Actin and Endocytosis in Budding Yeast

Bruce L. Goode,*,1 Julian A. Eskin,* and Beverly Wendland†,1

*Brandeis University, Department of Biology, Rosenstiel Center, Waltham, Massachusetts 02454, and †The Johns Hopkins University, Department of Biology, Baltimore, Maryland 21218

ABSTRACT Endocytosis, the process whereby the plasma membrane invaginates to form vesicles, is essential for bringing many substances into the cell and for membrane turnover. The mechanism driving clathrin-mediated endocytosis (CME) involves > 50 different protein components assembling at a single location on the plasma membrane in a temporally ordered and hierarchal pathway. These proteins perform precisely choreographed steps that promote receptor recognition and clustering, membrane remodeling, and force-generating actin-filament assembly and turnover to drive membrane invagination and vesicle scission. Many critical aspects of the CME mechanism are conserved from yeast to mammals and were first elucidated in yeast, demonstrating that it is a powerful system for studying endocytosis. In this review, we describe our current mechanistic understanding of each step in the process of yeast CME, and the essential roles played by actin polymerization at these sites, while providing a historical perspective of how the landscape has changed since the preceding version of the *YeastBook* was published 17 years ago (1997). Finally, we discuss the key unresolved issues and where future studies might be headed.

KEYWORDS *S. cerevisiae*; Arp2/3 complex; clathrin; membrane; endocytosis

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ENDOCYTOSIS is a key mechanism that regulates plasma membrane composition and thus fundamentally supports cellular physiology and homeostasis. It is the process by which cells collect extracellular material and plasma membrane-associated surface proteins, such as receptors, channels, and signaling proteins, and package them into vesicles that are pinched off and enter the cytosol (Figure 1). Internalized vesicles then fuse with other internal compartments so that their contents can be recycled or degraded.

In most steps of intracellular traffic, including endocytosis, proteins (referred to as "cargo") move between intracellular organelles and the extracellular milieu via formation of vesicles or tubules. This process begins when transmembrane cargo recruit cytosolic proteins to the membrane surface. These proteins then assemble in a temporally ordered pattern suggesting layers or coats (Figure 1). The inner "adaptor" layer of coat proteins binds to sorting signals in the cytosolic portions of the transmembrane cargo proteins to collect and concentrate the cargo and also helps to recruit and organize subsequent outer coat layers. Among the earliest proteins recruited to sites of vesicle formation are the namesake "coat" proteins (e.g., clathrin, COPI, COPII), which polymerize into spherical cages of different sizes to promote vesicle budding. A final set of components acts on the membrane itself to facilitate scission and release of the vesicle into the cytosol (Figure 1).

Clathrin-mediated endocytosis (CME) is the best characterized of all the endocytic pathways and requires a large number of cytosolic proteins that concentrate transmembrane cargo proteins and form clathrin-coated vesicles (McMahon and Boucrot 2011). In CME, adaptor proteins recognize sorting signals in the cargo proteins and directly link the cargo to clathrin. The basic subunit of clathrin is a triskelion, which is a trimer of clathrin heavy chains, each with an associated clathrin light chain. The controlled formation of a dense and branched actin-filament network provides forces critical for driving subsequent membrane tubulation and vesicle scission (Figure 1). Although there is an absolute requirement for dynamic rearrangements of the actin cytoskeleton to support CME in yeast, there is a more variable requirement in animal cells, discussed below.

Our goal in this review is to summarize the current understanding of endocytosis and its relationship with actin dynamics in budding yeast, while highlighting the major advances that have been made since publication of chapters on this topic in the last edition of the YeastBook (Botstein et al. 1997; Kaiser et al. 1997), and finally to point out some of the remaining open questions in this field. We hope that this information will be useful to newcomers and experts alike and to those not directly working on endocytosis or actin biology. Looking back, it becomes strikingly evident that in 1997 our knowledge was in its infancy and that major leaps have since been made in our understanding of actin and endocytosis. There is now a wealth of information on the >50 components of endocytic sites, the temporal order of their arrival, their biochemical activities, physical

interactions, and relative abundances, along with recent ultrastructural analysis clarifying the successive steps in endocytic internalization of vesicles from the plasma membrane. While much of this work was initially pioneered in *Saccharomyces cerevisiae*, many critical insights as well as important quantitative information (e.g., numbers of molecules per actin patch and mathematical modeling of actin networks; see below) have been contributed by studies in *Schizosaccharomyces pombe* (e.g., Berro et al. 2010; Sirotkin et al. 2010; Arasada and Pollard 2011). We attempt to compile all of this information together into a working model for the formation and internalization of endocytic vesicles. We apologize to all those whose work we did not highlight. Even in a review of this length, it was not possible to discuss this subject and its literature base comprehensively, and we were faced with difficult choices regarding where to focus. However, we refer readers to a series of excellent recent reviews on actin and endocytosis that provide complementary information and more in-depth discussion of specific areas that we do not (Boettner et al. 2012; Mooren et al. 2012; Weinberg and Drubin 2012; Idrissi and Geli 2014; Schmid et al. 2014).

Cortical actin patches represent the final stages of endocytic internalization

It is now clear that the plasma membrane-associated structures referred to as "cortical actin patches" correspond to sites of endocytosis in the late (actin-dependent) stages of vesicle formation. However, in 1997, the equivalency of actin patches to endocytic sites had not yet been established. A few proteins genetically linked to endocytosis and actin were known to localize to cortical sites on the plasma membrane (e.g., Abp1 and Sac6) (Drubin et al. 1988), and immuno-electron microscopy (immuno-EM) had suggested an association between these proteins and membrane invaginations (Figure 3, A and B), leading to preliminary models for how actin might contribute to endocytosis (Mulholland et al. 1994) (Figure 3C). However at the time, there was minimal understanding of the ultrastructure, dynamics, or function of these actin structures.

An important breakthrough came in 1996, when two studies used live-cell imaging with green fluorescent protein (GFP) fusions to study actin patch dynamics (Doyle and Botstein 1996; Waddle et al. 1996). Both groups concluded that cortical actin patches move within the plane of the membrane, in some cases for long distances. It was suggested that individual patches might assemble *de novo* and later disassemble, but it was difficult to gather further evidence for this model, given technological limitations; e.g., the intervals in time-lapse imaging were too long to permit accurate tracking of individual actin patches. These early studies set the stage for subsequent paradigm-shifting discoveries, in which two-color live-cell imaging of pairs of cortical patch components revealed that endocytic and actin machinery colocalize but arrive at sites of endocytosis at different times (Kaksonen et al. 2003). It remained unclear though, how actin assembly was initiated at endocytic sites and how actin organization
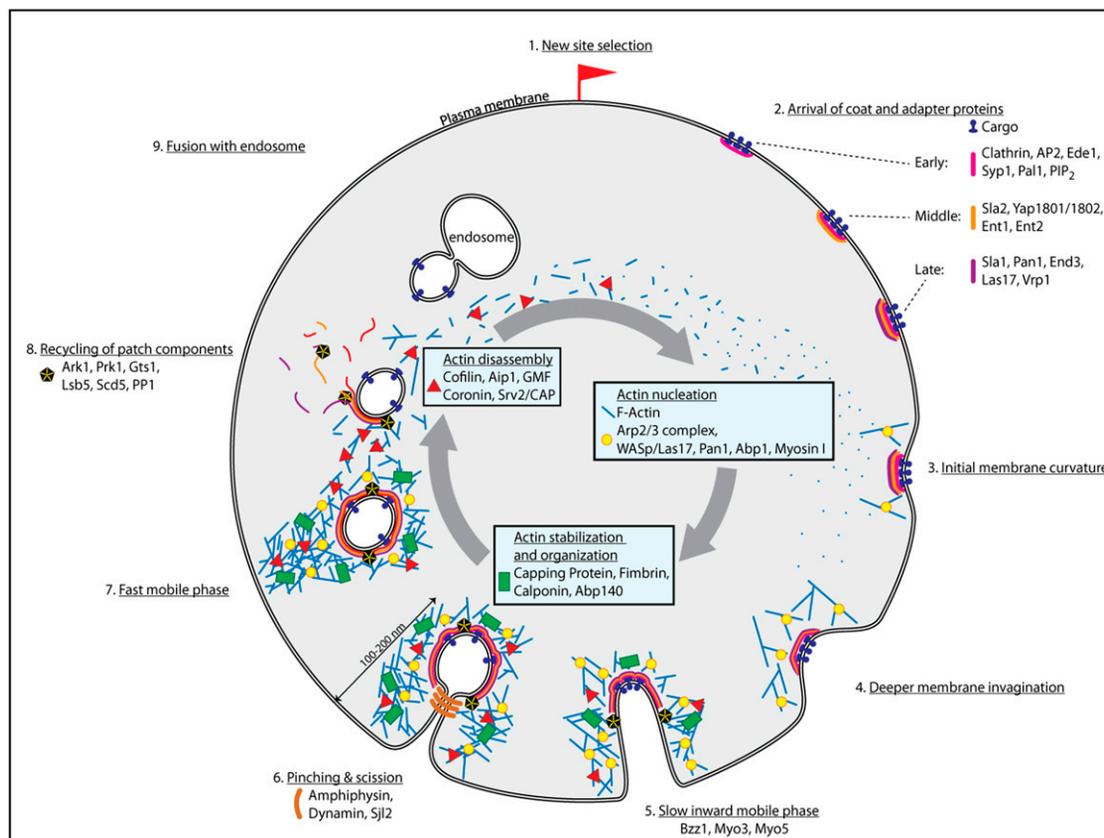
Figure 1 Stages of endocytic patch initiation, maturation, and turnover. The stages of progression (numbered 1–9) of an endocytic patch from initiation to membrane invagination, followed by scission and disassembly/uncoating, make components available for new rounds of patch formation (recycling). Boxes describe the concurrent sequential behavior of the actin cytoskeleton and actin-associated proteins during this process. Each icon represents a group of proteins with related functions, activities, and/or localization.

and dynamics were controlled. Efforts to connect actin with endocytosis were further confounded by unresolved differences in the molecular requirements for animal *vs.* yeast endocytosis, including inconsistent requirements for clathrin, the scission-promoting GTPase dynamin, and actin itself.

Genetic screens for endocytosis mutants

An obvious strength of the yeast system is the ability to conduct forward genetic screens to identify the genes involved in a cellular process. Depending on how the screen is performed, different types of genes will be identified while others will be missed (*e.g.*, redundant gene pairs). A wide variety of genetic screens designed to identify factors important for endocytosis have been conducted over the years, with a subset of factors being found in more than one screen (Table 1). These screens have provided the platform for many subsequent reverse genetic and biochemical studies dissecting the roles of candidate genes/proteins in endocytosis and defining their physical and genetic interaction partners. Together, these approaches have yielded a rich set of information on the endocytic machinery in budding yeast (Table 2). Below, we describe several of the early studies that identified endocytic factors, which illustrates the value in performing a variety of different genetic screens to probe a single process.

The first set of endocytosis mutants came from a collection of temperature-sensitive yeast strains produced by random EMS mutagenesis. The Riezman lab initially tested this collection for strains deficient in uptake of the bulk fluid phase marker Lucifer Yellow, identifying the *end1* and *end2* mutants (Chvatchko *et al.* 1986); however, these mutations were later found to have no direct effect on the internalization process (Dulic and Riezman 1989; Hamburger *et al.* 1995). After switching to measuring uptake of radiolabeled α-factor (a pheromone peptide secreted by yeast), the first *bona fide* yeast endocytosis mutants, *end3* and *end4*, were discovered from the same strain collection (Raths *et al.* 1993). Further screening identified *end5*, *end6*, and *end7* (Munn *et al.* 1995). Interestingly, *end7* is an allele of the *ACT1* (actin) gene, which had been shown previously to be defective in endocytosis (Kubler and Riezman 1993). In addition, the proteins encoded by *END3–END7* were later shown to localize to cortical actin patches (Benedetti *et al.* 1994; Vaduva *et al.* 1997; Wesp *et al.* 1997; Huh *et al.* 2003).

Another screen for endocytosis mutants was based on the observation that strains carrying a deletion of a vacuolar ATPase subunit such as *vma2Δ* grow only on low-pH media. It was speculated that the vacuoles of these mutants become acidified via the endocytic pathway (Nelson and Nelson

Table 1 Genes identified in screens for endocytosis mutants

Gene	Official name:	Also known as	Screen	Reference
END1	PEP5	VAM1, VPL9, VPS11, VPT11, YMR231w	Lucifer yellow internalization defect (EMS mutagenesis)	Chvatchko et al. (1986)
END2	GAA1	YLR088w		
END3	END3	YNL084c	α-Factor uptake defect (EMS mutagenesis)	Raths et al. (1993)
END4	SLA2	MOP2, YNL243w, RCB432		
END5	VRP1	MDP2, YLR337w		Munn et al. (1995)
END6	RVS161	FUS7, SPE161, YCR009c		
END7	ACT1	ABY1, YFL039c		
END8	LCB1	TSC2, YMR296c	Synthetic lethal with V-ATPase mutation (EMS mutagenesis)	Munn and Riezman (1994)
END9	ARC35	YNR035c		
END10	Unknown	Unknown		
END11	ERG2	YMR202w		
END12	VPS34	PEP15, VPL7, VPT29, STT8, VPS7, YLR240w		
END13	VPS4	CSC1, GRD13, VPL4, VPT10, DID6, YPR173c		
DIM1	SHE4	YOR035c	FM4-64 uptake defect (EMS mutagenesis)	Wendland et al. (1996)
DIM2	PAN1	MDP3, MIP3, YIR006c		
DIM4	FAB1	SVL7, YFR019w		
SYS1	SYS1	YJL004c	Reduced Lucifer Yellow accumulation (Nonessential gene deletion collection)	Shaw et al. (2003)
YNL177c	MRPL22	YNL177c		Wiederkehr et al. (2001)
YNL227c	JJ1	YNL227c		
EDE1	EDE1	BUD15, YBL047c		
TLG2	TLG2	YOL018c		
RCY1	RCY1	YJL204c		
YNR075w	COS10	YNR075w		
SDS24	SDS24	YBR214w		
YDR036c	EHD3	YDR036c		
FTH1	FTH1	YBR207w		
YBR266c	REI1?	YBR267w? (YBR266c = dubious ORF)		
YDL231c	BRE4	YDL231c		
YNL297c	MON2	YSL2, YNL297c		
VPS53	VPS53	YJL029c		
YPK1	YPK1	SLI2, YKL126w	Ubiquitin-dependent internalization of alpha-factor (EMS mutagenesis of strain with Ste2-378Stop)	Dehart et al. (2002)
TOR2	TOR2	DRR2, YKL203c		Dehart et al. (2003)

A partial list of hits from the screen (only genes predicted to be involved in endocytosis)

Gene	Official name	Also known as	Rank	Screen	Reference
RVS161	RVS161	END6, FUS7, SPE161, YCR009c	1	Elevated Snc1 levels at plasma membrane (Nonessential gene deletion collection)	Burston et al. (2009)
VRP1	VRP1	END5, MDP2, YLR337w	4		
RVS167	RVS167	YDR388w	8		
LDB17	LDB17	YDL146w	10		
SLA1	SLA1	YBL007c	12		
ARC18	ARC18	YLR370c	18		
YAP1801	YAP1801	YHR161c	29		
EDE1	EDE1	BUD15, YBL047c	31		
YAP1802	YAP1802	YGR241c	42		
CAP1	CAP1	YKL007w	45		

(continued)

