (e.g. Triton X-100-insoluble) microdomains within the plasma membrane [154–158]. A subset of these domains are flask-shaped invaginations (distinct from clathrin-coated pits) and bear a novel cytoplasmic coat composed of a protein known as caveolin. These microdomains are known as caveolae [155,157,159]. Sphingolipids have been shown

to concentrate in caveolae in higher eukaryotes [160]. Sterol- and sphingolipid-enriched detergent-insoluble microdomains (caveolae as well as other microdomains lacking caveolin) have been implicated in clathrin-independent endocytosis in mammalian cells [117,118,156,161–163]. The requirement for specific sterols and sphingolipids in yeast may indicate that internalisation in lower eukaryotic cells also occurs through sterol- and sphingolipid-containing microdomains (although yeast lacks a caveolin homologue and hence these domains are unlikely to have the same morphology as mammalian caveolae) [73]. A possible involvement of sterol- and sphingolipid-containing microdomains might be consistent with the observation that endocytosis in yeast is largely non-clathrin-dependent. The classification of endocytosis as either clathrin-dependent or sterol- and sphingolipid-dependent may not always be so clear, however. Recent evidence suggests that internalisation through clathrin-coated pits in mammalian cells may also be dependent on sterols [164,165].

The internalisation of many plasma membrane proteins in *S. cerevisiae* is dependent on their prior modification by phosphorylation and ubiquitination. The classical mammalian tyrosine-based and dileucine-based motifs are not commonly used as internalisation motifs in yeast. Interestingly, it has been known for some time that several mammalian plasma membrane receptors are ubiquitinated, e.g. the EGF, platelet-derived growth factor (PDGF), and human growth hormone (HGH) receptors. Recent evidence shows that in some cases the ubiquitination is dependent on ligand binding. The discovery that ubiquitin is a signal for internalisation in yeast led to investigations into whether the same is true in mammals. So far there is only one case (HGH receptor) where evidence exists that ubiquitination is important for internalisation. Nevertheless, ubiquitination may well turn out to be an important general signal for internalisation in both higher and lower eukaryotes [55].

Studies using yeast have contributed, not only to our understanding of the machinery involved, but also to our understanding of the types of surface proteins which are subject to endocytic internalisation. Most endocytosed proteins characterised so far in yeast are polytopic and the best characterised is Ste2p, a G-protein-coupled receptor. The majority of mammalian proteins whose endocytic internalisation has been well-characterised have only a single membrane-spanning domain [14]. Recently, several types of mammalian polytopic G-protein-coupled receptor have been shown to undergo endocytosis through clathrin-coated pits [166,167].

6. Yeast plasma membrane endocytic pits

The fact that many *end* mutants of *S. cerevisiae* have a defective cortical actin patch localisation, together with the observation that these patches are often seen close to the plasma membrane, led to the idea that the cortical actin patches are the sites of endocytic internalisation. Initially, this hypothesis also seemed to be supported by the first ultrastructural studies on yeast cortical actin patches. These studies showed that cortical actin patches are associated with plasma membrane invaginations [168]. More recent studies, however, show that during internalisation Ste2p does not localise to cortical actin patches. Instead, a novel type of furrow-like structure is the primary site at which Ste2p localises during internalisation. These structures contain some actin, but less than cortical actin patches [169]. The finding that these novel structures do contain some actin is consistent with the requirement for actin and actin-associated proteins in internalisation. Moreover, the fact that these endocytic structures are distinct from cortical actin patches may help to explain why not all mutations that affect cortical actin patches also affect endocytosis. Some actin-associated proteins may function in cortical actin patches but not in these furrow-like invaginations important for endocytosis. The protein composition of these furrow-like plasma membrane subdomains has not yet been described. It will be interesting to see whether they contain the actin-associated End proteins described above which are required for internalisation.

7. Models for endocytic internalisation

The study of endocytosis using both lower and higher eukaryotes has led to a widening of our perspective and a more complete knowledge of the key

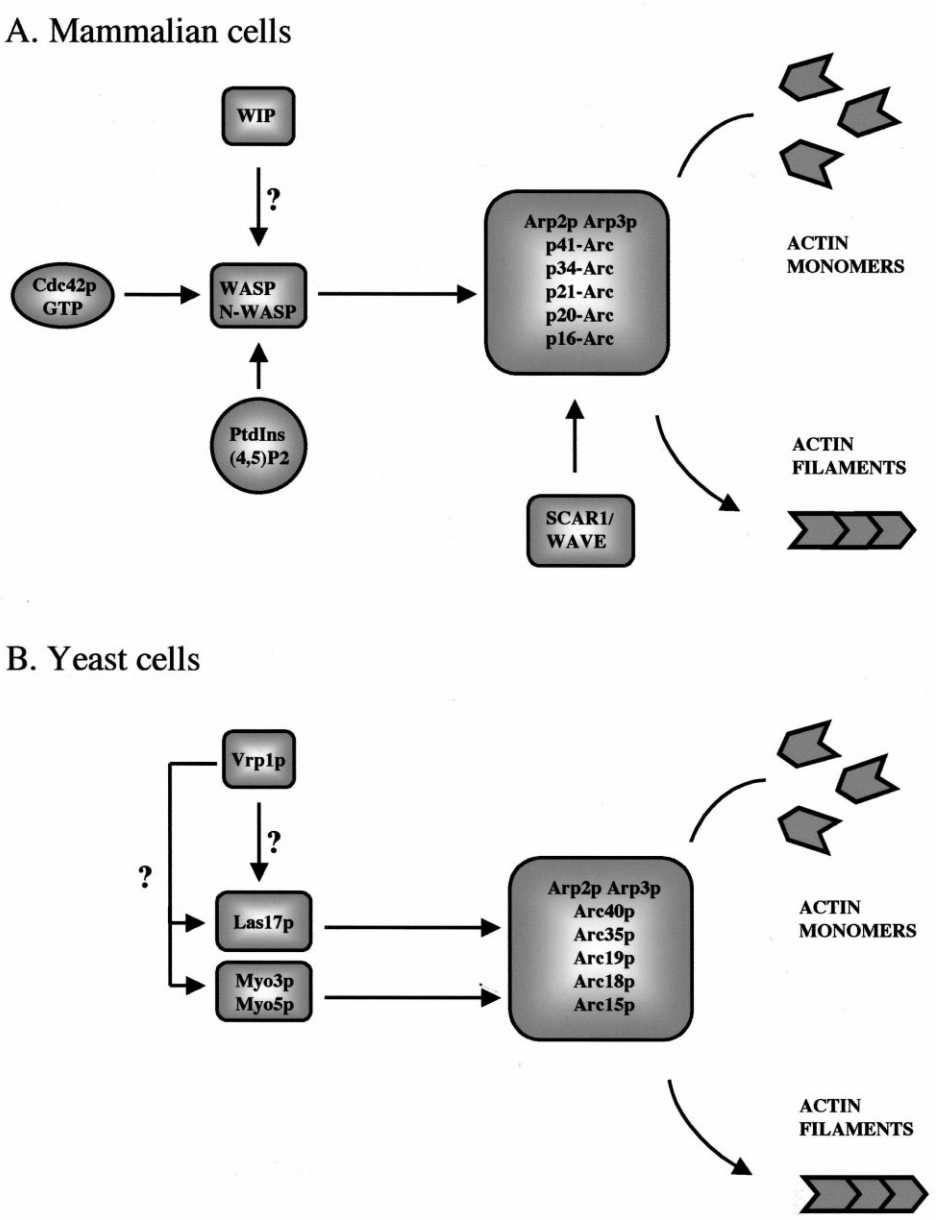

Fig. 2. Regulation of the actin filament nucleation activity of the Arp2/3p complex (A) in mammalian cells and (B) in yeast cells. Arrows indicate known interactions. The regulatory significance of the interaction of WIP with WASP and N-WASP in mammalian cells and the interaction of Vrp1p with Las17p, Myo3p, and Myo5p in yeast cells, is not yet clear.

molecules involved. How do these various molecules work together to bring about the budding of a vesicle from the plasma membrane? A number of exciting recent findings have given us insight into the function of some of the key players. One major advance has been in understanding the function of the WASP-family proteins and how they interact with the Arp2/3p complex. The Arp2/3p complex comprises seven proteins of which the two larger proteins are Arp2p and Arp3p [110,170]. The subunit composition and function of the Arp2/3p complex is highly conserved between higher and lower eukaryotes. A key finding was that the Arp2/3p complex is responsible for nucleation (i.e. initiation) of new actin filaments, which is the slow step in actin filament assembly [170,171]. A second advance was the demonstration that the purified Arp2/3p complex has only limited activity in vitro, but is strongly

stimulated by interaction with WASP-family proteins [147,170,172]. Las17p has been shown to have a similar effect on the yeast Arp2/3p complex [110,173]. It is likely that WASP-family proteins also regulate actin filament assembly in vivo. Ectopic WASP expression induces the formation of cytoplasmic clusters of F-actin in mammalian cells and WASP localises to these F-actin clusters [174–176]. Expression of N-WASP in mammalian cells induces the formation of F-actin-containing cell-surface projections [177]. WASP and N-WASP have Cdc42p/Rac interaction binding (CRIB) (also called GTPase binding domain (GBD)) domains which bind the Rho-family GTPase Cdc42p [144,174–176]. Both WASP and N-WASP also have amino-terminal pleckstrin homology (PH) domains which can potentially bind phosphatidylinositides such as PtdIns4,5P2 [144,172]. The GTP-bound form of Cdc42p and PtdIns4,5P2 have recently been shown to function together in regulating N-WASP activity on the Arp2/3p complex [172]. Mammalian and *Dictyostelium* SCAR1/WAVE and yeast Las17p lack the consensus sequence for interaction with Rho-like GTPases [99,145,146]. The various interactions between the Arp2/3p complex and known regulatory proteins and lipids is depicted in Fig. 2. WIP has also been implicated in actin filament assembly. High-level expression of WIP in mammalian cells causes accumulation of what appear to be F-actin bundles at the cortex and the formation of F-actin-containing cell-surface projections [152].

In *S. cerevisiae* Arp2p is required for endocytic internalisation [82,83]. Furthermore, the *END9* gene product (End9p/Arc35p), which is also required for endocytosis, is one of the smaller (35 kDa) subunits of the yeast Arp2/3p complex [72,110,111]. This, together with the additional requirements for a WASP-related protein, Las17p [76,77], and a WIP-related protein, End5p/Vrp1p [70,76], for endocytosis in yeast suggests a model in which nucleation and rapid actin polymerisation provides a force pushing vesicles away from the plasma membrane and into the cytoplasm (Fig. 3). Whether actin filament polymerisation by the Arp2/3p complex plays a direct role in vesicle budding from the plasma membrane in mammalian cells is not yet known. However, in support of this possible mechanism, N-WASP has recently been implicated in endocytic vesicle formation [149–151]. Moreover, there is recent evidence that in some mammalian cells primary endocytic vesicles move through the cytoplasm leaving behind a trail of polymerised actin [178]. Recently, a role for

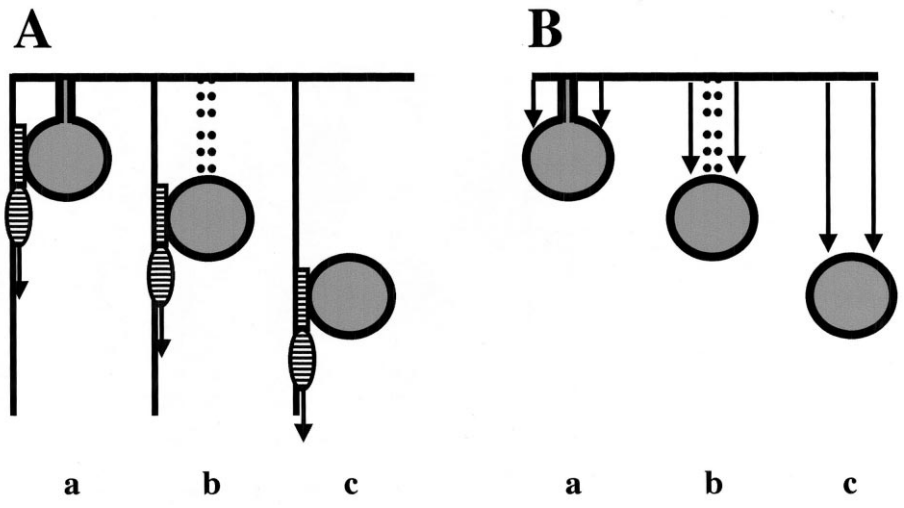

Fig. 3. Models for the internalisation step of endocytosis. In model (A) the endocytic pits invaginate (a), undergo fission to form vesicles (b), and the vesicles move (c) because of their attachment to type I myosins (striped). The type I myosins bind to membranes via their basic tail domain and to actin filaments (vertical lines) via their ATPase head domain and move the membrane with respect to the actin filaments. In model (B) the endocytic pits undergo the same events as in model (A) (a–c), but driven by the formation (nucleation) and extension (by polymerisation) of new actin filaments (vertical lines) which push the vesicles off the plasma membrane and into the cell interior. Nucleation of actin filaments may occur upon activation of the Arp2/3p complex by proteins such as Las17p. The arrowheads depict the direction of actin filament extension.

sterol- and PtdIns4,5P2-enriched membrane microdomains in this type of actin-based motility was reported [179]. This is interesting in light of the requirement for specific sterols and phosphoinositides in endocytic internalisation in yeast [73,84,85]. Perhaps similar microdomains in the plasma membrane are required for Arp2/3p-dependent actin polymerisation during membrane invagination or vesicle budding [73]. A lipid requirement for this actin-based movement may play a role in controlling vesicle release spatially and temporally. High sterol content in microdomains may provide sufficient localised rigidity to ensure that the force of actin polymerisation is channelled into driving vesicles inwards rather than into formation of outwards cell-surface projections.