Table 1, *continued*

Gene	Official name:	Also known as	Screen	Reference
TPM1	TPM1	YNL079c	52	
ABP1	ABP1	YCR088w	54	
MYO5	MYO5	YMR109w	61	
INP52	INP52	SIL2, YNL106c	68	
AIM21	AIM21	YIR003w	78	
CAP2	CAP2	YIL034c	127	
TWF1	TWF1	YGR080w	155	
SAC6	SAC6	ABP67, YDR129c	209	
SYP1	SYP1	YCR030c, YCR029c-A	216	
BBC1	BBC1	MTI1, YJL021c	231	
ENT1	ENT1	YDL161w	281	
END3	END3	YNL084c	315	
LSB3	LSB3	YFR024c-A, YFR024c	338	
CRN1	CRN1	YLR429w	365	
AIP1	AIP1	YMR092c	368	
BZZ1	BZZ1	LSB7, YHR114w	383	
PKH2	PKH2	YOL100w	396	

A partial list of hits from the screen (only those genes associated with endocytosis based on gene ontology are included)

Gene	Official name:	Also known as	Screen	Reference
APL1	APL1	YAP80, YJR005w	Killer toxin resistance	Carroll et al. (2009)
APL3	APL3	YBL037w	(Nonessential gene deletion + ts allele collection)	
APM4	APM4	AMP1, YOL062c		
APS2	APS2	YAP17, YJR058c		
EDE1	EDE1	BUD15, YBL047c		
SLA1	SLA1	YBL007c		
SLA2	SLA2	END4, MOP2, YNL243w, RCB432		
END3	END3	YNL084c		
SHE4	SHE4	DIM1, YOR035c		
VRP1	VRP1	END5, MDP2, YLR337w		
MYO5	MYO5	YMR109w		
ABP1	ABP1	YCR088w		
ARC18	ARC18	YLR370c		
SAC6	SAC6	ABP67, YDR129c		
RVS161	RVS161	END6, FUS7, SPE161, YCR009c		
RVS167	RVS167	YDR388w		
APP1	APP1	YNL094w		
SRV2	SRV2	CAP, YNL138w		
DRS2	DRS2	FUN38, SWA3, YAL026c		
YPK1	YPK1	SLI2, YKL126w		
ARV1	ARV1	YLR242c		

Table 2 Yeast endocytosis proteins, partners, domains and mammalian homologs

Yeast protein	Physical interaction partners	Key domains, *motifs*	Homolog, common name[a]
	Early coat proteins		
Ede1	Syp1, Yap1801/2, Ent1/2	EH, CC, UBA	Eps15
Syp1	Ede1	F-BAR, μHD, PRD	FCHO1/2
Chc1	Clc1, Yap1801/2, Ent1/2, Sla1	β-propeller	Clathrin heavy chain
Clc1	Chc1, Sla2		Clathrin light chain
Pal1	Ede1	NPF	Unknown
Apl1	Apl3, Apm4, Aps2		AP2 complex β2 subunit
Apl3	Apl1, Apm4, Aps2		AP2 complex α subunit
Apm4	Apl1, Apl3, Aps2		AP2 complex μ2 subunit
Aps2	Apl1, Apl3, Apm4	μ	AP2 complex σ2 subunit
	Middle coat proteins		
Sla2	Sla1, End3, Pan1, Clc1, Act1	ANTH, CC, THATCH	Hip1R, Hip1
Yap1801, Yap1802	Pan1, Ede1, Chc1	ANTH, *NPF, CBM*	CALM, AP180
Ent1, Ent2	Pan1, Ede1, Chc1, ubiquitin	ENTH, *UIM, NPF, CBM*	Epsin
	Late coat proteins		
Sla1	Pan1, End3, Sla2, Chc1, Scd5	SH3, SHD1, SHD2, SR repeats, *CBM*	CIN85
Pan1	Sla1, End3, Sla2, Yap1801/2, Ent1/2, Myo3/5, Scd5	EH, CC, PRD, *WH2, acidic*	Intersectin
End3	Sla1, Pan1, Scd5	EH, CC	
Lsb3	Las17, Ldb17	SH3	SH3YL1a
Ysc84/Lsb4	Las17, Ldb17	SH3	SH3YL1a
Lsb5	Sla1, Las17	VHS, GAT	GGA?
WASP/Myosin I			
Las17	Vrp1, Sla1, Bbc1, Syp1, Lsb1-5, Bzz1, Myo3/5, Rvs167	WH1/EVH1, PRD, *WH2, acidic*	WASP/N-WASP
Vrp1	Las17, Myo3/5, Act1	PRD, *WH2*	WIP (WASP interacting protein)
Bzz1	Las17, Ldb17	F-BAR, SH3	Syndapin
Scd5	Glc7, Sla1, End3, Pan1		Unknown
Myo3, Myo5	Act1, Pan1, Cmd1, Pan1, Vrp1, Las17	Motor, TH1, TH2, SH3, *acidic*	Type I Myosin
Bbc1	Las17	SH3, PRD	Unknown
Ldb17	Sla1, Bzz1, Lsb3, Ysc84/Lsb4, Bem1	PRD	DIP/WISH/SPIN90
	Actin[b]		
Act1	Srv2, Bni1, Bnr1		Actin
Pfy1	Las17, Myo3/5, Pan1, Abp1, Gmf1, Crn1		Profilin
Arp2, Arp3, Arc15/18/19/35/40	Arp2/3, Rvs167, Aim3, Srv2, Scp1, Ark1, Prk1, Sjl2	ARP, β-propeller	Arp2/3 complex
Abp1	Abp1, Rvs167	ADFH, SH3, PRD, *acidic*	ABP1
Aim3		PRD, *NPF*	Unknown
Srv2/CAP	Cof1, Pfy1, Abp1, Twf1, Aip1	HFD, β-sheet, PRD, *WH2*	Cyclase associated protein
Ark1, Prk1, Akl1	Abp1	Ser/Thr kinase, PRD	AAK1, GAK
Cap1, Cap2	Twf1		Capping protein (αβ subunits[a])
Sac6		CH	Fimbrin
Scp1	Abp1	CH, PRD, *CLR*	Calponin/Transgelin
Abp140			Unknown
Cof1	Srv2, Crn1, Aip1	ADFH	Cofilin

(continued)

Yeast protein	Physical interaction partners	Key domains, motifs	Homolog, common name[a]
Twf1	Cap1/2, Srv2	ADFH	Twinfilin
Crn1	Arp2/3, Cof1	β-propeller, CC	Coronin
Gmf1	Arp2/3	ADFH	GMF (glial maturation factor)
Aip1	Cof1, Srv2	β-propeller	Aip1
Scission			
Rvs161	Rvs167	N-BAR	Amphiphysin
Rvs167	Rvs161, Abp1, Las17	N-BAR, SH3	Amphiphysin/endophilin
Vps1	Sla1	GTPase	Dynamin
Inp52/Sjl2	Abp1	phosphatase XYZ	Synaptojanin 1
Less-well-characterized proteins			
Lsb1	Las17	SH3	
Pin3/Lsb2		SH3	
Bsp1			Unknown
Gts1			Unknown
App1			Unknown
Ypp1			
Aim21			Unknown
Swf1			Palmitoyl transferase 1

[a] Any gene for which a clearly identifiable homolog is currently unknown is indicated as such, even if presently seems to be specific to fungi.
[b] Act1 is not listed as a binding partner for proteins in this subsection.

1990), and the Riezman lab found that combining an *end* mutant with *vma2Δ* led to inviability (*i.e.*, synthetic lethality). By performing EMS mutagenesis in a *vma2Δ* background, they isolated *end8* through *end13* as synthetic lethal mutations (Munn and Riezman 1994). The *END9* gene encodes a protein later recognized as the Arc35 subunit of the Arp2/3 complex (Machesky *et al.* 1994; Welch *et al.* 1997a), the first hint that branched actin structures are important for endocytosis (see *Mechanisms of Actin Assembly and Turnover*). This screen also highlighted the critical importance of membrane composition, since it identified enzymes that are required for synthesizing both sphingolipids (*END8/LCB1*) and the yeast cholesterol-like molecule ergosterol (*END11/ERG2*) (see *Vesicle Scission and Uncoating*).

A growing appreciation for the role of ubiquitin in endocytosis (Volland *et al.* 1994) led the Hicke lab to modify the original screen of random mutants defective for radiolabeled α-factor uptake. They mutagenized a strain expressing an α-factor receptor that entirely relied upon ubiquitin modification for its internalization (Terrell *et al.* 1998) to find ubiquitin-dependent internalization (*udi*) mutants. This approach yielded protein kinases Ypk1 and Tor2, which turned out to be required for both ubiquitin-dependent and -independent receptor internalization (Dehart *et al.* 2002; Dehart *et al.* 2003; Tenay *et al.* 2013).

Another marker for endocytosis is the fluorescent lipophilic (membrane-binding) dye FM4-64, which can be used to trace the endocytic pathway from the plasma membrane through endosomes to the vacuole (Vida and Emr 1995). EMS-mutagenized cells were subjected to a pulse/chase labeling with FM4-64, followed by flow cytometry selection of "dim" cells that had internalized the least amount of dye. This screen yielded the myosin chaperone She4/Dim1 and the EH domain scaffold protein Pan1/Dim2 (Wendland *et al.* 1996). A role for the lipid kinase Fab1 in late stages of the endocytic pathway was subsequently uncovered using the same approach (Shaw *et al.* 2003).

After the yeast genome was sequenced in 1996 (Goffeau *et al.* 1996), a strain collection was generated with deletions of nonessential genes, which provided the yeast community with another valuable tool for identifying endocytosis mutants. For example, a subset of these nonessential deletion strains was tested for deficiency in Lucifer Yellow accumulation, which identified the endosomal F-Box protein Rcy1, among others (Wiederkehr *et al.* 2000; Wiederkehr *et al.* 2001). Further, the Conibear lab screened several different gene deletion collections for strains with elevated plasma membrane levels of the v-SNARE protein Snc1 (Burston *et al.* 2009), an endocytic cargo that must be included in the membranes of secretory/endocytic vesicles for their delivery and fusion to the subsequent compartment (Gurunathan *et al.* 2000). This approach uncovered many of the expected genes, validating the general strategy, and identified the SH3-domain protein Ldb17 as an endocytosis protein. It also revealed quantitative endocytic defects for mutants in a number of actin binding proteins that reside on actin patches but had not previously

been linked functionally to endocytosis (*e.g.*, Aip1, Crn1, Gmf1). The Drubin lab then used a larger set of systematic gene deletions combined with temperature-sensitive alleles to screen for mutants that were resistant to the yeast killer toxin virus, some of which were resistant due to the inability to internalize the toxin (Carroll *et al.* 2009). While the toxin receptor remains undefined, an exciting discovery from this screen was a requirement for the yeast homolog of the AP-2 adaptor complex. Until this finding, an endocytic role for the yeast AP-2 complex had been lacking (Huang *et al.* 1999; Yeung *et al.* 1999).

Through the combined efforts of many labs, genetic screens, interaction studies, and biochemical analyses have uncovered the existence of dozens of proteins involved in endocytosis. Subsequent experimentation has shown that these components are sequentially recruited as early, middle, and late coats, leading to the formation of a colossal molecular assemblage that culminates in a burst of F-actin polymerization and the reshaping of plasma membrane into a vesicle. This multistep process is depicted in Figure 1 and described in more detail in *Cargo Selection and Coat Formation, Mechanisms of Actin Assembly and Turnover*, and *Vesicle Scission and Uncoating*.

Cargo Selection and Coat Formation

Clathrin was linked to endocytosis in animal cells in 1975 (Pearse 1975), but only in the last decade has a role for clathrin in yeast become fully accepted (Kaksonen *et al.* 2005; Newpher *et al.* 2005). Whether budding yeast had a CME pathway was initially fraught with controversy, beginning with confusion about the requirements of clathrin for viability. In one commonly used lab strain background, the clathrin heavy chain (*CHC1*) gene was found to be essential, while in another it was nonessential. Although the viable clathrin deletion cells were extremely sick (Lemmon and Jones 1987; Payne *et al.* 1987) they exhibited only partial endocytosis defects, retaining 30–50% uptake of mating pheromone (Payne *et al.* 1988). Later, using a *chc1* temperature-sensitive allele, it became possible to acutely disrupt clathrin function, which led to reduced endocytosis within 2 min of temperature shift (Tan *et al.* 1993). The chronic *chc1Δ* and acute *chc1-ts* endocytosis defects were similar, indicating that clathrin might play a direct role in endocytic internalization in yeast.

Two other issues fueled suspicions about the importance of clathrin for yeast endocytosis. First, the prevailing view was that the endocytic mechanisms used in yeast were likely to differ significantly from those used in mammalian cells (Baggett and Wendland 2001), since actin had been so closely linked to yeast endocytosis while apparently playing only a minor or specialized role in mammalian cells (Gottlieb *et al.* 1993; Fujimoto *et al.* 2000). Second, the majority of clathrin in yeast cells had been localized to large, concentrated intracellular punctae that are likely Golgi associated, with no apparent localization at the cell surface (Pishvaee *et al.* 2000). These two observations, along with the incomplete

block of internalization in clathrin mutants, led some investigators to argue against a direct role of clathrin in facilitating endocytosis in yeast.

Two key developments then led to the direct visualization of clathrin at the yeast plasma membrane. First, following on the pioneering work from the Keen and Almers labs (Gaidarov *et al.* 1999; Merrifield *et al.* 2002), Kaksonen *et al.* (2003) established the use of two-color live-cell imaging techniques in yeast to analyze the dynamics of pairs of components of cortical actin/endocytic patches. Through this approach, they made the landmark discovery that cortical patches form in discrete stages following a stereotypical, temporally ordered program in which endocytic components (including the clathrin heavy and light chains) are subsequently joined by actin and actin-associated proteins (Figure 1). Further, mutating the endocytic protein Sla2/End4 led to aberrantly long actin "comet tails" at endocytic sites, which allowed the measurement of both the rate of actin turnover (Kaksonen *et al.* 2003) and the relative positions of key actin regulatory proteins within the actin networks, *i.e.*, at the front, at the rear, or evenly distributed (Okreglak and Drubin 2007; Gandhi *et al.* 2009; Michelot *et al.* 2013). Next, the Lemmon lab found that *sla2Δ* mutants and/or treatment with the actin depolymerizing drug Latrunculin-A stabilized clathrin at the plasma membrane. In contrast, they found that clathrin adaptor mutants (two epsins and two AP180 proteins) had significantly diminished clathrin accumulation at the plasma membrane (Newpher *et al.* 2005). These observations were supported by the use of total internal reflection fluorescence (TIRF) microscopy, which reduced the signal from bright clathrin-positive internal structures and revealed the existence of transient patches of plasma membrane-associated clathrin in wild-type cells. Importantly, these clathrin patches subsequently recruited Abp1, a well-established component of both the endocytic machinery and actin cytoskeleton (Newpher *et al.* 2005). Thus, clathrin was finally accepted as an important and early arriving component of the endocytic machinery in yeast, and connected CME to the actin cytoskeleton.

Overview of the stages of yeast endocytosis

A major concept revealed by the above-mentioned studies and elaborated on in studies that followed was that an endocytic site develops or matures over time and that numerous proteins are sequentially recruited to this site and assembled into a structure that collects cargo and sculpts the plasma membrane into a vesicle. This process can be broadly divided, into three operational steps: (1) an immobile phase (Figure 1, steps 1–4; coat assembly), (2) a mobile phase (Figure 1, steps 5–7; actin assembly, membrane invagination, and vesicle scission), and (3) a patch dissolution phase (Figure 1, steps 8 and 9; vesicle uncoating and fusion with endosome). Note that uncoating of certain components may also be a prerequisite to scission.

For organizational purposes, the remainder of *Cargo Selection and Coat Formation* focuses on the immobile phase of
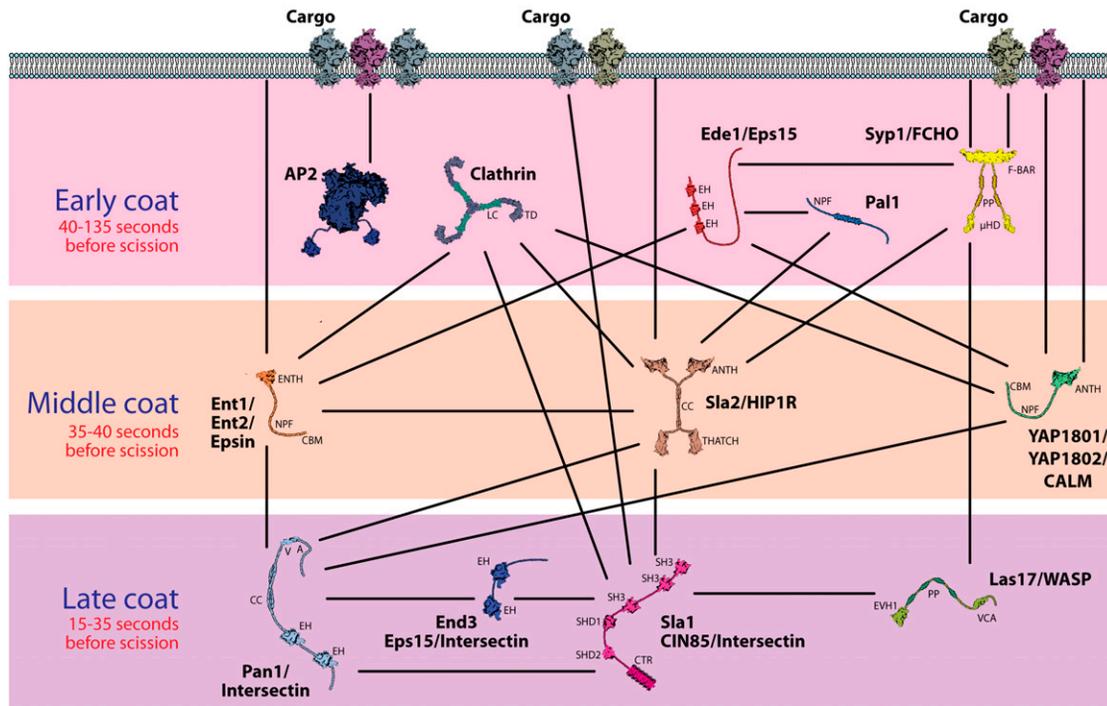
Figure 2 Endocytic adaptor and coat proteins. Depicted are the key factors in the three stages of endocytic coat formation, early, middle, and late coat proteins, which are recruited at distinct stages of endocytosis and thus represented as separate layers. Importantly, these represent temporal and not necessarily spatially segregated layers. Lines indicate known binding interactions among components. See *Cargo Selection and Coat Formation* for details. Domains abbreviations: LC, light chain; TD, terminal domain; EH, Eps15 homology domain; NPF, Asn–Pro–Phe motif; F-BAR, FER/Cip4 homology-Bin/Amphiphysin/Rvs domain; PP, polyproline region; μHD, μ homology domain; ENTH, Epsin N-terminal homology domain; CBM, clathrin-binding motif; ANTH, AP180 N-terminal homology domain; CC, coiled coil; THATCH, talin-HIP1/R/Sla2p actin-tethering C-terminal homology domain; V, verprolin homology motif; A, acidic motif ; SHD, Sla1-homology domain; SH3, Src homology 3 domain; CTR, C-terminal repeats; EVH1, Ena/VASP homology 1 domain; VCA, verprolin homology/connector/acidic motif.

patch assembly. We first discuss the selection of cargo proteins and then coat assembly (Figure 1, steps 1–4), including the recruitment and functions of early, middle, and late coat complexes. As discussed in detail below, the best-characterized adaptors are members of the middle and late coat complexes. In the process of these coat layers being recruited and ultimately forming a vesicle, the protein constituents form a dense network of interactions with each other, with the membrane, and with transmembrane cargo proteins, although there are undoubtedly more interactions to be defined (Figure 2). The coats both organize cargo and generate a structural platform to transmit forces generated by F-actin polymerization for vesicle formation, as described in *Mechanisms of Actin Assembly and Turnover* and *Vesicle Scission and Uncoating*.

Cargo sorting signals and adaptors

Endocytic adaptors recognize cytoplasmic sorting signals in cargo proteins and couple the cargo to the coat. There are generally two types of sorting signals: short peptide motifs and covalently attached ubiquitin molecules (Reider and Wendland 2011). Different adaptors recognize different sorting signals on transmembrane proteins at the plasma membrane. Key open questions include uncovering the code that matches each adaptor with each cargo and learning whether particular classes of cargo are targeted by adaptors in response to specific environmental changes.

Ubiquitin as a sorting signal: Ubiquitin (Ub) is a conserved, 76 amino acid protein that can be covalently linked to substrate proteins via a cascade of three enzymes: E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligase) (Varshavsky 2012). Commonly, Ub is linked to a lysine residue in the substrate protein, and since Ub itself has lysines on its surface, it can act as a target for Ub-ligases to produce poly-Ub chains. In mammalian cells, Ub modifications are important for intracellular sorting events at endosomal compartments (Erpapazoglou *et al.* 2012). There were hints that Ub might contribute to endocytic regulation in yeast starting in the mid-1990s; high-copy Ub was found to suppress the lethality associated with clathrin deletion (Nelson and Lemmon 1993), and some plasma membrane cargoes were found to be ubiquitylated prior to their internalization (Kolling and Hollenberg 1994; Hein *et al.* 1995; Hicke and Riezman 1996; Roth and Davis 1996). A key role of Ub as a signal for endocytic internalization was shown by the translational fusions of Ub to a mating pheromone receptor (Terrell *et al.* 1998). We now know that Ub-labeled endocytic cargo can either be mono-ubiquitylated (note that more than one lysine can be modified, generating a multi-ubiquitylated protein) or bear K63-linked poly-Ub chains. The major enzyme responsible for covalent attachment of Ub to endocytic transmembrane cargo proteins is the E3 ligase Rsp5 (Springael *et al.* 1999). Rsp5 substrate selection is influenced by protein kinases such as Npr1

(Kaouass et al. 1998; De Craene et al. 2001; Macgurn et al. 2011; Merhi and Andre 2012), raising the question of how the kinase activities are controlled.

α-Arrestins bridge Rsp5 and cargo substrates: Rsp5 contains three WW domains that promote binding to [L/P]PxY motifs in its substrates (Kasanov et al. 2001); however, many Rsp5-dependent substrates, including transmembrane proteins, lack [L/P]PxY motifs. Recent work has uncovered an important role for the [L/P]PxY motif-containing α-arrestin family in bridging Rsp5 to specific transmembrane cargo destined for covalent Ub modification and internalization. This family consists of 13 known members, many with partially redundant functions; additionally, many cargo proteins are able to functionally interact with multiple α-arrestins (Lin et al. 2008; Nikko and Pelham 2009). The precise mechanisms by which cells regulate binding of transmembrane cargo to α-arrestins are unclear, but may be dictated by physiological or environmental conditions (Nikko and Pelham 2009; Macgurn et al. 2011; Merhi and Andre 2012). Another recent finding involves the action of the N-terminal "LID" domain found in permeases, a transmembrane cargo family; this LID domain undergoes a conformational change when the permease is nonfunctional or misfolded, triggering an Rsp5-dependent (but α-arrestin independent) Ub modification (Keener and Babst 2013). It will be interesting to learn more about α-arrestin-dependent and -independent routes that govern plasma membrane protein turnover.

Ub-binding endocytic adaptors: The first ubiquitin interaction motif (UIM) identified found was a conserved α-helical motif in the Rpn10/S5a subunit of the proteasome S19 domain (Hofmann and Falquet 2001). The realization that a UIM could predict Ub-binding activity stimulated the study of a number of endocytic proteins with UIMs, in particular the epsin proteins. The two epsin proteins in *S. cerevisiae*, Ent1 and Ent2, are members of the middle coat. Initial studies suggested that the epsins might act as endocytic adaptors for binding to covalent Ub modifications on cargo (Aguilar et al. 2003). More recent work suggests that these and other Ub-binding proteins (*e.g.*, Ede1 and its UBA domain) may instead utilize their Ub-binding regions for self-regulation, similar to what has been proposed for their mammalian counterparts (Dores et al. 2010). Another Ub-binding adaptor protein is the late coat protein Sla1, which uses one of its SH3 domains to bind Ub via an unconventional interaction (Stamenova et al. 2007). Interestingly, Sla1 and its mammalian homolog CIN85 also bind to Ub E3 ligases, a requirement for the internalization of certain receptors (Szymkiewicz et al. 2002; Tonikian et al. 2009).

Peptide-based sorting motifs and their adaptors: The concept of peptide-based sorting motifs originally emerged from studies of familial hypercholesterolemia, in which it was discovered that the Tyr-x-x-hydrophobic signal in the cytosolic tail of the LDL (low density lipoprotein) receptor directs internalization of LDL particles (Anderson et al. 1977). Since then, numerous other peptide-based sorting motifs have been recognized in the tails of many animal cell cargo proteins, mediating selection and sorting at various organelles along the secretory and endocytic pathways.

The only endocytic peptide sorting motif characterized in yeast to date is the NPF$(x)_{1,2}$D sorting motif (Tan et al. 1996). NPF tripeptide motifs are hereafter labeled as NPF$_{motif}$, to distinguish them from the NPF acronym used later (see *Mechanisms of Actin Assembly and Turnover*) in reference to actin nucleation promoting factors (NPFs). Although most NPF$_{motifs}$ are bound by EH domains, typically the NPF$(x)_{1,2}$D sorting motif is not (Paoluzi et al. 1998; Howard et al. 2002). Instead, the NPF$(x)_{1,2}$D motif binds the adaptor protein Sla1, via its SH3-like SHD1 (Sla1 homology domain 1) (Howard et al. 2002; Mahadev et al. 2007). One possible reason for this distinction may be the influence of other Sla1 binding partners, such as membrane or cargo (via Ub), but more work is needed to resolve this. Sla1-interacting transmembrane cargo bearing the NPF$(x)_{1,2}$D motif (or a variant) include the mating pheromone receptors Ste2 and Ste3, the cell-wall stress sensor Wsc1, and the phospholipid flipases Drs2 and Dnf1 (Howard et al. 2002; Liu et al. 2007; Piao et al. 2007).

In animal cells, the C-terminal extension of the μ-subunit of the heterotetrameric AP complexes (and related domains), known as the μHD domain, has well-known peptide-sorting motif-binding activities (Owen and Evans 1998; Jung et al. 2007; Michelsen et al. 2007). The yeast early coat protein Syp1 contains a μHD domain, suggesting a role for Syp1 as an adaptor (Reider et al. 2009). It is interesting to define the sorting motif recognized by the Syp1–μHD and to learn whether other yeast adaptors similarly use peptide sorting motifs. In addition, it is important to continue the search for new adaptors.

Initiation of endocytic sites

Endocytic proteins in the early coat are the first to arrive at a nascent endocytic site during the initiation phase (Figure 1, step 2), and include clathrin, AP-2, Ede1, Syp1, and Pal1 (Kaksonen et al. 2005; Boettner et al. 2009; Carroll et al. 2009; Reider et al. 2009; Carroll et al. 2012). There are many questions about the early coat proteins, including what their functions are (both individually and as a group), what triggers their initial recruitment to the membrane, and why the majority of endocytic sites occur at/near sites of polarized growth. Another question is how yeast clathrin is recruited to these sites, since none of the early coat proteins are known to bind clathrin (Figure 2), with the possible exception of YAP180 (whose arrival time is debated in the literature; see below). Interestingly, among all the stages of endocytosis, the initiation phase is the most variable in length (Newpher and Lemmon 2006; Toret et al. 2008; Boettner et al. 2009; Carroll et al. 2012), raising questions about what leads to this variability and what triggers or checkpoints govern progression to subsequent recruitment of middle coat proteins.

Early coat proteins: Two of the earliest-arriving coat proteins, Ede1 and Syp1, directly bind each other (Boettner *et al.* 2009; Reider *et al.* 2009; Stimpson *et al.* 2009). Ede1 most closely resembles the mammalian endocytic protein Eps15, which is classified as a combination scaffold/ubiquitin adaptor (Gagny *et al.* 2000; Polo *et al.* 2003). The N-terminal region contains three EH domains (which bind NPF_{motifs}), and the C-terminal region includes an ubiquitin-binding UBA domain. The mammalian proteins FCHO1 and FCHO2 are homologs of Syp1 and have been implicated in stabilizing nascent clathrin-coated pit structures (Henne *et al.* 2010; Cocucci *et al.* 2012). Members of this family have a domain layout consisting of an N-terminal F-BAR domain (an anti-parallel homodimerizing membrane-binding domain), a central unstructured region that contains a proline-rich domain, and a C-terminal cargo-binding µHD (see above; Owen and Evans 1998; Reider *et al.* 2009). While the F-BAR domain binds to membranes with a particular curvature (Heath and Insall 2008), the unstructured central region contains a high-affinity membrane-binding activity that is sufficient to mediate strong membrane association. This region of Syp1 also directly binds and inhibits the actin polymerization functions of Las17 ((Boettner *et al.* 2009; Feliciano and Di Pietro 2012); see *Mechanisms of Actin Assembly and Turnover*). Although *ede1Δ* or *syp1Δ* cells have relatively mild endocytic phenotypes, these defects are consistent with the observed roles in mammalian cells, such as influencing the localization of clathrin and reducing the number and stability of endocytic patches (Kaksonen *et al.* 2005; Reider *et al.* 2009; Stimpson *et al.* 2009; Carroll *et al.* 2012).

The early coat protein Pal1 is another member of an Ede1-containing complex (Gavin *et al.* 2002; Carroll *et al.* 2012). Pal1 appears to be specific to fungi and has no characterized domains that hint at its functions, although it does have two copies of the NPF_{motif}. The *S. pombe* homolog of Pal1 is implicated in regulating cell polarity (Ge *et al.* 2005), which is interesting since endocytic sites in *S. cerevisiae* initiate in a polarized manner (Adams and Pringle 1984). The *S. cerevisiae* genome encodes a paralog of Pal1, called Yhr097c, but it seems unlikely to contribute to endocytosis given its localization to intracellular sites and its relative lack of genetic or physical interactions with other endocytic machinery (*Saccharomyces* Genome Database, http://www.yeastgenome.org).

Yeast AP-2 complex is also an early coat component. It is homologous to the mammalian AP-2 complex, except that the yeast complex does not bind clathrin (Yeung *et al.* 1999). As mentioned above, yeast AP-2 has a role in killer toxin internalization. In addition, it was recently shown to be involved in the maintenance of cell-wall integrity by acting as the endocytic cargo adaptor for the cell-wall stress-sensor Mid2 (Chapa-Y-Lazo *et al.* 2014). Beyond these roles, little else about the function and regulation of AP-2 in yeast is known. Thus, many questions remain open about its recruitment to the early coat and/or the plasma membrane and how it interacts with other early coat proteins.

Early coat protein dynamics are variable: While the temporal behaviors of most endocytic protein "modules" (Figure 1) are invariant in wild-type cells (Kaksonen *et al.* 2005), the early coat is an exception, ranging in lifetime from 30 to 180 sec (Newpher and Lemmon 2006; Toret *et al.* 2008; Boettner *et al.* 2009; Carroll *et al.* 2012). This variability may reflect a putative checkpoint that monitors the packaging of cargo at an endocytic site (Loerke *et al.* 2009; Carroll *et al.* 2012) and restricts progress until cargo is suitably concentrated or organized. Recently, it was found that the regions of the cell with the highest concentrations of newly delivered exocytic proteins (some of which are undoubtedly endocytic cargo) colocalize with the most rapidly maturing early coat patches (Layton *et al.* 2011), consistent with the checkpoint model. However, the major cargo-binding components of the endocytic patch are not recruited until later steps (see below). Whatever the precise cause of temporal variability, it is clear that some trigger causes a transition within the early coat proteins and/or the underlying membrane that allows the next wave of proteins to join the endocytic site.