Another possibility is that the cytoskeleton is required in a structural way to provide a framework against which force can be applied to pull endocytic vesicles into the cellular interior. Unconventional myosins of the type I class (e.g. Myo3p and Myo5p) have been implicated in endocytic internalisation [80,112]. These type I myosins have a conserved head domain which binds actin and has ATPase activity as in conventional type II myosins, but their characteristic tail domain (which contains a higher content of basic amino acids) can associate with phospholipid membranes. This domain structure makes them ideal for attaching to the cytoskeleton and plasma membrane and applying contractile force to the membrane (see Fig. 3) [80,112].

Interestingly, type I myosins may be able to function in more than one way to promote membrane invagination. It has recently been reported that the yeast type I myosins (Myo3p and Myo5p), like Las17p, can activate the Arp2/3p complex [180–182]. Thus, this family of myosins may be able to function as motors or as stimulators of actin filament assembly, at least in lower eukaryotes. It has been difficult to test the relative importance of the motor activity and Arp2/3p activation activity in endocytosis because Arp2/3p activation is dependent on the motor domain [180].

While the two models shown in Fig. 3 invoke a direct mechanical role for the actin cytoskeleton in endocytic vesicle formation, it is also possible that the actin cytoskeleton plays a regulatory role. In support of this idea is the recent finding that depolymerisation of F-actin by latrunculin A treatment does not abolish endocytosis in all cultured mammalian cell lines. Even in those cell lines in which endocytosis does appear to require F-actin, this requirement can be overcome by altering the cell culture conditions, e.g. growing the cells in suspension instead of monolayers [183].

8. Future directions

Over the past few years our understanding of endocytosis has improved enormously. Molecular genetic studies using *S. cerevisiae* have led to a better idea of the types of proteins and lipids required for endocytosis in this lower eukaryote. Recent studies have suggested that similar classes of protein and lipid may be required for endocytosis in at least some higher eukaryotic cells. Actin, the Arp2/3p complex, WASP- and WIP-related proteins, type I myosins, phosphatidylinositides, sphingolipids, and sterols may turn out to be general requirements for endocytic vesicle formation in higher as well as lower eukaryotes. This realisation, together with recent progress in understanding the biochemical activity of the Arp2/3p complex in actin polymerisation and its regulation by WASP-related proteins, Rho-family GTPases, and lipids has made this a very exciting time to study endocytosis. The next challenge is to determine which of these new requirements reflect a mechanical role for the corresponding protein or lipid in endocytosis and which reflect a regulatory role. If the involvement of actin, the Arp2/3p complex, type I myosins, WASP, and WIP in endocytosis is indeed mechanical, we will need to fit these 'new' players together with 'older' players like clathrin, dynamin, and AP-2 into a coherent model that describes how they all work together to create an endocytic vesicle at the plasma membrane.

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References

[1] R. Schekman, Protein localization and membrane traffic in yeast, Annu. Rev. Cell Biol. 1 (1985) 115–143.

[2] N.K. Pryer, L.J. Wuestehube, R. Schekman, Vesicle-mediated protein sorting, Annu. Rev. Biochem. 61 (1992) 471–516.

[3] H. Riezman, A. Munn, M.I. Geli, L. Hicke, Actin-, myosin- and ubiquitin-dependent endocytosis, Experientia 52 (1996) 1033–1041.

[4] M.I. Geli, H. Riezman, Endocytic internalization in yeast and animal cells: similar and different, J. Cell Sci. 111 (1998) 1031–1037.

[5] N.J. Bryant, T.H. Stevens, Vacuole biogenesis in *Saccharomyces cerevisiae*: protein transport pathways to the yeast vacuole, Microbiol. Mol. Biol. Rev. 62 (1998) 230–247.

[6] B. Wendland, S.D. Emr, H. Riezman, Protein traffic in the yeast endocytic and vacuolar protein sorting pathways, Curr. Opin. Cell Biol. 10 (1998) 513–522.

[7] H.R.B. Pelham, SNAREs and the secretory pathway – lessons from yeast, Exp. Cell Res. 247 (1999) 1–8.

[8] A.L. Munn, The yeast endocytic membrane transport system, Microsc. Res. Tech. 51 (2000) 547–562.

[9] J.R. Pringle, J.R. Broach, E.W. Jones, (Eds.), The Molecular and Cellular Biology of the Yeast *Saccharomyces*, Cell Cycle and Cell Biology, Cold Spring Harbor Laboratory Press, Plainview, NY, 1997.

[10] A. Goffeau, B.G. Barrell, H. Bussey, R.W. Davis, B. Dujon, H. Feldmann, F. Galibert, J.D. Hoheisel, C. Jacq, M. Johnston, E.J. Louis, H.W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, S.G. Oliver, Life with 6000 genes, Science 274 (1996) 546–567.

[11] R.M. Steinman, I.S. Mellman, W.A. Muller, Z.A. Cohn, Endocytosis and the recycling of plasma membrane, J. Cell Biol. 96 (1983) 1–27.

[12] B.M.F. Pearse, M.S. Bretscher, Membrane recycling by coated vesicles, Annu. Rev. Biochem. 50 (1981) 85–101.

[13] T.F. Roth, K.R. Porter, Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti*L, J. Cell Biol. 20 (1964) 313–332.

[14] J.L. Goldstein, M.S. Brown, R.G.W. Anderson, D.W. Russell, W.J. Schneider, Receptor-mediated endocytosis: concepts emerging from the LDL receptor system, Annu. Rev. Cell Biol. 1 (1985) 1–39.

[15] M.S. Marks, H. Ohno, T. Kirchhausen, J.S. Bonifacino, Protein sorting by tyrosine-based signals: adapting to the Ys and wherefores, Trends Cell Biol. 7 (1997) 124–128.

[16] I. Mellman, Endocytosis and molecular sorting, Annu. Rev. Cell Dev. Biol. 12 (1996) 575–625.

[17] S.L. Schmid, Clathrin-coated vesicle formation and protein sorting: an integrated process, Annu. Rev. Biochem. 66 (1997) 511–548.

[18] J. Hirst, M.S. Robinson, Clathrin and adaptors, Biochim. Biophys. Acta 1404 (1998) 173–193.

[19] H. Riezman, Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis, Cell 40 (1985) 1001–1009.

[20] V. Dulic, M. Egerton, I. Elguindi, S. Raths, B. Singer, H. Riezman, Yeast endocytosis assays, Methods Enzymol. 194 (1991) 697–710.

[21] L. Bardwell, J.G. Cook, C.J. Inouye, J. Thorner, Signal propagation and regulation in the mating pheromone response pathway of the yeast *Saccharomyces cerevisiae*, Dev. Biol. 166 (1994) 363–379.

[22] Y. Chvatchko, I. Howald, H. Riezman, Two yeast mutants defective in endocytosis are defective in pheromone response, Cell 46 (1986) 355–364.

[23] D.D. Jenness, P. Spatrick, Down regulation of the α-factor pheromone receptor in *S. cerevisiae*, Cell 46 (1986) 345–353.

[24] K.A. Schandel, D.D. Jenness, Direct evidence for ligand-induced internalization of the yeast α-factor pheromone receptor, Mol. Cell. Biol. 14 (1994) 7245–7255.

[25] N.G. Davis, J.L. Horecka, G.F. Sprague Jr., Cis- and trans-acting functions required for endocytosis of the yeast pheromone receptors, J. Cell Biol. 122 (1993) 53–65.

[26] B. Zanolari, S. Raths, B. Singer-Krüger, H. Riezman, Yeast pheromone receptor endocytosis and hyperphosphorylation are independent of G protein-mediated signal transduction, Cell 71 (1992) 755–763.

[27] C. Volland, D. Urban-Grimal, G. Géraud, R. Haguenauer-Tsapis, Endocytosis and degradation of the yeast uracil permease under adverse conditions, J. Biol. Chem. 269 (1994) 9833–9841.

[28] J. Horak, D.H. Wolf, Catabolite inactivation of the galactose transporter in the yeast *Saccharomyces cerevisiae*: ubiquitination, endocytosis, and degradation in the vacuole, J. Bacteriol. 179 (1997) 1541–1549.

[29] E. Riballo, M. Herweijer, D.H. Wolf, R. Lagunas, Catabolite inactivation of the yeast maltose transporter occurs in the vacuole after internalization by endocytosis, J. Bacteriol. 177 (1995) 5622–5627.

[30] S. Krampe, O. Stamm, C.P. Hollenberg, E. Boles, Catabolite inactivation of the high-affinity hexose transporters Hxt6 and Hxt7 of *Saccharomyces cerevisiae* occurs in the vacuole after internalization by endocytosis, FEBS Lett. 441 (1998) 343–347.

[31] K. Lai, C.P. Bolognese, S. Swift, P. McGraw, Regulation of inositol transport in *Saccharomyces cerevisiae* involves inositol-induced changes in permease stability and endocytic degradation in the vacuole, J. Biol. Chem. 270 (1995) 2525–2534.

[32] J.Y. Springael, B. André, Nitrogen-regulated ubiquitination of the Gap1 permease of *Saccharomyces cerevisiae*, Mol. Biol. Cell 9 (1998) 1253–1263.

[33] T. Beck, A. Schmidt, M.N. Hall, Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast, J. Cell Biol. 146 (1999) 1227–1237.

[34] R.S. Gitan, H. Luo, J. Rodgers, M. Broderius, D. Eide, Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation, J. Biol. Chem. 273 (1998) 28617–28624.

[35] C. Berkower, D. Loayza, S. Michaelis, Metabolic instability and constitutive endocytosis of STE6, the **a**-factor transporter of *Saccharomyces cerevisiae*, Mol. Biol. Cell 5 (1994) 1185–1198.
[36] R. Egner, Y. Mahé, R. Pandjaitan, K. Kuchler, Endocytosis and vacuolar degradation of the plasma membrane-localized Pdr5 ATP-binding cassette multidrug transporter in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 15 (1995) 5879–5887.
[37] J.S. Chuang, R.W. Schekman, Differential trafficking and timed localization of two chitin synthase proteins, Chs2p and Chs3p, J. Cell Biol. [Erratum: J. Cell Biol. (1996) 135, 1925] 135 (1996) 597–610.
[38] M. Ziman, J.S. Chuang, R.W. Schekman, Chs1p and Chs3p, two proteins involved in chitin synthesis, populate a compartment of the *Saccharomyces cerevisiae* endocytic pathway, Mol. Biol. Cell 7 (1996) 1909–1919.
[39] M.J. Lewis, B.J. Nichols, C. Prescianotto-Baschong, H. Riezman, H.R.B. Pelham, Specific retrieval of the exocytic SNARE Snc1p from early yeast endosomes, Mol. Biol. Cell 11 (2000) 23–38.
[40] M. Ziman, J.S. Chuang, M. Tsung, S. Hamamoto, R. Schekman, Chs6p-dependent anterograde transport of Chs3p from the chitosome to the plasma membrane in *Saccharomyces cerevisiae*, Mol. Biol. Cell 9 (1998) 1565–1576.
[41] J. Rohrer, H. Bénédetti, B. Zanolari, H. Riezman, Identification of a novel sequence mediating regulated endocytosis of the G protein-coupled α-pheromone receptor in yeast, Mol. Biol. Cell 4 (1993) 511–521.
[42] P.K. Tan, J.P. Howard, G.S. Payne, The sequence NPFXD defines a new class of endocytosis signal in *Saccharomyces cerevisiae*, J. Cell Biol. 135 (1996) 1789–1800.
[43] L. Hicke, H. Riezman, Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis, Cell 84 (1996) 277–287.
[44] J. Terrell, S. Shih, R. Dunn, L. Hicke, A function for monoubiquitination in the internalization of a G protein-coupled receptor, Mol. Cell 1 (1998) 193–202.
[45] M. Hochstrasser, Ubiquitin-dependent protein degradation, Annu. Rev. Genet. 30 (1996) 405–439.
[46] S.C. Shih, K.E. Sloper-Mould, L. Hicke, Monoubiquitin carries a novel internalization signal that is appended to activated receptors, EMBO J. 19 (2000) 187–198.
[47] J.E. Reneke, K.J. Blumer, W.E. Courchesne, J. Thorner, The carboxy-terminal segment of the yeast α-factor receptor is a regulatory domain, Cell 55 (1988) 221–234.
[48] L. Hicke, B. Zanolari, H. Riezman, Cytoplasmic tail phosphorylation of the α-factor receptor is required for its ubiquitination and internalization, J. Cell Biol. 141 (1998) 349–358.
[49] J.M. Galan, V. Moreau, B. Andre, C. Volland, R. Haguenauer-Tsapis, Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease, J. Biol. Chem. 271 (1996) 10946–10952.
[50] A.F. Roth, N.G. Davis, Ubiquitination of the yeast **a**-factor receptor, J. Cell Biol. 134 (1996) 661–674.
[51] R. Egner, K. Kuchler, The yeast multidrug transporter Pdr5 of the plasma membrane is ubiquitinated prior to endocytosis and degradation in the vacuole, FEBS Lett. 378 (1996) 177–181.
[52] D. Loayza, S. Michaelis, Role for the ubiquitin–proteasome system in the vacuolar degradation of Ste6p, the **a**-factor transporter in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 18 (1998) 779–789.
[53] J.M. Galan, R. Haguenauer-Tsapis, Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein, EMBO J. 16 (1997) 5847–5854.
[54] C. Marchal, R. Haguenauer-Tsapis, D. Urban-Grimal, A PEST-like sequence mediates phosphorylation and efficient ubiquitination of yeast uracil permease, Mol. Cell. Biol. 18 (1998) 314–321.
[55] L. Hicke, Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins, FASEB J. 11 (1997) 1215–1226.
[56] C. Hein, J.Y. Springael, C. Volland, R. Haguenauer-Tsapis, B. André, *NPI1*, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase, Mol. Microbiol. 18 (1995) 77–87.
[57] C. Hein, B. André, A C-terminal di-leucine motif and nearby sequences are required for NH_4^+-induced inactivation and degradation of the general amino acid permease, Gap1p, of *Saccharomyces cerevisiae*, Mol. Microbiol. 24 (1997) 607–616.
[58] C.A. Wilcox, K. Redding, R. Wright, R.S. Fuller, Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole, Mol. Biol. Cell 3 (1992) 1353–1371.
[59] J.J. Vowels, G.S. Payne, A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole, EMBO J. [Erratum: EMBO J. (1998) 17, 4211] 17 (1998) 2482–2493.
[60] G.S. Payne, D. Baker, E. van Tuinen, R. Schekman, Protein transport to the vacuole and receptor-mediated endocytosis by clathrin heavy chain-deficient yeast, J. Cell Biol. 106 (1988) 1453–1461.
[61] D.S. Chu, B. Pishvaee, G.S. Payne, The light chain subunit is required for clathrin function in *Saccharomyces cerevisiae*, J. Biol. Chem. 271 (1996) 33123–33130.
[62] P.K. Tan, N.G. Davis, G.F. Sprague, G.S. Payne, Clathrin facilitates the internalization of seven transmembrane segment receptors for mating pheromones in yeast, J. Cell Biol. 123 (1993) 1707–1716.
[63] K.M. Huang, K. D'Hondt, H. Riezman, S.K. Lemmon, Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast, EMBO J. 18 (1999) 3897–3908.
[64] B.G. Yeung, H.L. Phan, G.S. Payne, Adaptor complex-independent clathrin function in yeast, Mol. Biol. Cell 10 (1999) 3643–3659.
[65] K.A. Shepard, M.P. Yaffe, The yeast dynamin-like protein, Mgm1p, functions on the mitochondrial outer membrane to