Early coat proteins appear to have differing roles in regulating later-arriving endocytic proteins. Both *ede1Δ* and *chc1Δ* cells display shorter residence times of the late coat protein Sla1 (see below) and a reduced number of Sla1-containing patches at steady state (Kaksonen *et al.* 2005; Newpher and Lemmon 2006). Intriguingly, these effects on late coat behavior are apparently specific to *chc1* and *ede1* mutants and are not seen in *pal1*, *syp1*, or AP-2 mutants (Carroll *et al.* 2009; Carroll *et al.* 2012). Further analysis may shed light on how clathrin and Ede1 influence later-arriving endocytic proteins and/or other early coat components and may uncover a link to the putative cargo checkpoint.

Another feature of the early coat is the differential spatial behavior of its constituents. Clathrin, AP-2, and Pal1 all move inward with the coat during the mobile phase, while Ede1 and Syp1 remain at the plasma membrane (Stimpson *et al.* 2009). This is consistent with the activity of Syp1, which inhibits the polymerization of actin (see below); perhaps Ede1 also negatively regulates an activity required for the later stages and must disperse to promote the mobile phase.

Maturation of endocytic sites

"Maturation" refers to the events that occur between the end of the variable early coat initiation phase and the beginning of the mobile invagination/scission phase and typically lasts for about 30 sec. During this interval, the coat transitions to a more stereotypical behavior and prepares for assembly of the actin network. Initially, characterization of the temporal and spatial dynamics of endocytic proteins had not included the early coat proteins and was focused on the immobile coat proteins that became mobile when F-actin appeared (Kaksonen *et al.* 2003). However, subsequent studies with higher-resolution time-lapse analyses of many more proteins revealed a need to further classify endocytic components into middle coat *vs.* late coat proteins (Chi *et al.* 2012). This

more refined classification should facilitate the identification of which proteins influence the transitions from initiation to maturation to scission. The middle coat proteins represent major links to clathrin and cargo (Figure 2), while the late coat proteins are regulators of actin dynamics (see *Mechanisms of Actin Assembly and Turnover*). As discussed in more detail below, there is some uncertainty as to whether the membrane curvature/invagination stage precedes or coincides with the appearance of actin.

Middle coat proteins: The first middle coat proteins to join the early coat patch are likely to regulate the transition between the end of the early coat-based initiation phase and the next step in endocytosis. Several observations point to Sla2 playing a role in this transition. First, Sla2 physically interacts with three early coat proteins (Ede1, Syp1, and Pal1) (Gavin *et al.* 2002). Second, Sla2 arrives ∼6 sec before the late coat protein Sla1 (Newpher and Lemmon 2006). Third, an interaction between Sla2 and clathrin light chain in turn affects the timing of Sla1 recruitment (Newpher and Lemmon 2006; Boettner *et al.* 2011), suggesting that the early coat together with Sla2 influences later stages of endocytosis.

Sla2 is the yeast homolog of HIP1R (Huntingtin interacting protein-1 related). Sla2 and HIP1R are both homodimeric proteins and have N-terminal membrane-binding ANTH (AP180 N-terminal homology) domains, central coiled-coil regions that bind clathrin light chain, and C-terminal F-actin-binding THATCH (talin-HIP1/R/Sla2p actin-tethering C-terminal homology) domains (Wesp *et al.* 1997; Yang *et al.* 1997; Henry *et al.* 2002). In both yeast and mammals, Sla2/HIP1R has been implicated in linking the endocytic coat to F-actin and may direct actin polymerization and force generation to facilitate membrane invagination. In the absence of Sla2/HIP1R, uncontrolled polymerization of F-actin occurs without associated vesicle formation, resulting in actin comet tail structures (Kaksonen *et al.* 2003). This is now called an uncoupling phenotype, where actin polymerization appears to be unlinked from membrane invagination (Brady *et al.* 2010; Skruzny *et al.* 2012). However, the comet tails in *sla2Δ* cells are likely associated with at least small membrane invaginations, as suggested by immuno-EM studies and by the observation that FM4-64 accumulates in plasma membrane-associated punctae in these cells (Stefan *et al.* 2002; Idrissi *et al.* 2012). Intriguingly, clathrin mutants also exhibit actin comet tails; they are distinct in shape and size from those found in *sla2Δ* cells, but this still suggests that clathrin may also be involved in the coupling of the coat to actin, perhaps via the Sla2/Clc1 interaction described above (Newpher and Lemmon 2006).

The yeast epsins Ent1 and Ent2 have been defined as middle coat proteins based on both the timing of their arrival at patches and their functional links to Sla2 (Toret *et al.* 2008; Skruzny *et al.* 2012). Ent1 and Ent2 are an essential pair of clathrin adaptor proteins whose epsin N-terminal homology (ENTH) domains bind PtdIns(4,5)P$_2$ and play a crucial role in signaling to the polarity-regulating GTPase

Cdc42 (Wendland *et al.* 1999; Aguilar *et al.* 2006). Ent1 and Ent2 each contain two ubiquitin-interaction motifs (UIMs) adjacent to their ENTH domains; further, their intrinsically disordered C-terminal domains harbor an NPF$_{motif}$ and a variant clathrin box motif that binds the terminal domain of clathrin (Wendland *et al.* 1999; Kalthoff *et al.* 2002; Wendland 2002). The epsin C-terminal domain also binds actin (Skruzny *et al.* 2012).

Recent evidence has implicated Ent1 and Ent2 in the transmission of force from actin polymerization to the plasma membrane, in complex with Sla2. First, the Sla2 THATCH domain was found to be required for endocytosis in *ent1 ent2* mutant cells (Baggett *et al.* 2003). Second, the lipid binding ANTH domain of Sla2 heterodimerizes with the lipid-binding ENTH domain of Ent1 or Ent2 and cooperatively binds the plasma membrane (Skruzny *et al.* 2012).

Two additional coat proteins are Yap1801 and Yap1802, yeast homologs of the clathrin assembly lymphoid myeloid leukemia protein (CALM), a ubiquitously expressed clathrin adaptor (Dreyling *et al.* 1996; Chi *et al.* 2012). There is some disagreement in the literature about the precise temporal recruitment of the Yap180 proteins, as different groups observed them in either the early or middle coat, possibly influenced by the different positions of the GFP tags (Carroll *et al.* 2012; Chi *et al.* 2012). Yap1801/2 in yeast and CALM homologs in other organisms have conserved functions in endocytosis and in recycling of the v-SNARE Snc1/VAMP (Maritzen *et al.* 2012). Like Sla2, the Yap1801/2 proteins have N-terminal lipid-binding ANTH domains, and like the epsins they have intrinsically disordered C termini; Yap1801/2 and the epsins also have common binding partners (clathrin and EH domain scaffolds) (Wendland and Emr 1998). Further, yeast cells lacking all four of these related proteins (Ent1, Ent2, Yap1801, and Yap1802) exhibit temperature-sensitive growth and endocytic defects (Maldonado-Baez *et al.* 2008).

Late coat proteins: Another cohort of endocytic proteins defines the late coat (Kaksonen *et al.* 2005; Chi *et al.* 2012). Many late coat proteins associate with each other, with early and middle coat proteins, and with actin regulatory factors recruited in the final invagination/scission stage (Figure 2). As described below, Sla1 is currently thought to shepherd the transition from the middle to late coat stages.

Sla1 is a critical late coat protein that was among the first clear links discovered between the endocytic and actin machineries (Warren *et al.* 2002). Sla1 has numerous binding partners and regulatory interactions and the capacity to both positively and negatively regulate endocytosis. Its domain layout consists of three SH3 domains, two distinct conserved Sla1 homology domains (SHD1 and SHD2), a clathrin-binding motif, and a C-terminal region that is heavily phosphorylated by actin-regulating kinase (ARK) family kinases (see *Vesicle Scission and Uncoating*). Sla1 is subject to autoinhibition, as its SHD2 domain binds to and inhibits the clathrin-binding motif (Di Pietro *et al.* 2010). Sla1 forms a complex with two EH domain-containing late coat proteins, Pan1 and End3

(Tang *et al.* 1997; Tang *et al.* 2000). Recently, the specific domains in Pan1 and End3 mediating their interaction were identified, and it was shown that this interaction is required for Pan1 phosphoregulation (Whitworth *et al.* 2014). This Sla1–Pan1–End3 trio may be the yeast functional counterpart to mammalian endocytic scaffold/signaling protein Intersectin, which has EH and SH3 domains in a single polypeptide (Yamabhai *et al.* 1998).

Studies have shown that once Sla1 is recruited, an endocytic patch is highly likely to assemble F-actin and undergo internalization (Newpher and Lemmon 2006). Thus, the mechanism of Sla1 recruitment appears to be linked to the mechanism that deems a patch ready to transition from the immobile to the mobile phase. What underlies this key transition step remains unknown, but is likely affected by phosphoregulation (see below). It is possible that Sla1 monitors patch maturation by "sensing" the status or concentration of clathrin. As noted above, Sla1 residence in patches is reduced in *chc1Δ* cells (Kaksonen *et al.* 2005), and cells expressing a *sla1* mutant that cannot bind clathrin are defective for internalization of the Sla1-dependent cargo protein Wsc1 and have significantly longer lived patches (Di Pietro *et al.* 2010). However, further work is needed to fully understand this mechanism.

Pan1 is another critically important late coat protein; surprisingly, it is the only component of the endocytic coat that is essential for viability (in addition to the clathrin heavy chain in certain strain backgrounds mentioned above) (Sachs and Deardorff 1992; Wendland *et al.* 1996). Whether the essential function of Pan1 is tied to its role in endocytosis has not yet been determined, but given its subcellular localization to cortical patches, the phenotypes of *pan1* mutant cells, and numerous physical and genetic interactions (Miliaras and Wendland 2004), it is clear that it plays a central, and perhaps essential, role in endocytosis.

Pan1 was originally misidentified as a poly(A)-binding protein-dependent poly(A) nuclease (hence its name, Pan1), but Pan2 was later shown to harbor this nuclease activity (Sachs and Deardorff 1992; Boeck *et al.* 1996). After realizing that Pan1 in fact is an endocytic protein, the clathrin-binding adaptor proteins Yap1801/2 and Ent1/2 were identified as binding partners of Pan1's conserved EH domains (Wendland and Emr 1998; Wendland *et al.* 1999). The Cai lab independently connected Pan1 to endocytosis through suppressor screens, which also identified the late coat proteins Sla1 and End3 and the regulatory protein kinase Prk1 (a member of the ARK kinase family) (Tang *et al.* 1997, 2000). The Drubin lab later showed that Pan1 binds the Arp2/3 complex and can stimulate its actin nucleation activity, thereby linking Pan1 to actin regulation (Duncan *et al.* 2001). Further, Sla2 interactions with Pan1 were shown to inhibit its effects on Arp2/3 complex, providing a potentially important regulatory mechanism (Toshima *et al.* 2007). Pan1 also binds to the type I myosins Myo3/5 and facilitates their ability to stimulate Arp2/3 complex (Barker *et al.* 2007). These many binding partners and activities raise a key question

about Pan1, which extends more generally to other coat proteins, which is how do these proteins interact with their binding partners in the correct temporal sequence when the partners are often recruited at distinct stages of the endocytic process?

A final late coat protein, Las17, arrives coincidentally with Pan1. Since Las17 is one of the most potent activators of Arp2/3 complex-mediated actin polymerization, it is discussed below (*Mechanisms of Actin Assembly and Turnover*).

Mechanisms of Actin Assembly and Turnover

In this section we focus on events that occur at endocytic sites after the recruitment of the early, middle, and late coats (above) and that produce a short and critically required burst of actin assembly that drives vesicle scission and internalization (Kaksonen *et al.* 2003, 2005) (Figure 1 and Figure 3). Although this phase of endocytosis lasts only 15–20 sec, it is the major force-generating step in the endocytic process and results in all of the extreme membrane remodeling events that accompany endocytic internalization. Patches also reach their peak of molecular complexity at this phase, since most earlier components remain present and >30 new proteins arrive (Table 2).

Over the past 17 years, there have been major advances in defining the activities, mechanisms, and structures of actin-associated components, as well as their physiological interacting partners, dynamics, and cellular roles. Moreover, from quantitative live-cell imaging studies we know the timing of arrival of most actin-associated protein components, their molar ratios in cells, and even the number of molecules per endocytic site for many of these proteins. Finally, EM studies have defined the shape and dimensions of endocytic invaginations and surrounding actin networks (Figure 3, G and H). With this information, it has been possible to begin answering central mechanistic questions about this actin-based phase of endocytosis, including: How is the timing of actin assembly regulated at endocytic sites, and what mechanisms initiate actin polymerization? How are the size, shape, and geometry of the actin networks determined? How is a particular set of actin-associated proteins recruited to the actin filaments in patches, while a different set is recruited to the other major actin structures in yeast: actin cables and the cytokinetic ring (Figure 4)? Finally, how does the actin patch network provide directional force to drive vesicle invagination and scission?