mediate mitochondrial inheritance, J. Cell Biol. 144 (1999) 711–719.
[66] A.E. Gammie, L.J. Kurihara, R.B. Vallee, M.D. Rose, *DNM1*, a dynamin-related gene, participates in endosomal trafficking in yeast, J. Cell Biol. 130 (1995) 553–566.
[67] S.F. Nothwehr, E. Conibear, T.H. Stevens, Golgi and vacuolar membrane proteins reach the vacuole in *vps1* mutant yeast cells via the plasma membrane, J. Cell Biol. 129 (1995) 35–46.
[68] H. Sesaki, R.E. Jensen, Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape, J. Cell Biol. 147 (1999) 699–706.
[69] S. Raths, J. Rohrer, F. Crausaz, H. Riezman, *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*, J. Cell Biol. 120 (1993) 55–65.
[70] A.L. Munn, B.J. Stevenson, M.I. Geli, H. Riezman, *end5*, *end6*, and *end7*: mutations that cause actin delocalization and block the internalization step of endocytosis in *Saccharomyces cerevisiae*, Mol. Biol. Cell 6 (1995) 1721–1742.
[71] H. Nelson, N. Nelson, Disruption of genes encoding subunits of yeast vacuolar H^+-ATPase causes conditional lethality, Proc. Natl. Acad. Sci. USA 87 (1990) 3503–3507.
[72] A.L. Munn, H. Riezman, Endocytosis is required for the growth of vacuolar H^+-ATPase-defective yeast: identification of six new *END* genes, J. Cell Biol. 127 (1994) 373–386.
[73] A.L. Munn, A. Heese-Peck, B.J. Stevenson, H. Pichler, H. Riezman, Specific sterols required for the internalization step of endocytosis in yeast, Mol. Biol. Cell 10 (1999) 3943–3957.
[74] A. Wesp, L. Hicke, J. Palecek, R. Lombardi, T. Aust, A.L. Munn, H. Riezman, End4p/Sla2p interacts with actin-associated proteins for endocytosis in *Saccharomyces cerevisiae*, Mol. Biol. Cell 8 (1997) 2291–2306.
[75] P.J. Plant, M.F. Manolson, S. Grinstein, N. Demaurex, Alternative mechanisms of vacuolar acidification in H^+-ATPase-deficient yeast, J. Biol. Chem. 274 (1999) 37270–37279.
[76] S.N. Naqvi, R. Zahn, D.A. Mitchell, B.J. Stevenson, A.L. Munn, The WASp homologue Las17p functions with the WIP homologue End5p/verprolin and is essential for endocytosis in yeast, Curr. Biol. 8 (1998) 959–962.
[77] A. Madania, P. Dumoulin, S. Grava, H. Kitamoto, C. Schärer-Brodbeck, A. Soulard, V. Moreau, B. Winsor, The *Saccharomyces cerevisiae* homologue of human Wiskott–Aldrich syndrome protein Las17p interacts with the Arp2/3 complex, Mol. Biol. Cell 10 (1999) 3521–3538.
[78] E. Kübler, H. Riezman, Actin and fimbrin are required for the internalization step of endocytosis in yeast, EMBO J. 12 (1993) 2855–2862.
[79] E. Kübler, F. Schimmöller, H. Riezman, Calcium-independent calmodulin requirement for endocytosis in yeast, EMBO J. 13 (1994) 5539–5546.
[80] M.I. Geli, H. Riezman, Role of type I myosins in receptor-mediated endocytosis in yeast, Science 272 (1996) 533–535.
[81] P. Lappalainen, D.G. Drubin, Cofilin promotes rapid actin filament turnover in vivo, Nature [Erratum: Nature (1997) 389, 211] 388 (1997) 78–82.
[82] V. Moreau, A. Madania, R.P. Martin, B. Winsor, The *Saccharomyces cerevisiae* actin-related protein Arp2 is involved in the actin cytoskeleton, J. Cell Biol. 134 (1996) 117–132.
[83] V. Moreau, J.M. Galan, G. Devilliers, R. Haguenauer-Tsapis, B. Winsor, The yeast actin-related protein Arp2p is required for the internalization step of endocytosis, Mol. Biol. Cell 8 (1997) 1361–1375.
[84] W.J. Luo, A. Chang, Novel genes involved in endosomal traffic in yeast revealed by suppression of a targeting-defective plasma membrane ATPase mutant, J. Cell Biol. 138 (1997) 731–746.
[85] B. Singer-Krüger, Y. Nemoto, L. Daniell, S. Ferro-Novick, P. De Camilli, Synaptojanin family members are implicated in endocytic membrane traffic in yeast, J. Cell Sci. 111 (1998) 3347–3356.
[86] B. Winsor, E. Schiebel, Review: an overview of the *Saccharomyces cerevisiae* microtubule and microfilament cytoskeleton, Yeast 13 (1997) 399–434.
[87] H. Bénédetti, S. Raths, F. Crausaz, H. Riezman, The *END3* gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast, Mol. Biol. Cell 5 (1994) 1023–1037.
[88] M.I. Geli, A. Wesp, H. Riezman, Distinct functions of calmodulin are required for the uptake step of receptor-mediated endocytosis in yeast: the type I myosin Myo5p is one of the calmodulin targets, EMBO J. 17 (1998) 635–647.
[89] S.E. Brockerhoff, T.N. Davis, Calmodulin concentrates at regions of cell growth in *Saccharomyces cerevisiae*, J. Cell Biol. 118 (1992) 619–629.
[90] F. Fazioli, L. Minichiello, B. Maťošková, W.T. Wong, P.P. Di Fiore, eps15, a novel tyrosine kinase substrate, exhibits transforming activity, Mol. Cell. Biol. 13 (1993) 5814–5828.
[91] W.T. Wong, C. Schumacher, A.E. Salcini, A. Romano, P. Castagnino, P.G. Pelicci, P.P. Di Fiore, A protein-binding domain, EH, identified in the receptor tyrosine kinase substrate Eps15 and conserved in evolution, Proc. Natl. Acad. Sci. USA 92 (1995) 9530–9534.
[92] H.Y. Tang, A. Munn, M. Cai, EH domain proteins Pan1p and End3p are components of a complex that plays a dual role in organization of the cortical actin cytoskeleton and endocytosis in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 17 (1997) 4294–4304.
[93] H.Y. Tang, M. Cai, The EH-domain-containing protein Pan1 is required for normal organization of the actin cytoskeleton in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 16 (1996) 4897–4914.
[94] B. Wendland, J.M. McCaffery, Q. Xiao, S.D. Emr, A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15, J. Cell Biol. 135 (1996) 1485–1500.
[95] G. Zeng, M. Cai, Regulation of the actin cytoskeleton organization in yeast by a novel serine/threonine kinase Prk1p, J. Cell Biol. 144 (1999) 71–82.
[96] D.A. Holtzman, S. Yang, D.G. Drubin, Synthetic-lethal interactions identify two novel genes, *SLA1* and *SLA2*, that

control membrane cytoskeleton assembly in *Saccharomyces cerevisiae*, J. Cell Biol. 122 (1993) 635–644.
[97] R. Li, Y. Zheng, D.G. Drubin, Regulation of cortical actin cytoskeleton assembly during polarized cell growth in budding yeast, J. Cell Biol. 128 (1995) 599–615.
[98] T.S. Karpova, J.G. McNally, S.L. Moltz, J.A. Cooper, Assembly and function of the actin cytoskeleton of yeast: relationships between cables and patches, J. Cell Biol. 142 (1998) 1501–1517.
[99] R. Li, Bee1, a yeast protein with homology to Wiskott–Aldrich syndrome protein, is critical for the assembly of cortical actin cytoskeleton, J. Cell Biol. 136 (1997) 649–658.
[100] S.F. Donnelly, M.J. Pocklington, D. Pallotta, E. Orr, A proline-rich protein, verprolin, involved in cytoskeletal organization and cellular growth in the yeast *Saccharomyces cerevisiae*, Mol. Microbiol. 10 (1993) 585–596.
[101] G. Vaduva, N.C. Martin, A.K. Hopper, Actin-binding verprolin is a polarity development protein required for the morphogenesis and function of the yeast actin cytoskeleton, J. Cell Biol. 139 (1997) 1821–1833.
[102] M. Crouzet, M. Urdaci, L. Dulau, M. Aigle, Yeast mutant affected for viability upon nutrient starvation: characterization and cloning of the *RVS161* gene, Yeast 7 (1991) 727–743.
[103] F. Bauer, M. Urdaci, M. Aigle, M. Crouzet, Alteration of a yeast SH3 protein leads to conditional viability with defects in cytoskeletal and budding patterns, Mol. Cell. Biol. 13 (1993) 5070–5084.
[104] P. Sivadon, F. Bauer, M. Aigle, M. Crouzet, Actin cytoskeleton and budding pattern are altered in the yeast *rvs161* mutant: the Rvs161 protein shares common domains with the brain protein amphiphysin, Mol. Gen. Genet. 246 (1995) 485–495.
[105] B. Lichte, R.W. Veh, H.E. Meyer, M.W. Kilimann, Amphiphysin, a novel protein associated with synaptic vesicles, EMBO J. [Erratum: EMBO J. 11 (1992) 3809] 11 (1992) 2521–2530.
[106] A. Balguerie, P. Sivadon, M. Bonneu, M. Aigle, Rvs167p, the budding yeast homolog of amphiphysin, colocalizes with actin patches, J. Cell Sci. 112 (1999) 2529–2537.
[107] P. Navarro, P. Durrens, M. Aigle, Protein–protein interaction between the *RVS161* and *RVS167* gene products of *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1343 (1997) 187–192.
[108] D.C. Amberg, E. Basart, D. Botstein, Defining protein interactions with yeast actin in vivo, Nat. Struct. Biol. 2 (1995) 28–35.
[109] V. Brizzio, A.E. Gammie, M.D. Rose, Rvs161p interacts with Fus2p to promote cell fusion in *Saccharomyces cerevisiae*, J. Cell Biol. 141 (1998) 567–584.
[110] D. Winter, A.V. Podtelejnikov, M. Mann, R. Li, The complex containing actin-related proteins Arp2 and Arp3 is required for the motility and integrity of yeast actin patches, Curr. Biol. [Erratum: Curr. Biol. 7 (1997) R593] 7 (1997) 519–529.
[111] C. Schaerer-Brodbeck, H. Riezman, Functional interactions between the p35 subunit of the Arp2/3 complex and calmodulin in yeast, Mol. Biol. Cell 11 (2000) 1113–1127.
[112] H.V. Goodson, B.L. Anderson, H.M. Warrick, L.A. Pon, J.A. Spudich, Synthetic lethality screen identifies a novel yeast myosin I gene (MYO5): myosin I proteins are required for polarization of the actin cytoskeleton, J. Cell Biol. 133 (1996) 1277–1291.
[113] B.L. Anderson, I. Boldogh, M. Evangelista, C. Boone, L.A. Greene, L.A. Pon, The Src homology domain 3 (SH3) of a yeast type I myosin, Myo5p, binds to verprolin and is required for targeting to sites of actin polarization, J. Cell Biol. 141 (1998) 1357–1370.
[114] B. Zanolari, S. Friant, K. Funato, C. Sütterlin, B.J. Stevenson, H. Riezman, Sphingoid base synthesis requirement for endocytosis in *Saccharomyces cerevisiae*, EMBO J. 19 (2000) 2824–2833.
[115] K.R. Ayscough, J. Stryker, N. Pokala, M. Sanders, P. Crews, D.G. Drubin, High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A, J. Cell Biol. 137 (1997) 399–416.
[116] T.A. Gottlieb, I.E. Ivanov, M. Adesnik, D.D. Sabatini, Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells, J. Cell Biol. 120 (1993) 695–710.
[117] R.G. Parton, B. Joggerst, K. Simons, Regulated internalization of caveolae, J. Cell Biol. 127 (1994) 1199–1215.
[118] M. Deckert, M. Ticchioni, A. Bernard, Endocytosis of GPI-anchored proteins in human lymphocytes: role of glycolipid-based domains, actin cytoskeleton, and protein kinases, J. Cell Biol. 133 (1996) 791–799.
[119] C. Lamaze, L.M. Fujimoto, H.L. Yin, S.L. Schmid, The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells, J. Biol. Chem. 272 (1997) 20332–20335.
[120] O. Shupliakov, P. Löw, D. Grabs, H. Gad, H. Chen, C. David, K. Takei, P. De Camilli, L. Brodin, Synaptic vesicle endocytosis impaired by disruption of dynamin–SH3 domain interactions, Science 276 (1997) 259–263.
[121] C. David, P.S. McPherson, O. Mundigl, P. De Camilli, A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals, Proc. Natl. Acad. Sci. USA 93 (1996) 331–335.
[122] D. Grabs, V.I. Slepnev, Z. Songyang, C. David, M. Lynch, L.C. Cantley, P. De Camilli, The SH3 domain of amphiphysin binds the proline-rich domain of dynamin at a single site that defines a new SH3 binding consensus sequence, J. Biol. Chem. 272 (1997) 13419–13425.
[123] P. Wigge, Y. Vallis, H.T. McMahon, Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain, Curr. Biol. 7 (1997) 554–560.
[124] K. Takei, V. Haucke, V. Slepnev, K. Farsad, M. Salazar, H. Chen, P. De Camilli, Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes, Cell 94 (1998) 131–141.
[125] K. Takei, V.I. Slepnev, V. Haucke, P. De Camilli, Func-