The working model we arrive at (described below) helps to explain how >30 different actin regulatory proteins arrive at patches within 5–7 sec of each other and work in concert to initiate the rapid formation of an actin network, stimulate its dynamic turnover and remodeling, and ultimately dismantle the network and recycle its components (Figure 4). In many ways, this process can be compared to *Listeria* actin-based motility, which requires both focused nucleation at the bacterial surface and rapid turnover of the network (Tilney and Portnoy 1989). Similarly, latex beads coated with actin nucleators found at yeast endocytic
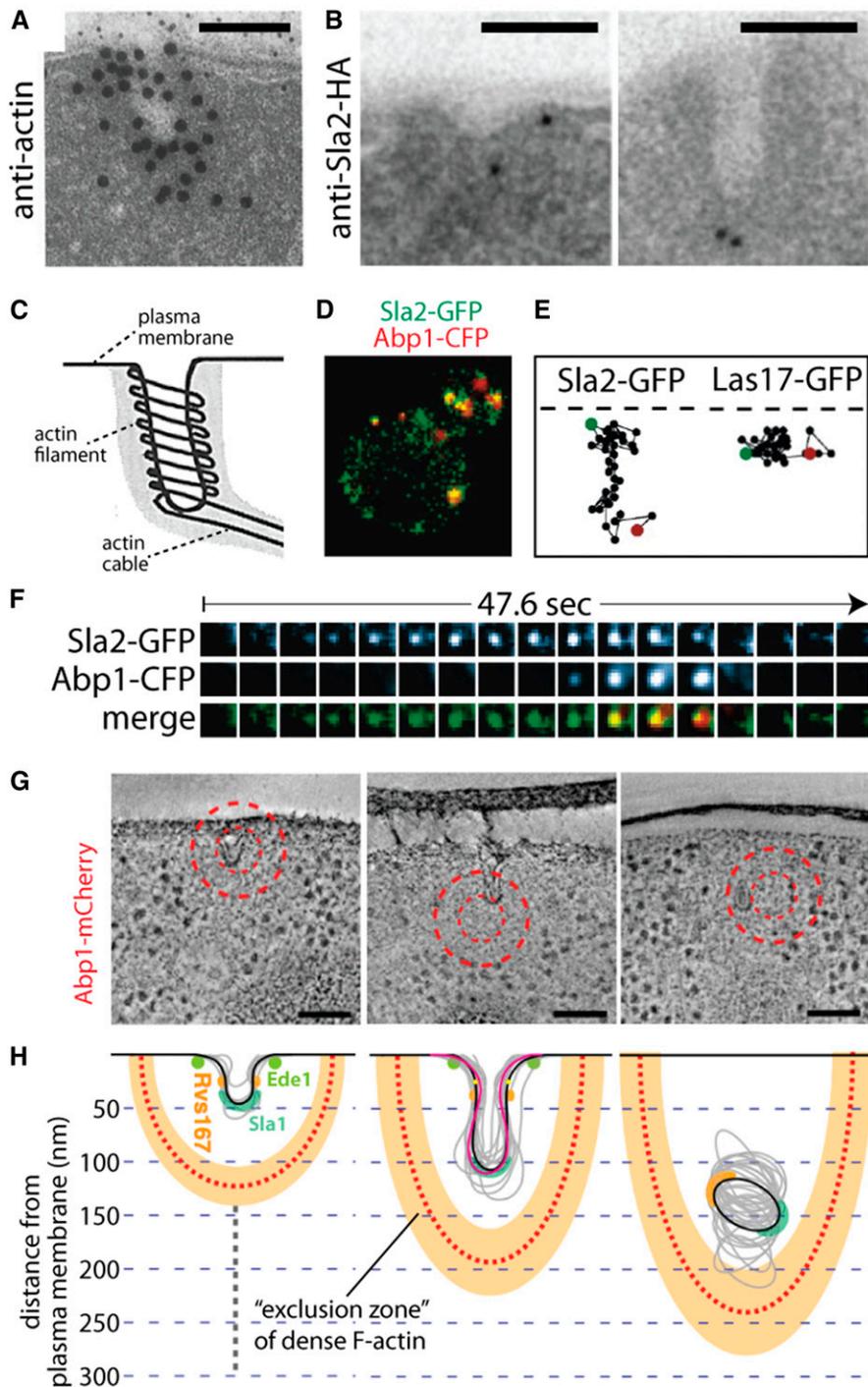
Figure 3 Endocytic patch dynamics and ultrastructure. (A and B) Longitudinal EM sections through cortical actin patches in fixed yeast cells visualized with anti-actin and anti-HA-Sla2 immunogold labeling respectively (Mulholland *et al.* 1994; Idrissi *et al.* 2008). (C) Scheme of F-actin at a cortical actin patch circa 1994, adapted from Mulholland *et al.* 1994. (D–F) Localization of Sla2–GFP and Abp1–CFP by live cell fluorescence imaging (Kaksonen *et al.* 2003). (D) A single frame from a time-lapse series; Sla2-GFP in green, Abp1–CFP in red. (E) Tracking of consecutive positions of the indicated proteins from consecutive frames; dashed line, plasma membrane. Green dot, starting position when the patch appears; red dot, final position when the patch dissipates. (F) Individual frames from a time-lapse series showing distinct arrival/departure of Sla2 *vs.* Abp1 at an individual endocytic patch; frames acquired every 2.8 sec. (G) Electron tomograms of plasma membrane structures at various stages of endocytosis (Kukulski *et al.* 2012b). Dashed circles are zones of 50 and 80% prediction accuracy for the correlated position of fluorescent Abp1–mCherry signal, independently acquired. Dark electron densities, corresponding to ribosomes, are excluded from the region around the membrane invagination, presumably due to the dense F-actin network. (H) Schematic of electron tomograms, presenting the shape and positions of endocytic structures and the ribosome "exclusion zone" (interpreted as the actin-rich zone) at selected stages of endocytosis. Shaded lines overlay the data from individual EM tomograms; solid outline is the average shape at each stage. Adapted from Kukulski *et al.* 2012b.

sites (see below), when mixed with yeast-cell extracts, reconstitutes *in vitro* bead motility (Michelot *et al.* 2010). Force generation by actin networks in both motility and endocytosis can be explained by the Brownian ratchet and elastic Brownian ratchet models, in which actin polymerization produces work by polymerizing to fill small gaps between the actin network and membrane created by Brownian motion (Kaksonen *et al.* 2006). In these models, actin polymerization must be focused at the membrane, consistent with nucleation and elongation machinery being typically found at these sites.

Capping proteins are additionally required to focus polymerization at new nucleation sites and to maintain network architecture, and filament branching and crosslinking factors are necessary to hold a network together to enable force production. The precise organization and orientation of actin filaments in yeast actin patches is still being worked out, but most evidence suggests that the barbed ends face the cell cortex, at least at early stages of membrane invagination (details below).

In the subsections below, we describe in detail the molecular machinery and mechanisms underlying each major step in actin
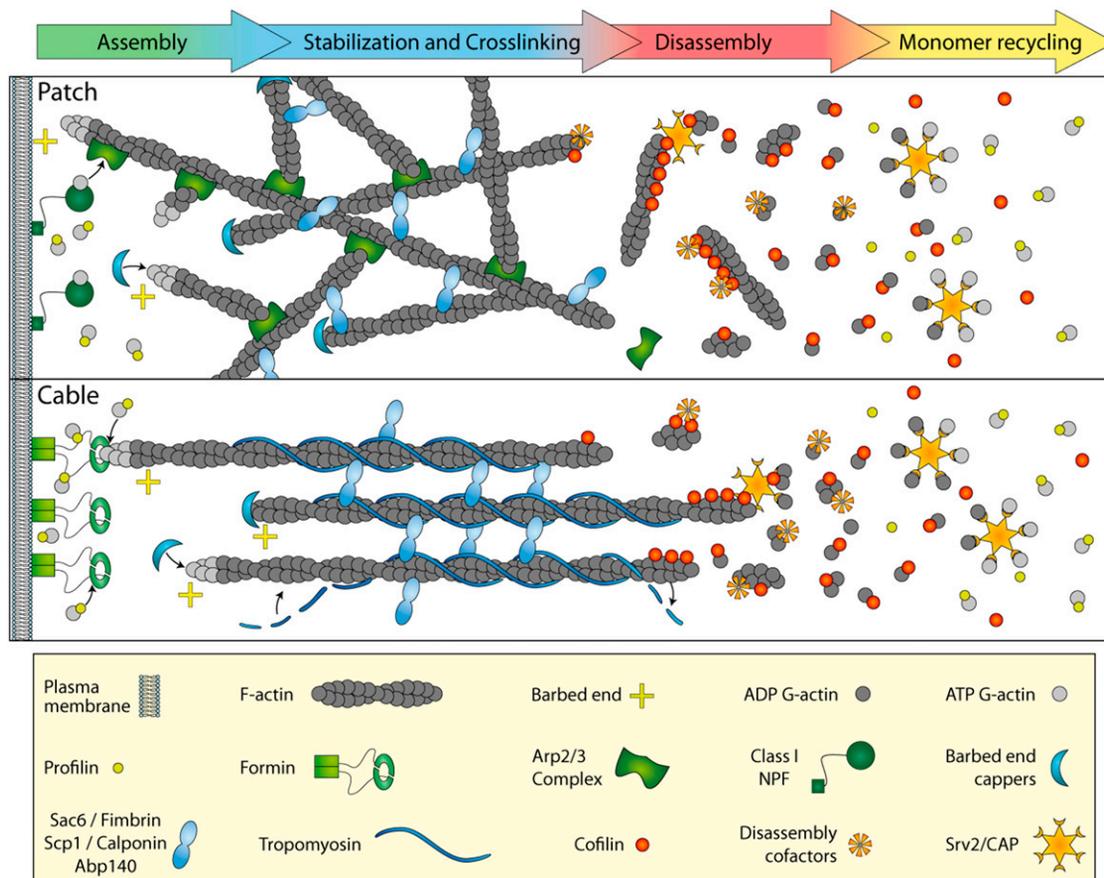
Figure 4 A comparison of the four stages in formation and turnover of actin patches *vs.* cables. Scheme of the ultrastructure of a cortical actin patch (top) and cable (middle); legend defining the icons (bottom). Both structures are initiated by the nucleation of filaments from ATP-bound G-actin (left). Then, F-actin is stabilized/crosslinked and subsequently severed and disassembled. The released ADP-actin monomers undergo nucleotide exchange to replenish the pool of polymerization-competent ATP-G-actin (right side). Some icons represent groups of proteins with related activities (*e.g.*, Arp2/3 complex NPFs, disassembly cofactors of cofilin). This cycle takes ∼5 sec to complete in each type of actin structure; due to distinct nucleation rates, it occurs within the <300 nm ribosome exclusion zone in an actin patch *vs.* >5000 nm along the length of an actin cable. See *Mechanisms of Actin Assembly and Turnover* for details.

regulation that occurs during yeast endocytosis: (A) actin nucleation, (B) actin-filament capping, crosslinking, and stabilization, and (C) actin-filament disassembly and turnover (Figure 4). Genetic analysis has shown that each step is crucial for endocytosis, presumably because the rapid assembly and turnover of the actin networks provides: (i) directional force to drive plasma membrane tubulation and vesiculation and (ii) plasticity necessary for the actin network surrounding the membrane invagination to be dynamically resculpted as the geometry of the membrane changes (Figure 1, G and H).

Actin nucleation

Actin is a ubiquitous, abundant, and conserved 42-kDa protein that binds ATP and exists in either a globular (monomeric) form called G-actin or a filamentous (polymeric) form called F-actin. Actin filaments are polarized, helical, two-stranded polymers with a fast-growing "barbed" end and a slow-growing "pointed" end, named for their appearances in electron micrographs after decoration with myosin S1 fragment. The critical concentration for assembly

of yeast and animal actins is ∼0.15 μM, yet their cytosols maintain micromolar levels of actin monomers; thus cellular conditions strongly favor actin assembly over disassembly. What limits the growth of actin filaments in yeast and animal cells is the production and/or availability of "free" (uncapped) barbed ends of filaments onto which actin subunits can be rapidly added. Because free ends are limiting, a reservoir of ATP-actin monomers builds up in cells, poised for growth once free ends become available. Profilin is a small, abundant cellular protein that preferentially binds ATP-actin monomers and blocks their addition onto pointed ends but freely allows addition onto barbed ends (Tilney *et al.* 1983; Pollard and Cooper 1984; Haarer *et al.* 1990; Haarer *et al.* 1993; Eads *et al.* 1998). Thus, actin polymerization *in vivo* is restricted to barbed ends, and the generation of free barbed ends is a key regulatory step in actin network formation.

In principle, there are three distinct mechanisms by which free barbed ends can be generated. Existing capped filaments can be uncapped, existing filaments can be severed to produce new ends, and new filaments can be formed *de novo* from

monomers. Although there is some evidence for uncapping and severing contributing to the growth of actin networks in animal cells (Condeelis 2001), rich evidence demonstrates that the primary mechanism for stimulating actin network growth is regulated nucleation (Pollard and Borisy 2003; Pollard and Cooper 2009).

While actin monomers are rapidly added to existing filaments barbed ends (11.6 subunits sec^{-1} μM^{-1}; Pollard 1986), they do not readily self-nucleate to form new filaments in the absence of other factors. This is because polymerization intermediates, actin dimers and trimers, are extremely short lived and rapidly dissociate back into monomers (Sept and McCammon 2001). In addition, profilin further suppresses nucleation by blocking longitudinal (long-pitch) actin–actin contacts (Schutt et al. 1993). In yeast and other organisms, these kinetic barriers to nucleation are overcome by activation of specialized actin regulators, called nucleators, which structurally mimic a filament end, stabilize polymerization intermediates, or recruit and organize monomers into filament seeds (Chesarone and Goode 2009; Campellone and Welch 2010).

Two distinct sets of actin assembly-promoting machinery have been identified in yeast, the Arp2/3 complex and formins (Figure 4). Endocytic actin patches are nucleated by the combined activities of Arp2/3 complex and its cofactors, called nucleation-promoting factors (NPFs, unrelated to the NPF$_{motif}$ discussed in Cargo Selection and Coat Formation). Actin cables and cytokinetic rings are nucleated instead by formins (Bni1 and Bnr1) working in combination with a different set of cofactors, which includes Bud6, profilin, and tropomyosin. Yeast actin structures (patches, cables, and rings) each have a distinct shape, function, architecture, and molecular composition (Moseley and Goode 2006). How these differences are programmed is not yet understood, but may stem in part from which nucleator is used to initiate the actin network (Michelot and Drubin 2011). Further, the relative amount of actin distributed between different types of actin networks (e.g., patches and cables) is controlled by homeostatic mechanisms, in which the nucleators in these different structures compete for a limiting pool of G-actin in the cytosol (Gao and Bretscher 2008; Burke et al. 2014). This balance can also be shifted by any of the complex sets of stimulatory and inhibitory interactions that control the activity states of the nucleator systems (see below).

The Arp2/3 complex: The Arp2/3 complex ($\sim$200 kDa), which was first isolated from Acanthamoeba cell extracts (Machesky et al. 1994), consists of seven subunits and is conserved across distant species of animals, plants, and fungi (Welch et al. 1997b, 1998). It was also the first bona fide actin nucleator identified (Welch et al. 1997b; Mullins et al. 1998) and remains the only one that nucleates branched filaments, polymerizing new "daughter" filaments from the sides of existing "mother" filaments at a 70° angle (Mullins et al. 1998). Thus, the Arp2/3 complex forms branched networks, such as those found at the leading edge of motile cells (Svitkina

and Borisy 1999) and at endocytic sites in yeast and mammalian cells (Young et al. 2004; Rodal et al. 2005a; Collins et al. 2011). Given its two actin-related protein (Arp) subunits (Figure 5A), there was early speculation that the complex might directly nucleate actin polymerization (Mullins et al. 1996), and shortly thereafter seminal work from the Mitchison and Pollard labs demonstrated that this is the case (Welch et al. 1997b, 1998; Machesky et al. 1999). Importantly though, the Arp2/3 complex alone has minimal activity, and strong nucleation requires binding of an NPF. Four NPFs have been identified in yeast (see below), with the Wiskott–Aldrich syndrome protein (WASP), called Las17 in yeast, being the best characterized. Las17/WASP contains a C-terminal VCA (or WCA) region (detailed below), which is sufficient to bind Arp2/3 complex and actin monomers and stimulate actin nucleation in vitro.

A critical advance in our understanding of the Arp2/3 complex mechanism came with publication of its crystal structure, solved for bovine Arp2/3 (Robinson et al. 2001) (Figure 5A). This provided a wealth of information to guide subsequent genetic and biochemical dissections of individual subunits defining Arp2/3 function, mechanism, and regulation in yeast (Beltzner and Pollard 2004; D'agostino and Goode 2005; Martin et al. 2005; Daugherty and Goode 2008; Balcer et al. 2010; Goley et al. 2010). The structure of the complex also revealed that the Arp2 and Arp3 subunits were well separated from each other, suggesting that an activation step might be required to bring Arp2 and Arp3 into register for nucleation. This hypothesis was confirmed both by EM and FRET studies, which showed that WASP triggers a conformational change in yeast or human Arp2/3 complex, shifting it from an "open" (inactive) state to a "closed" (primed for nucleation) state (Figure 5B) (Goley et al. 2004; Rodal et al. 2005b). Further insights came from electron micrographs and 3D reconstructions of yeast Arp2/3 complex at filament branch sites, which revealed that additional conformational rearrangements in the complex occur upon mother filament binding (Rouiller et al. 2008). Moreover, addition of preformed filaments was shown to catalyze nucleation by VCA and bovine Arp2/3 complex (Higgs et al. 1999). These results suggest that robust nucleation by Arp2/3 complex requires interactions with both an NPF and a mother filament.

Studies comparing yeast and mammalian Arp2/3 complex activities have revealed some differences that may be relevant, including partial nucleation activity by yeast Arp2/3 complex in the absence of NPFs, i.e., weak activation by F-actin alone (Winter et al. 1999; Goode et al. 2001; Rodal et al. 2005b; Wen and Rubenstein 2005). However, genetic studies show that NPFs are essential for cell viability and endocytosis (Evangelista et al. 2000; Lechler et al. 2000); thus, nucleation by Arp2/3 without NPFs is not sufficient in vivo. Still, this low-level NPF-independent nucleation activity of yeast Arp2/3 complex could be important during endocytosis; e.g., NPFs may control network initiation as well as ongoing polymerization at the membrane, while NPF-independent nucleation may contribute to reinforcing the network away from the membrane.