tional partnership between amphiphysin and dynamin in clathrin-mediated endocytosis, Nat. Cell Biol. 1 (1999) 33–39.
[126] A.R. Ramjaun, P.S. McPherson, Multiple amphiphysin II splice variants display differential clathrin binding: identification of two distinct clathrin-binding sites, J. Neurochem. 70 (1998) 2369–2376.
[127] M.H. Butler, C. David, G.C. Ochoa, Z. Freyberg, L. Daniell, D. Grabs, O. Cremona, P. De Camilli, Amphiphysin II (SH3P9; BIN1), a member of the amphiphysin/Rvs family, is concentrated in the cortical cytomatrix of axon initial segments and nodes of Ranvier in brain and around T tubules in skeletal muscle, J. Cell Biol. 137 (1997) 1355–1367.
[128] A. Benmerah, J. Gagnon, B. Bègue, B. Mégarbané, A. Dautry-Varsat, N. Cerf-Bensussan, The tyrosine kinase substrate eps15 is constitutively associated with the plasma membrane adaptor AP-2, J. Cell Biol. 131 (1995) 1831–1838.
[129] A. Benmerah, B. Bègue, A. Dautry-Varsat, N. Cerf-Bensussan, The ear of α-adaptin interacts with the COOH-terminal domain of the Eps15 protein, J. Biol. Chem. 271 (1996) 12111–12116.
[130] F. Tebar, T. Sorkina, A. Sorkin, M. Ericsson, T. Kirchhausen, Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits, J. Biol. Chem. 271 (1996) 28727–28730.
[131] S. van Delft, C. Schumacher, W. Hage, A.J. Verkleij, P.M.P. van Bergen en Henegouwen, Association and colocalization of Eps15 with adaptor protein-2 and clathrin, J. Cell Biol. 136 (1997) 811–821.
[132] A. Benmerah, C. Lamaze, B. Bègue, S.L. Schmid, A. Dautry-Varsat, N. Cerf-Bensussan, AP-2/Eps15 interaction is required for receptor-mediated endocytosis, J. Cell Biol. 140 (1998) 1055–1062.
[133] A. Benmerah, M. Bayrou, N. Cerf-Bensussan, A. Dautry-Varsat, Inhibition of clathrin-coated pit assembly by an Eps15 mutant, J. Cell Sci. 112 (1999) 1303–1311.
[134] A.E. Salcini, S. Confalonieri, M. Doria, E. Santolini, E. Tassi, O. Minenkova, G. Cesareni, P.G. Pelicci, P.P. Di Fiore, Binding specificity and in vivo targets of the EH domain, a novel protein–protein interaction module, Genes Dev. 11 (1997) 2239–2249.
[135] H. Chen, S. Fre, V.I. Slepnev, M.R. Capua, K. Takei, M.H. Butler, P.P. Di Fiore, P. De Camilli, Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis, Nature 394 (1998) 793–797.
[136] B. Wendland, K.E. Steece, S.D. Emr, Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis, EMBO J. 18 (1999) 4383–4393.
[137] A.E. Engqvist-Goldstein, M.M. Kessels, V.S. Chopra, M.R. Hayden, D.G. Drubin, An actin-binding protein of the Sla2/Huntingtin interacting protein 1 family is a novel component of clathrin-coated pits and vesicles, J. Cell Biol. 147 (1999) 1503–1518.
[138] P.S. McPherson, E.P. Garcia, V.I. Slepnev, C. David, X. Zhang, D. Grabs, W.S. Sossin, R. Bauerfeind, Y. Nemoto, P. De Camilli, A presynaptic inositol-5-phosphatase, Nature 379 (1996) 353–357.
[139] E. de Heuvel, A.W. Bell, A.R. Ramjaun, K. Wong, W.S. Sossin, P.S. McPherson, Identification of the major synaptojanin-binding proteins in brain, J. Biol. Chem. 272 (1997) 8710–8716.
[140] K.D. Micheva, B.K. Kay, P.S. McPherson, Synaptojanin forms two separate complexes in the nerve terminal. Interactions with endophilin and amphiphysin, J. Biol. Chem. 272 (1997) 27239–27245.
[141] G. Cestra, L. Castagnoli, L. Dente, O. Minenkova, A. Petrelli, N. Migone, U. Hoffmüller, J. Schneider-Mergener, G. Cesareni, The SH3 domains of endophilin and amphiphysin bind to the proline-rich region of synaptojanin 1 at distinct sites that display an unconventional binding specificity, J. Biol. Chem. 274 (1999) 32001–32007.
[142] S.B. Snapper, F.S. Rosen, The Wiskott–Aldrich syndrome protein (WASP): roles in signaling and cytoskeletal organization, Annu. Rev. Immunol. 17 (1999) 905–929.
[143] J.M. Derry, H.D. Ochs, U. Francke, Isolation of a novel gene mutated in Wiskott–Aldrich syndrome [Erratum: Cell (1994) 79, following page 922], Cell 78 (1994) 635–644.
[144] H. Miki, K. Miura, T. Takenawa, N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases, EMBO J. 15 (1996) 5326–5335.
[145] H. Miki, S. Suetsugu, T. Takenawa, WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac, EMBO J. 17 (1998) 6932–6941.
[146] L.M. Machesky, R.H. Insall, Scar1 and the related Wiskott–Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex, Curr. Biol. 8 (1998) 1347–1356.
[147] L.M. Machesky, R.D. Mullins, H.N. Higgs, D.A. Kaiser, L. Blanchoin, R.C. May, M.E. Hall, T.D. Pollard, Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex, Proc. Natl. Acad. Sci. USA 96 (1999) 3739–3744.
[148] J. Zhang, A. Shehabeldin, L.A. da Cruz, J. Butler, A.K. Somani, M. McGavin, I. Kozieradzki, A.O. dos Santos, A. Nagy, S. Grinstein, J.M. Penninger, K.A. Siminovitch, Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott–Aldrich syndrome protein-deficient lymphocytes, J. Exp. Med. 190 (1999) 1329–1342.
[149] B. Qualmann, J. Roos, P.J. DiGregorio, R.B. Kelly, Syndapin I, a synaptic dynamin-binding protein that associates with the neural Wiskott–Aldrich syndrome protein, Mol. Biol. Cell 10 (1999) 501–513.
[150] F. Simpson, N.K. Hussain, B. Qualmann, R.B. Kelly, B.K. Kay, P.S. McPherson, S.L. Schmid, SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation, Nat. Cell Biol. 1 (1999) 119–124.
[151] B. Qualmann, R.B. Kelly, Syndapin isoforms participate in