One of the ultimate goals in studying Arp2/3 complex mechanism is to define the precise order and timing of each step in the nucleation pathway. This is extremely challenging however, because it involves a complex set of interactions involving Arp2/3 complex, NPFs, F-actin, and G-actin. Some of the individual steps in this pathway were elegantly defined by measuring the rate constants of specific interactions involved, allowing investigators to build models of the pathway (Beltzner and Pollard 2008). However, key steps in such models required speculation where direct measurements were not available, which left gaps in our understanding. More recently, multi-wavelength single-molecule analysis of fluorescently labeled actin, yeast Arp2/3 complex, and dimerized mammalian WASP–VCA (di-VCA) molecules has allowed direct visualization of the events in real time, making it possible to quantitatively define each step in the pathway (Figure 5C) (Smith *et al.* 2013a,b). These studies demonstrated that di-VCA first binds Arp2/3 complex in solution and then diVCA-bound Arp2/3 complex associates with the side of a mother filament, and finally a daughter filament begins to polymerize, but only after di-VCA dissociates from Arp2/3 complex (Figure 5D).

Importantly, these studies identified several highly inefficient steps in the nucleation pathway, leaving generous room for positive regulation by additional cellular factors. For example, *in vitro* the great majority of complexes that bind the mother filament rapidly dissociate without ever nucleating a daughter filament, so factors that stabilize Arp2/3 complex on mother filament sides could dramatically enhance nucleation. Single-molecule studies now offer a powerful strategy by which to define the precise spatial and temporal effects of each NPF, alone and together, on nucleation and branching (Figure 5C).

Nucleation-promoting factors: NPFs directly associate with Arp2/3 complex and actin and serve as cofactors in stimulating nucleation. As mentioned above, there are four known NPFs in yeast: (1) Las17/WASP, (2) myosin I (Myo3 or Myo5) in a complex with Vrp1/WIP, (3) Pan1/Intersectin, and (4) Abp1 (Winter *et al.* 1999; Evangelista *et al.* 2000; Lechler *et al.* 2000; Duncan *et al.* 2001; Goode *et al.* 2001) (Figure 6). Each of these NPFs localizes to actin patches, binds to actin and to Arp2/3 complex, stimulates Arp2/3 nucleation activity to some degree *in vitro*, and exhibits genetic interactions with Arp2/3 complex and/or other NPFs. Las17/WASP and myosin I (with Vrp1) are categorized as class I NPFs because they bind actin monomers and have strong NPF effects (Sun *et al.* 2006). Pan1 and Abp1 are categorized as class II NPFs because they bind to F-actin and have comparably weak NPF effects (Figure 6C). Consistent with this classification, Arp2/3-inactivating mutations in Las17 combined with mutations in either myosin I or Vrp1 are synthetic lethal, demonstrating that class I NPF function is essential (Evangelista *et al.* 2000; Lechler *et al.* 2000). The roles of Pan1 and Abp1 in regulating Arp2/3 complex during endocytosis are less well understood (see below).

Las17/WASP: Las17 (also known as Bee1) is the yeast homolog of WASP and was first identified as a factor essential for reconstituted actin patch assembly in a permeabilized cell assay (Li 1997) and then as a multicopy suppressor of *vrp1* mutants (Naqvi *et al.* 1998). Las17 contains an N-terminal WASP-homology 1 (WH1)/EVH1 domain that binds to Vrp1/WIP, followed by a central proline-rich region that binds to a number of SH3 domain-containing proteins (*e.g.*, Sla1, Bbc1, Bzz1, Myo3, Myo5, Lsb1, Lsb2, Ysc84, Sho1, and Rvs167), and finally a C-terminal VCA region (Figure 6). The ∼10-kDa VCA region consists of a WASP-homology 2 (WH2 or V) domain that binds G-actin, a short "connecting" (C) domain that binds Arp2/3 complex, and an "acidic" (A) domain that binds Arp2/3 complex. Biochemical analyses of yeast and animal WASP homologs have demonstrated two critical mechanistic roles for VCA during nucleation: (i) it induces conformational changes in Arp2/3 complex that promote nucleation, and (ii) it delivers the first actin monomer of the daughter filament via its WH2 (or V) domain (Higgs *et al.* 1999; Rohatgi *et al.* 1999; Rodal *et al.* 2003) (Figure 5D). Early models of nucleation proposed binding of a single VCA to a single site on Arp2/3 complex. However, it was later shown that dimerization of human VCA elevates NPF effects substantially and increases VCA affinity for Arp2/3 complex by >100-fold compared to monomeric VCA (Padrick *et al.* 2008). More recently, it was found that there are two separate VCA-binding sites on bovine Arp2/3 complex (Padrick *et al.* 2011; Ti *et al.* 2011). Together, these studies suggest that maximal nucleation is achieved by Arp2/3 complex binding two VCAs simultaneously. Another implication of these findings is that the activity of WASP, and possibly other NPFs, can be enhanced by ligand-induced dimerization, and indeed, specific binding partners of Las17 (*e.g.*, Bzz1, which has a dimeric F-BAR domain, Figure 6C) may function in this capacity.

While the VCA region is critical for yeast Las17 NPF effects, even dimerized it has substantially weaker NPF effects compared to full-length Las17 (Rodal *et al.* 2003). These observations suggest that other domains in Las17 outside of VCA contribute to its robust NPF activity. One recent study identified sequences in the proline-rich (PR) region of Las17, adjacent to the VCA, that bind actin and contribute to NPF activity (Urbanek *et al.* 2013). However, the NPF activity of this PR-VCA fragment was still much weaker than that of full-length Las17, suggesting that there is still much to learn about Las17 activity. Importantly, this study also reported that some Las17-stimulated actin nucleation was Arp2/3 independent; such activity could contribute to filament initiation at the patch (see below). Purified Las17 is also constitutively active rather than autoinhibited like mammalian N-WASP (Rodal *et al.* 2003). So, in this regard Las17 is more similar to mammalian SCAR/WAVE proteins, other members of the larger WASP/SCAR/WAVE family (Welch and Mullins 2002). WAVE proteins are inhibited *in trans* by a large multisubunit WAVE-regulatory complex (WRC) (Eden *et al.* 2002; Gautreau *et al.* 2004; Chen
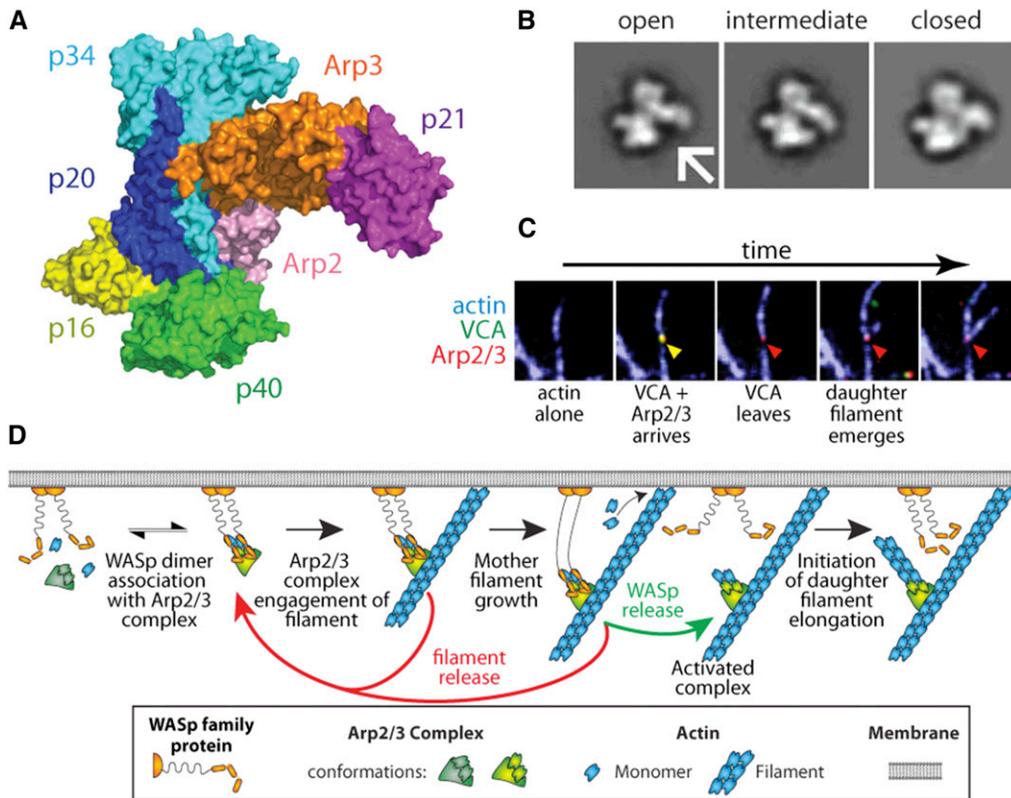
Figure 5 Mechanism of Arp2/3 complex-mediated actin branch formation stimulated by WASP/Las17. (A) Crystal structure of bovine Arp2/3 complex (Robinson *et al.* 2001), in the conformation corresponding to open labeled with the arrow in B. Individual subunits are labeled and color-coded. (B) Distinct conformations of purified *S. cerevisiae* Arp2/3 complex, visualized by negative-stain EM and single particle averaging (Rodal *et al.* 2005b). The open conformation corresponds to the inactive state, and the closed conformation is primed for nucleation. (C) Single-molecule time-lapse TIRFM imaging of Arp2/3-mediated branch formation (adapted from Smith *et al.* 2013b). Actin daughter filament (blue) nucleated by SNAP-tagged Arp2/3 complex (red) stimulated by dimeric Cy3-labeled WASP (VCA domain, green). A selection of frames is shown from time points at −20, 0, 5, 40, and 100 sec, with 0 sec defined when VCA releases from Arp2/3. (D) Scheme describing the reaction steps leading to branched actin network formation near cellular membranes. WASP/Las17 binding to Arp2/3 closes the complex to bind the side of an existing F-actin mother filament. Elongation of the mother filament against the plasma membrane causes release of WASP/Las17 from Arp2/3 and nucleation/elongation of the daughter filament branch (adapted from Smith *et al.* 2013b).

et al. 2010). Although homologs of WRC genes are not found in yeast, a distinct set of Las17-binding partners appears to mediate *trans*-inhibition (see below). Therefore, while the domain layout of Las17 resembles WASP, its regulation more closely resembles SCAR/WAVE.

Myosin I (Myo3 and Myo5): Type I myosins are considered unconventional within the myosin superfamily because they are single headed, nonprocessive, and lipid associated. There are two yeast homologs of myosin I, called Myo3 and Myo5. Each has a motor domain, lipid and F-actin binding domains, an SH3 domain, and an Arp2/3-interacting acidic (A) domain (Figure 6C). Myosin I NPF activities require both the A domains and interactions of their SH3 domains with Vrp1/WIP, which in turn binds G-actin (Sirotkin *et al.* 2005; Sun *et al.* 2006). Thus, the combination of myosin I and Vrp1 provides a functional equivalent to VCA and delivers actin monomers to Arp2/3 complex. Single deletions of *MYO3* or *MYO5* cause relatively minor defects *in vivo*, but double mutants show pronounced defects in endocytosis and actin organization (Geli and Riezman 1996). Both the motor and NPF activities of myosin I are required for its endocytic functions (Sun *et al.* 2006).

Pan1: In addition to its roles as a scaffold protein in organizing other components of the endocytic machinery (see *Cargo Selection and Coat Formation*; Figure 2), Pan1 contains an Arp2/3-interacting A domain and an unconventional WH2 (V) domain that binds F-actin rather than G-actin (Figure 6). Pan1 has weak NPF effects on Arp2/3 complex compared to those of Las17 or myosin I and Vrp1. The NPF activities of Pan1 depend on its WH2 and A domains (Duncan *et al.* 2001; Toshima *et al.* 2007). Pan1 is categorized as a class II NPF (Welch and Mullins 2002), but its functions in regulating Arp2/3 complex are still not well understood.

Abp1: Abp1 (actin binding protein 1) was first isolated from yeast-cell extracts on an F-actin affinity column (Drubin *et al.* 1988) and later found to be conserved in mammals (Ensenat *et al.* 1999; Larbolette *et al.* 1999; Kessels *et al.* 2000). It has an N-terminal actin depolymerization factor (ADF)/cofilin-homology (ADFH) domain through which it binds F-actin but not G-actin and two acidic (A) domains that bind Arp2/3 complex (Kessels *et al.* 2000; Goode *et al.* 2001) (Figure 6C). The C-terminal half of Abp1 contains a proline-rich region that binds the SH3 domain of Rvs167 (Friesen *et al.* 2006) and an SH3 domain that binds Srv2, Scp1, and the kinases Ark1 and Prk1 (Freeman *et al.* 1996; Lila and Drubin 1997; Fazi *et al.* 2002; Haynes *et al.* 2007); the functions of these proteins are described below. Like Pan1, the NPF effects of Abp1 are weak compared to those of class I NPFs (Sun *et al.* 2006). Further, because Abp1 binds to F-actin, it is categorized as a class II NPF. Although *abp1*Δ mutants have no discernable defects in actin organization and only subtle defects in endocytosis (Lila and Drubin
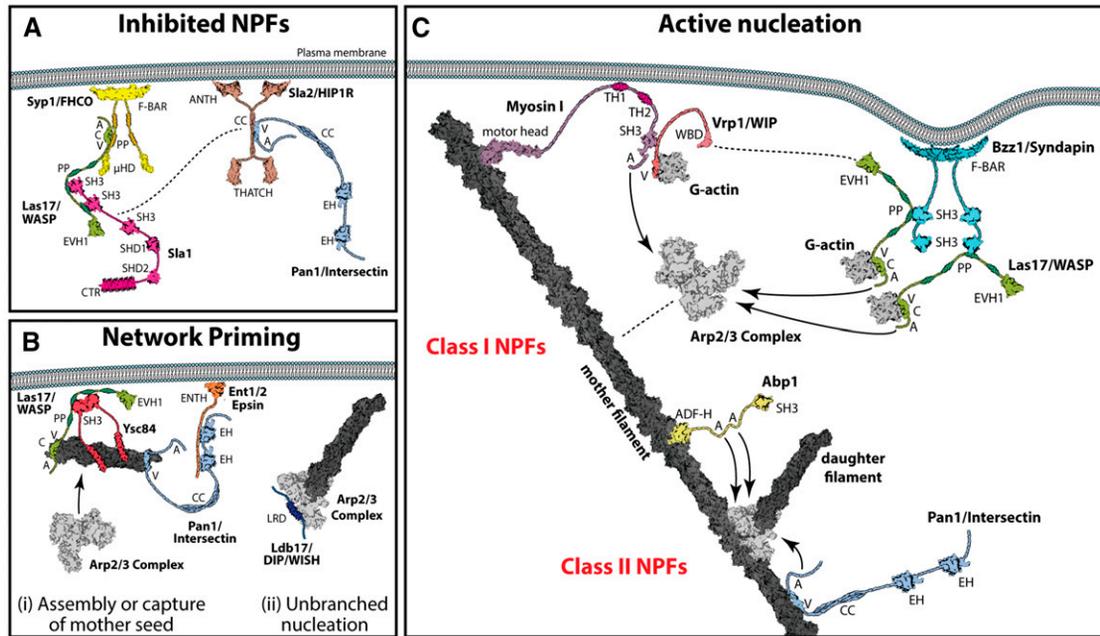
Figure 6 Arp2/3 complex nucleation-promoting factors (NPFs). The figure depicts three proposed stages of NPF activity. (A) Inhibited NPFs. In earlier stages of patch development well before the arrival of Arp2/3 complex, the NPFs Las17/WASP and Pan1/Intersectin are maintained at patches in an inactive state by direct binding partners and are unable to recruit Arp2/3 complex. (B) Network priming. After subsequent deactivation of their inhibitors, Las17/WASP and Pan1/Intersectin are released to recruit and activate Arp2/3 complex. However, network nucleation also requires priming by one of two proposed mechanisms: (i) the capture or *de novo* assembly of an initial mother filament, which may depend on the Arp2/3-independent activities of Las17, Pan1, and/or Ysc84/Lsb4, or (ii) the stimulation of unbranched actin nucleation by Arp2/3 complex, as Nolen and colleagues have recently demonstrated can be induced by the *S. pombe* homolog of Ldb17/DIP/WISH (Wagner *et al.* 2013). (C) Active nucleation. After the priming stage, the Arp2/3 complex and its NPFs rapidly assemble a densely branched actin-filament network that promotes membrane remodeling. Class I NPFs bind G-actin: Las17/WASP, Vrp1/WIP, and myosin I. Class II NPFs bind F-actin: Pan1/Intersectin and Abp1. Some of the proteins are shown in complexes with their known binding partners. Additional interactions are indicated by dotted lines. Arrows represent stimulatory effects. Illustrations are based on available structural and sequence data. Abbreviations: THATCH, talin-HIP1/R/Sla2p actin-tethering C-terminal homology domain; ANTH, AP180 N-terminal homology domain; F-BAR, FER/Cip4 homology-Bin/Amphiphysin/Rvs domain; μHD, μ homology domain; EH, Eps15 homology domain; LRD, leucine-rich domain; CC, coiled coil; V, verprolin homology motif; C, connector motif; A, acidic motif; SH3, Src homology 3 domain; SHD, Sla1 homology domain; CTR, C-terminal repeats; ADF-H, actin depolymerizing factor homology domain; TH, tail homology domain; WBD, WASP-binding domain; EVH1, Ena/Vasp homology 1 domain; PP, polyproline region.