receptor-mediated endocytosis and actin organization, J. Cell Biol. 148 (2000) 1047–1062.
[152] N. Ramesh, I.M. Antón, J.H. Hartwig, R.S. Geha, WIP, a protein associated with Wiskott–Aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells, Proc. Natl. Acad. Sci. USA 94 (1997) 14671–14676.
[153] G. Vaduva, N. Martinez-Quiles, I.M. Anton, N.C. Martin, R.S. Geha, A.K. Hopper, N. Ramesh, The human WASP-interacting protein, WIP, activates the cell polarity pathway in yeast, J. Biol. Chem. 274 (1999) 17103–17108.
[154] K.G. Rothberg, Y.S. Ying, B.A. Kamen, R.G.W. Anderson, Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate, J. Cell Biol. 111 (1990) 2931–2938.
[155] K.G. Rothberg, J.E. Heuser, W.C. Donzell, Y.S. Ying, J.R. Glenney, R.G.W. Anderson, Caveolin, a protein component of caveolae membrane coats, Cell 68 (1992) 673–682.
[156] J.E. Schnitzer, P. Oh, E. Pinney, J. Allard, Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules, J. Cell Biol. 127 (1994) 1217–1232.
[157] T. Harder, K. Simons, Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains, Curr. Opin. Cell Biol. 9 (1997) 534–542.
[158] D. Hailstones, L.S. Sleer, R.G. Parton, K.K. Stanley, Regulation of caveolin and caveolae by cholesterol in MDCK cells, J. Lipid Res. 39 (1998) 369–379.
[159] R.G. Parton, Caveolae and caveolins, Curr. Opin. Cell Biol. 8 (1996) 542–548.
[160] R.G. Parton, Ultrastructural localization of gangliosides; GM_1 is concentrated in caveolae, J. Histochem. Cytochem. 42 (1994) 155–166.
[161] W.J. Chang, K.G. Rothberg, B.A. Kamen, R.G.W. Anderson, Lowering the cholesterol content of MA104 cells inhibits receptor-mediated transport of folate, J. Cell Biol. 118 (1992) 63–69.
[162] D.P. Cerneus, E. Ueffing, G. Posthuma, G.J. Strous, A. van der Ende, Detergent insolubility of alkaline phosphatase during biosynthetic transport and endocytosis. Role of cholesterol, J. Biol. Chem. 268 (1993) 3150–3155.
[163] P.A. Orlandi, P.H. Fishman, Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains, J. Cell Biol. 141 (1998) 905–915.
[164] S.K. Rodal, G. Skretting, Ø. Garred, F. Vilhardt, B. van Deurs, K. Sandvig, Extraction of cholesterol with methyl-β-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles, Mol. Biol. Cell 10 (1999) 961–974.
[165] A. Subtil, I. Gaidarov, K. Kobylarz, M.A. Lampson, J.H. Keen, T.E. McGraw, Acute cholesterol depletion inhibits clathrin-coated pit budding, Proc. Natl. Acad. Sci. USA 96 (1999) 6775–6780.
[166] T.T. Cao, R.W. Mays, M. von Zastrow, Regulated endocytosis of G-protein-coupled receptors by a biochemically and functionally distinct subpopulation of clathrin-coated pits, J. Biol. Chem. 273 (1998) 24592–24602.
[167] N. Signoret, J. Oldridge, A. Pelchen-Matthews, P.J. Klasse, T. Tran, L.F. Brass, M.M. Rosenkilde, T.W. Schwartz, W. Holmes, W. Dallas, M.A. Luther, T.N. Wells, J.A. Hoxie, M. Marsh, Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4, J. Cell Biol. 139 (1997) 651–664.
[168] J. Mulholland, D. Preuss, A. Moon, A. Wong, D. Drubin, D. Botstein, Ultrastructure of the yeast actin cytoskeleton and its association with the plasma membrane, J. Cell Biol. 125 (1994) 381–391.
[169] J. Mulholland, J. Konopka, B. Singer-Krüger, M. Zerial, D. Botstein, Visualization of receptor-mediated endocytosis in yeast, Mol. Biol. Cell 10 (1999) 799–817.
[170] M.D. Welch, The world according to Arp: regulation of actin nucleation by the Arp2/3 complex, Trends Cell Biol. 9 (1999) 423–427.
[171] M.D. Welch, A. Iwamatsu, T.J. Mitchison, Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria monocytogenes*, Nature 385 (1997) 265–269.
[172] R. Rohatgi, L. Ma, H. Miki, M. Lopez, T. Kirchhausen, T. Takenawa, M.W. Kirschner, The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly, Cell 97 (1999) 221–231.
[173] D. Winter, T. Lechler, R. Li, Activation of the yeast Arp2/3 complex by Bee1p, a WASP-family protein, Curr. Biol. 9 (1999) 501–504.
[174] M. Symons, J.M.J. Derry, B. Karlak, S. Jiang, V. Lemahieu, F. McCormick, U. Francke, A. Abo, Wiskott–Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization, Cell 84 (1996) 723–734.
[175] P. Aspenström, U. Lindberg, A. Hall, Two GTPases, Cdc42 and Rac, bind directly to a protein implicated in the immunodeficiency disorder Wiskott–Aldrich syndrome, Curr. Biol. 6 (1996) 70–75.
[176] R. Kolluri, K.F. Tolias, C.L. Carpenter, F.S. Rosen, T. Kirchhausen, Direct interaction of the Wiskott–Aldrich syndrome protein with the GTPase Cdc42, Proc. Natl. Acad. Sci. USA 93 (1996) 5615–5618.
[177] H. Miki, T. Sasaki, Y. Takai, T. Takenawa, Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP, Nature 391 (1998) 93–96.
[178] C.J. Merrifield, S.E. Moss, C. Ballestrem, B.A. Imhof, G. Giese, I. Wunderlich, W. Almers, Endocytic vesicles move at the tips of actin tails in cultured mast cells, Nat. Cell Biol. 1 (1999) 72–74.
[179] A.L. Rozelle, L.M. Machesky, M. Yamamoto, M.H. Driessens, R.H. Insall, M.G. Roth, K. Luby-Phelps, G. Marriott, A. Hall, H.L. Yin, Phosphatidylinositol 4,5 bisphosphate induces actin based movement of raft enriched vesicles through WASP Arp2/3, Curr. Biol. 10 (2000) 311–320.
[180] T. Lechler, A. Shevchenko, A. Shevchenko, R. Li, Direct

involvement of yeast type I myosins in Cdc42-dependent actin polymerization, J. Cell Biol. 148 (2000) 363–373.
[181] M. Evangelista, B.M. Klebl, A.H.Y. Tong, B.A. Webb, T. Leeuw, E. Leberer, M. Whiteway, D.Y. Thomas, C. Boone, A role for myosin-I in actin assembly through interactions with Vrp1p, Bee1p, and the Arp2/3 complex, J. Cell Biol. 148 (2000) 353–362.
[182] M.I. Geli, R. Lombardi, B. Schmelzl, H. Riezman, An intact SH3 domain is required for myosin I-induced actin polymerization, EMBO J. 19 (2000) 4281–4291.
[183] L.M. Fujimoto, R. Roth, J.E. Heuser, S.L. Schmid, Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells, Traffic 1 (2000) 161–171.