1997; Kaksonen *et al.* 2005), double mutants with *sla1*, *sla2*, or *sac6* are lethal (Holtzman *et al.* 1993), demonstrating that the functions of Abp1 are important, although genetically redundant. Interestingly, all three shared functions of Abp1 depend on its SH3 domain, but only two of these (shared with Sla2 and Sac6) depend on its ability to bind F-actin (Quintero-Monzon *et al.* 2005). Recently, Abp1 was found to be essential for an alternative endocytic pathway in yeast, perhaps related to its synthetic lethality with factors central to the conventional CME pathway (Aghamohammadzadeh *et al.* 2014). While purified Abp1 alone shows no effects on actin-filament dynamics or organization *in vitro* (Goode *et al.* 2001; Quintero-Monzon *et al.* 2005), when bound to its ligand Aim3 it can cap actin-filament barbed ends (Michelot *et al.* 2013). Through this interaction, and its interactions with the Arp2/3 complex and the other binding partners mentioned above, Abp1 may influence actin-filament assembly and turnover in a number of ways.

Regulation of actin nucleation in endocytosis: How are the activities of Arp2/3 complex's four NPFs utilized and controlled during endocytosis? As mentioned above, the precise spatial recruitment and/or localized activation of NPFs at endo-

cytic sites is likely to be critical in enabling the polymerizing actin network to provide the appropriate directional force to drive plasma membrane invagination and tubulation. Recent EM studies have begun to define the locations of the NPFs to give us clues to how this works (Idrissi *et al.* 2008; Idrissi *et al.* 2012). These studies suggest that two different pools of NPFs, one at the plasma membrane (Las17, Myo3, and Myo5) and the other at the tip of the invagination (Pan1 and Abp1), might control two different phases of endocytosis, invagination and scission, respectively.

In addition to the proper spatial recruitment of NPFs at endocytic sites, it is critical that their activities be tightly controlled in a temporal manner. Las17 and Pan1 are the first NPFs to appear at patches, arriving at least 20 sec before the onset of actin assembly, and subsequently myosin I and Abp1 arrive shortly after the first appearance of F-actin. These observations beg the question, How are early arriving NPFs, Las17 and Pan1, kept inactive at endocytic sites until the required onset of actin assembly?

Control of early NPF localization and activity: Among the many binding partners of Pan1, so far only Sla2 has been shown to regulate Pan1 NPF activity, inhibiting its effects

in vitro (Toshima *et al.* 2007). This may be critical for blocking actin assembly until an endocytic patch has sufficiently matured, although much work is still needed to fully understand Pan1 regulation *in vivo*. In contrast, our understanding of Las17 regulation has expanded significantly in recent years. Five direct inhibitors of Las17 activity have been identified: Sla1, Bbc1, Syp1, Lsb1, and Lsb2 (Rodal *et al.* 2003; Boettner *et al.* 2009; Spiess *et al.* 2013). Syp1 arrives at patches well before Las17, and Sla1 arrives in approximately the same time frame as Las17, making these binding partners prime candidates for constraining Las17 activity (Kaksonen *et al.* 2003; Stimpson *et al.* 2009). In contrast, Bbc1 arrives later, suggesting that it may have a different role than that of a Las17 inhibitor, possibly in spatially restricting Las17 activity during vesicle internalization. Finally, the function and localization of Lsb1 and Lsb2 are unclear, but overexpressed Lsb1 redistributes Las17 into cytoplasmic puncta, and together with the Ub-ligase Rsp5 and Sla1, Lsb1 is implicated in the regulation of Las17 protein levels (Kaminska *et al.* 2011; Spiess *et al.* 2013). As mentioned earlier, Syp1 binds and inhibits Las17, which is achieved through its central region (Boettner *et al.* 2009). Sla1 contains multiple SH3 domains through which it binds to proline-rich motifs in Las17 to inhibit its NPF activity (Rodal *et al.* 2003; Feliciano and Di Pietro 2012). Syp1 and Sla1 also directly associate to form a stable complex in cells and together more effectively block Las17 activity *in vitro* (Feliciano and Di Pietro 2012) (Figure 6A). It should be noted that in biochemical assays testing inhibition of Las17, high ratios of the inhibitors to Las17 (likely higher than those found at endocytic sites *in vivo*) are required to block activity. However, this does not rule out the possibility that clustering at the membrane, lipid/scaffold protein binding, and/or post-translational modifications *in vivo* drive these interactions to improve the efficiency of Las17 inhibition.

How is inhibition of Las17 by Syp1 and Sla1, and possibly Lsb1 and Lsb2, subsequently released to allow actin assembly? The first clue is the observation that Syp1 disappears from patches just before the onset of actin assembly (Boettner *et al.* 2009; Stimpson *et al.* 2009). The mechanism for promoting Syp1 departure from patches is unknown, but one possibility is direct phosphorylation by Ark1 and Prk1 kinases (Huang *et al.* 2003; Albuquerque *et al.* 2008). The second clue is the arrival Bzz1 a few seconds before the onset of actin assembly (Sun *et al.* 2006). Bzz1 binds Las17 via its SH3 domains and is predicted to compete with Sla1 (Soulard *et al.* 2005; Tonikian *et al.* 2009). Further, *S. pombe* Bzz1 stimulates WASP–Arp2/3-mediated actin assembly *in vitro*, and mutants have reduced number of actin patches and impaired patch dynamics (Arasada and Pollard 2011). Bzz1 dimerizes using its F-BAR domain and thus could elevate Las17 NPF activity by organizing two Las17 molecules (Figure 6C). It is tempting to speculate that the combination of Syp1 departing and Sla1 facing new competition from Bzz1 is sufficient to trigger Las17 release at the base of the invagination, allowing it to recruit Arp2/3 complex and

initiate actin assembly. Consistent with this model, mutations in Bzz1 and myosin I are synthetic lethal (Soulard *et al.* 2002), as are mutations in Las17 and myosin I.

Recent studies combining light and electron microscopy have shown that Bzz1 is recruited to endocytic sites when membrane curvature is low, remains at the base of the invagination, and helps shape the tubule in coordination with the BAR domain proteins Rvs161 and Rvs167 (Kishimoto *et al.* 2011). Like F-BAR proteins, Bzz1 may self-assemble to form a rigid collar at the base of the invagination, providing resistance to actin-assembly forces, while Rvs proteins coat the tubule to help shape the vesicle. Bzz1 may also locally activate Las17 at the base of the invagination, directly coordinating membrane remodeling and actin assembly. Similar roles have been suggested for Sla2 and Rvs167 in coordinating membrane and actin remodeling; Sla2 links membranes to F-actin, Sla1, Vrp1, and Ysc84, while Rvs167 links membranes to Las17, Ysc84, Abp1, and Aim3.

The search for Eve: the first mother filament: Even after Las17 is activated, there is another requirement for initiation of an actin network by Arp2/3 complex, which is a first mother filament, or alternatively a mechanism for bypassing the requirement of a first filament. Two different models have been put forth to explain how this might be achieved. First, it has been suggested that short filament fragments produced by severing events at nearby patches or cables may diffuse to new sites of endocytosis and be captured to prime network assembly. This model is supported by one recent study analyzing the effects of cofilin mutants on patch formation (Chen and Pollard 2013). Second, it has been postulated that there is specialized machinery at patches that either (a) induces conformational changes in Arp2/3 complex similar to mother filament binding or (b) recruits and organizes actin monomers into a short F-actin seed that serves as the primer (Figure 6B). In this regard, as noted above, full-length Las17 has NPF activity at least 2 orders of magnitude stronger than just its VCA region (Rodal *et al.* 2003), suggesting that other domains in Las17 might contribute to such a function. Further, the Las17-binding partner Ysc84/Lsb4 binds F-actin and together with Las17 (no Arp2/3) can stimulate actin polymerization (Robertson *et al.* 2009). Further the *S. pombe* WISH/DIP/SPIN90 ortholog Dip1 (Ldb17 in *S. cerevisiae*) binds Arp2/3 complex and stimulates *unbranched* nucleation, suggesting that *in vivo* it may trigger formation by Arp2/3 complex of the first mother filament to initiate actin network assembly, a model that is strongly supported by analysis of its mutant phenotype (Basu and Chang 2011). Interestingly, Ldb17 has interactions with Sla1, Bzz1, and Ysc84/Lsb4, which further suggests that a number of these factors may contribute to a multicomponent regulatory mechanism controlling both the initial restraint and subsequent activation of actin network formation.

Orchestration of multiple NPFs: The next NPFs to arrive at sites of endocytosis, after Pan1 and Las17, are Vrp1 and myosin I, in that order. Live-imaging analysis has shown that Las17 recruits Vrp1, which together with F-actin in turn

recruits myosin I to patches (Soulard *et al.* 2002; Jonsdottir and Li 2004; Sirotkin *et al.* 2005; Sun *et al.* 2006). Current models suggest that myosin I remains anchored to the plasma membrane, while its nucleation activity and motor activity cooperate to produce directional force in facilitating membrane remodeling. Specifically, its barbed end-directed motor activity at the base of the invagination could provide an inward force on the entire actin network to drive tubulation. To facilitate this step, there may be patch components that help cluster and anchor myosin I at the base; one candidate for this role is Pan1, which binds myosin I through its C-terminal proline-rich domain (PRD) and increases the combined NPF activity of myosin I and Vrp1 (Barker *et al.* 2007). How the motor activity of myosin I is coordinated with its NPF effects and if/when myosin I functions in a complex with Las17 remain open questions. Las17 and myosin I activities also may not be restricted to the base of the patch, as originally thought, since more recent studies (using new tags that are possibly more functional) suggest that Las17 and myosin I might be positioned not only at the base of the patch but also at the tip of the invagination (Idrissi *et al.* 2008; Idrissi *et al.* 2012). What role these NPFs play at the tip of the invagination is unclear, but may involve molding the tubular invaginations into more bulbous vesicles (Kukulski *et al.* 2012a).

Last to arrive at patches is Abp1, another poorly understood NPF. *abp1* mutants show a pattern of genetic interactions with other NPFs and *arp2* alleles that suggests an antagonistic relationship with class I NPFs (D'agostino and Goode 2005). Consistent with this model, purified Abp1 competes directly with Las17 VCA region for binding Arp2/3 complex and attenuates VCA stimulatory effects. However, this may not be the full extent of Abp1's Arp2/3-regulating functions. Much like myosin I, which had little effect on Arp2/3 activity until it was combined with its ligand Vrp1 (Sun *et al.* 2006), Abp1 may have important effects on Arp2/3 complex that require the presence of other NPFs and/or Abp1 binding partners.

Actin-filament capping, cross-linking, and stabilization

Once nucleated, daughter filaments in the actin network must be capped rapidly at their barbed ends to (a) restrict filament length and produce a network consisting of densely branched, short interconnected filaments and (b) maintain the actin monomer pool in cells to support rapid growth at new sites of nucleation (Loisel *et al.* 1999; Blanchoin *et al.* 2000) (Figure 4). Thus, barbed end capping plays an instrumental role in enabling networks to generate directional force applied to membrane protrusion in motility and membrane invagination in endocytosis. In addition to being capped, newly formed filaments are decorated along their sides by a number of actin-associated proteins that stabilize, cross-link, and/or bundle F-actin. These proteins may be critical for holding the network together, and for transferring mechanical forces from actin polymerization and myosin activity across the network to deform the membrane (Carlsson and Bayly 2014).

Filament capping: For years, it was presumed that capping of Arp2/3 complex-generated branched networks was mediated solely by heterodimeric "capping protein," Cap1/Cap2 (CP), which has a well-defined structure and interactions with the filament end (reviewed in Cooper and Sept 2008). However, this left the nagging question—Why do mutations in CP cause only minor defects in actin patch organization or dynamics (Amatruda *et al.* 1990; Kim *et al.* 2006)? New insights into this question have emerged from genetic and biochemical studies demonstrating that the combination of the disassembly factors Aip1 and Cof1 (see below) blocks barbed end growth (Okada *et al.* 2002) and that mutations in CP and Aip1 are synthetic lethal (Balcer *et al.* 2003). Further, as mentioned above, a recent study showed that the combination of Abp1 and its ligand Aim3 caps barbed ends and that mutations in either Abp1 or Aim3 display synthetic genetic interactions with CP and Aip1 (Michelot *et al.* 2013). In addition, this study showed that CP and Aip1 localize to distinct regions of actin networks (newer and older, respectively, as visualized in the elongated actin structures of *sla2Δ* mutants) and that when CP is deleted Aip1 spreads into CP territory. Further, a complementary study in *S. pombe* shows that CP spreads into Aip1 territory when AIP1 is deleted (Berro and Pollard 2014). Thus, actin patches have at least three different barbed end-capping mechanisms (CP, Aip1–Cof1, and Abp1–Aim3), although there is much more to learn about their specific roles and mechanisms.

Intriguingly, the phosphoinositide PtdIns(4,5)P$_2$ (which is critical for endocytosis; see *Vesicle Scission and Uncoating*) can weaken interactions between CP and barbed ends in other systems (Schafer *et al.* 1996). Since barbed ends are thought to face the membrane at the base of the endocytic invagination, protection against capping at these locations by PtdIns(4,5)P$_2$ could allow polymerization toward the membrane and thus direct expansion of the actin network in the right orientation.

Filament bundling: Different F-actin crosslinking proteins can organize filaments into parallel and antiparallel bundles or orthogonal arrays, giving rise to networks of filaments with distinct geometries and mechanical properties (Bartles 2000; Pollard and Cooper 2009). Yeast cells have at least five different actin crosslinking proteins that potentially regulate the spatial organization of filaments at sites of endocytosis, Sac6/fimbrin, Scp1/calponin, Abp140, Crn1, and Tef1/Tef2, each discussed in detail below. As mentioned above, it is not yet clear where crosslinking of filaments occurs within the presumably densely branched actin patch networks. One possibility is that debranching effects rapidly remodel specific regions of a patch (see below), and then crosslinking proteins reorganize filaments into bundles or orthogonal arrays. Another possibility is that crosslinks are added to reinforce connections among already branched filaments, providing additional mechanical strength to the network (as illustrated in Figure 4). Since at least five crosslinking proteins potentially act on distinct regions of an actin patch,

they may function during specific temporal phases as the patch matures, elongates, and moves away from the plasma membrane. Further, their individual activities may help generate distinct architectural zones within the network during any given phase.

Sac6/fimbrin: Sac6 was first isolated from yeast-cell extracts on F-actin affinity columns (Drubin *et al.* 1988) and in parallel in genetic screens as a suppressor of an *act1* allele (Adams *et al.* 1989). It is the yeast homolog of the widely expressed protein fimbrin, which bundles filaments into parallel arrays *in vitro* (Bretscher 1981; Glenney *et al.* 1981). The crystal structures of *Arabidopsis thaliana* and *S. Pombe* fimbrin show that it consists of four tandem calponin homology (CH) domains organized into two separate actin-binding units (Klein *et al.* 2004). The 3D reconstructions from electron micrographs of fimbrin-decorated F-actin bundles suggest a regular pattern of crosslinks with a specific geometry (Hanein *et al.* 1998; Volkmann *et al.* 2001). However, there is currently only limited evidence to support a role for yeast Sac6 functioning as a bundler *in vivo*. One study found that a truncated *S. pombe* Sac6/fimbrin defective in bundling F-actin *in vitro* failed to rescue fimbrin function at actin patches *in vivo* (Skau *et al.* 2011), suggesting that bundling might be essential for fimbrin *in vivo* function. However, this truncation mutant reduced actin-binding affinity, leaving open alternative interpretations. There are at least two other biochemical activities of Sac6/fimbrin: (i) F-actin stabilization and (ii) competitive inhibition of tropomyosin decoration of F-actin at patches (Goodman *et al.* 2003; Skau and Kovar 2010). Thus, more work is needed to determine conclusively whether Sac6/fimbrin, or any of the other *in vitro* bundling proteins (below), actually crosslink filaments *in vivo* at actin patches.

Scp1/calponin: Scp1 is the yeast homolog of the mammalian actin-bundling protein calponin (22 kDa), also referred to as SM22 or transgelin. Vertebrate genomes contain three calponin genes, which have important functions in both muscle and nonmuscle cells (Rozenblum and Gimona 2008; Wu and Jin 2008). Yeast Scp1 is composed of an N-terminal CH domain, a short proline-rich segment, and a single C-terminal calponin-like repeat (CLR). Although CH domains bind actin in many proteins (*e.g.*, Sac6/fimbrin and α-actinin), the CH domain of calponin does not (Goodman *et al.* 2003) and instead may bind microtubules and/or other ligands (Rozenblum and Gimona 2008). The C-terminal half of Scp1 mediates actin bundling and harbors two distinct actin-binding sequences, one in the proline-rich region and one in the CLR (Goodman *et al.* 2003; Winder *et al.* 2003; Gheorghe *et al.* 2008). Although *scp1*Δ mutants alone do not cause obvious defects in actin organization or endocytosis, *scp1*Δ *sac6*Δ double mutants exhibit gross abnormalities in cell size, actin organization, and endocytosis, far more severe than defects in *sac6*Δ mutants (Goodman *et al.* 2003). Further, overexpression of Scp1 partially rescues the single-mutant defects of *sac6*Δ. Together, these observations suggest that Scp1 and Sac6 perform related, overlapping functions *in vivo*, which is consistent with bio-

chemical observations showing that each protein bundles and stabilizes F-actin. Sac6 is also upregulated in *scp1*Δ mutants, which may explain why the *scp1*Δ phenotype is not stronger (Gheorghe *et al.* 2008). Live-cell imaging analysis has revealed that Scp1 helps promote inward movement of patches from the plasma membrane (Gheorghe *et al.* 2008). These observations raise the intriguing possibility that Scp1 and Sac6 play a role in further crosslinking Arp2/3-generated branched networks and stiffened them to facilitate myosin I-dependent force transmission during invagination and scission.

Abp140: Abp140 was isolated from yeast cell extracts in an assay for novel F-actin bundling factors, and localizes to both actin patches and cables (Asakura *et al.* 1998). Although its molecular weight is 70 kDa, it was initially thought to have a molecular weight of 140 kDa (likely due to disulfide bond formation), giving rise to its name. To date, Abp140–GFP has provided the only effective marker for live-cell imaging of actin cables (Yang and Pon 2002). In fact, the popular actin marker Lifeact, used to label actin in live-cell imaging studies in a wide range of organisms, is a small peptide derived from Abp140 (Riedl *et al.* 2008). Because *abp140*Δ causes no obvious change in actin organization or dynamics, its role in the actin cytoskeleton may be redundant with other actin-binding proteins, but this has not been explored genetically. More recent studies have revealed that in addition to binding actin, Abp140 is a methyltransferase that modifies the tRNA anticodon loop (D'silva *et al.* 2011; Noma *et al.* 2011). Further, *ABP140* mRNA is tethered to nascent Abp140 protein, which binds actin and moves rapidly with actin cable retrograde flow to reach the back of the mother cell, accumulating at this pole (Kilchert and Spang 2011). These observations provide intriguing new links between actin and protein translational control, adding to the wealth of previous observations connecting actin and translation (Hesketh 1994).

Crn1: Crn1 is the yeast homolog of coronin and binds with high affinity to F-actin and homo-oligomerizes via its C-terminal coiled-coil domain to crosslink actin filaments *in vitro* (Goode *et al.* 1999). The actin-binding surfaces on Crn1's N-terminal β-propeller domain have been mapped to evolutionarily conserved residues and are required for its shared genetic functions with Cof1 (Gandhi *et al.* 2010a). Genetic interactions for *crn1*Δ, as well as the late arrival of Crn1 to actin patches (Lin *et al.* 2010), suggest that its primary role in actin regulation may be to promote turnover (see below); thus, more work is needed to determine whether the actin crosslinking activity is relevant to Crn1's *in vivo* functions.

Tef1/Tef2: Tef1 and Tef2 are the yeast homologs of protein translation elongation factor eEF1A (formerly known as eEF1α), which is one of the most abundant proteins in eukaryotic cells and delivers aminoacyl-tRNA to translating ribosomes. Tef1 and Tef2 are identical in sequence and genetically redundant. In addition to its canonical role, Tef1/Tef2/eEF1A has an important role in F-actin bundling

(Murray *et al.* 1996; Munshi *et al.* 2001). This function depends on a specific actin-binding surface, and mutations at this site abolish actin interactions without affecting protein translation (Gross and Kinzy 2005). Using these alleles, it was shown that Tef1-actin interactions are important for the proper formation of actin cables, which is also consistent with reported interactions between Tef1 and the formin Bni1 (Umikawa *et al.* 1998). Because of its abundance in the cytosol, it has been difficult to determine whether a subpopulation of Tef1 localizes to cables and/or patches, and there have been no investigations into the potential role of Tef1 in endocytosis.

Actin-filament disassembly and turnover

About 3–7 sec after actin assembly is initiated at the cortical patches, components of the disassembly and turnover machinery begin arriving (Okreglak and Drubin 2007; Lin *et al.* 2010). During the next 10–15 sec of the actin patch lifetime, there is continued actin assembly, likely concurrent with disassembly, which predicts a highly dynamic actin network. Maintaining a high rate of turnover may endow the actin network with plasticity, which may be especially important in processes such as endocytosis and lamellipodial protrusion, where the shape of the actin network changes dramatically with membrane invaginations or protrusions. In endocytosis, the shape and architecture of the actin network is likely to change drastically over the course of its 15-sec lifespan (Figure 1, G and H). Based on the measured rate of actin turnover in extended cortical actin patches (0.05 μm sec^{-1}, or about 70 subunits sec^{-1}) (Kaksonen *et al.* 2003), it is possible that the entire F-actin content of a patch turns over three to four times during the patch lifetime. This begs the question—What are the mechanisms that enable such rapid actin turnover, and how are they balanced with new actin assembly, to maintain a network of a particular size while allowing it to rapidly evolve in shape and architecture?

From knowledge gained over the past 17 years, an answer to the question above is that actin-filament turnover is controlled by two major factors: (i) the changing nucleotide state (age) of filaments and (ii) a complex set of machinery that responds to these changes and works collectively to efficiently dismantle older regions of filaments without affecting newer regions. Below, we first discuss the nucleotide state of F-actin and the components of the actin turnover machinery (Figure 7) and then provide an overview of their combined mechanisms in F-actin severing and depolymerization and G-actin recycling (Figure 4).

The nucleotide state of actin: Critical to the control of F-actin disassembly is the age of the filament, specified by its nucleotide state (Pollard *et al.* 2000; Dominguez and Holmes 2011). When each ATP-actin monomer is added to the barbed end of a filament, its noncovalently bound ATP undergoes very rapid hydrolysis to generate ADP and inorganic phosphate (P_i). Hydrolysis occurs at a rate of 0.3 sec^{-1} for rabbit muscle actin, and possibly much faster for yeast actin (Bryan and Rubenstein 2005). The products remain bound to actin,

as ADP + P_i-actin, and then in a subsequent and slower step, P_i is released from the filament to yield ADP-actin. The rate of P_i release for muscle actin is 0.0022 sec^{-1}, but has not been measured for yeast actin. Recent results show that each subunit in a filament formed by muscle actin releases P_i independently, without influence from its neighboring subunits (Jegou *et al.* 2011). Thus, each filament is thought to be a unique mosaic of subunits in different nucleotide states, with the growing end of the filament enriched in ATP and ADP + P_i-actin, and the older end of the filament enriched in ADP-actin. The relative amounts and distribution of each form of actin will depend on at least four factors: (a) the filament length, (b) the filament growth rate, (c) the species-specific rate constants for hydrolysis and P_i release, and (d) the influence of actin-binding proteins on these rates. Importantly, F-actin in each nucleotide state has a distinct conformation, which endows it with different dynamic properties and recruits a different set of actin-associated proteins (Dominguez and Holmes 2011). One unanswered question is what portion of a yeast endocytic actin network exists in each nucleotide state, but this is difficult to answer in the absence of more information on points c and d above. Clearly, this is an area ripe for future investigation.

The actin turnover machinery: Below, we discuss individually each of the seven known, and highly conserved, components of the actin turnover machinery (cofilin, GMF, Aip1, coronin, Srv2/CAP, profilin, and twinfilin), focusing on their protein structures, biochemical activities, and mutant phenotypes in yeast. We then discuss how the activities of these components are orchestrated to facilitate each step in actin turnover (Figure 4 and Figure 7).

Cof1: Cofilin, also called ADF, is one of the more abundant actin-associated proteins in yeast and mammalian cells. It is an 18-kDa globular protein that binds with higher affinity to ADP-actin monomers and filaments and lower affinity to ATP-actin (reviewed in Ono 2007). Cofilin binds cooperatively to F-actin (Blanchoin and Pollard 1999), leading to an uneven pattern of decoration that can be visualized by two-color TIRF microscopy (Suarez *et al.* 2011; Chaudhry *et al.* 2013). Decoration by cofilin "twists" filaments (McGough and Chiu 1999) and causes major conformational rearrangements (Galkin *et al.* 2011). Severing occurs at the boundaries between cofilin-decorated and undecorated regions on filaments due to mechanical discontinuities (Suarez *et al.* 2011). However, it remains to be seen how the severing mechanism is affected when cofilin functions in concert with other actin disassembly factors (below). The *COF1* gene is essential (Moon *et al.* 1993), but partial loss-of-function *cof1* alleles generated by scanning mutagenesis (Lappalainen *et al.* 1997) show defects in patch dynamics just before scission, pointing to an important role for cofilin-mediated actin turnover and remodeling in driving invagination (Lappalainen and Drubin 1997; Okreglak and Drubin 2007, 2010; Lin *et al.* 2010). Additional defects are observed at later stages of endocytosis, including a delayed uncoating of actin from internalized vesicles.
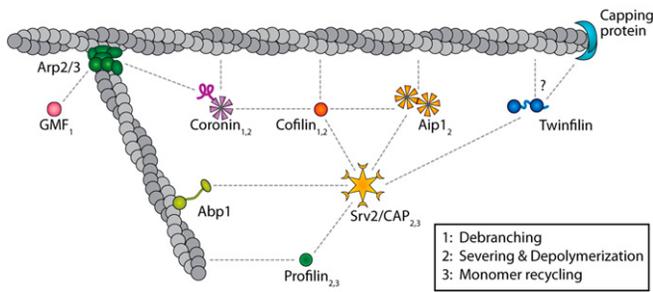
Figure 7 Actin turnover machinery. Scheme showing the proteins that regulate actin-filament debranching, filament severing and depolymerization, and monomer recycling. Dotted lines are physical interactions. Numbers correspond to the designated steps in actin turnover in which each protein participates.

Gmf1: Gmf1, the yeast homolog of mammalian GMF (glia maturation factor), is an 18-kDa protein with structural similarity to cofilin (Goroncy *et al.* 2009). GMF is one of four proteins in the ADFH domain superfamily conserved from yeast to humans: cofilin, Abp1, twinfilin, and GMF (Poukkula *et al.* 2011). Gmf1 has little if any binding affinity for F-actin, but binds strongly to Arp2/3 complex (Figure 7) and genetically interacts with cofilin suggesting a role in turnover (Ikeda *et al.* 2006; Gandhi *et al.* 2010b; Nakano *et al.* 2010). Gmf1 catalyzes debranching of filaments *in vitro* without severing filaments at nonbranch locations (Gandhi *et al.* 2010b). The debranching activity of yeast Gmf1 is conserved in *Drosophila* and mouse GMFs (Poukkula *et al.* 2014) and depends on separate binding interactions with Arp2 and the first actin subunit in the daughter filament, suggesting a modified cofilin-like severing mechanism for debranching (Luan and Nolen 2013; Ydenberg *et al.* 2013). GMF preferably binds to ADP-rather than ATP-Arp2/3 complex (Boczkowska *et al.* 2013), suggesting that it targets "aged" branches. GMF also inhibits Arp2/3-mediated actin nucleation by competing with VCA for binding Arp2/3 (Gandhi *et al.* 2010b; Nakano *et al.* 2010; Ydenberg *et al.* 2013). The *in vivo* relevance of this latter activity is unclear, but could help inhibit branched nucleation by Arp2/3 complex in the absence of NPFs or suppress branched nucleation at the older (ADP-rich) regions of networks to allow filament crosslinkers to act.

Aip1: Aip1 (actin-interacting protein 1) was first identified in a two-hybrid screen for novel interaction partners of yeast actin (Amberg *et al.* 1995) and subsequently as a binding partner of cofilin (Rodal *et al.* 1999). A deletion of *AIP1* causes only mild phenotypes, but is synthetic lethal with specific *cof1* alleles. Purified Aip1 alone has no effect on actin-filament dynamics, but in combination with cofilin promotes the net disassembly of F-actin into monomers and short oligomers (Okada *et al.* 1999; Rodal *et al.* 1999; Okreglak and Drubin 2010). The mechanism underlying these effects is thought to involve enhanced severing and blocking barbed end growth at severed ends (Okada *et al.* 2002; Balcer *et al.* 2003; Ono *et al.* 2004; Brieher *et al.* 2006; Tsuji *et al.* 2009; Michelot *et al.* 2013). Aip1 consists of two β-propellers in one polypeptide chain and its structure has an open clamshell appearance (Voegtli *et al.* 2003; Mohri *et al.* 2004) (Figure

7). The actin- and cofilin-binding surfaces on Aip1 have been defined by mutagenesis (Mohri *et al.* 2004; Clark *et al.* 2006; Okada *et al.* 2006) and are required for Aip1 functions *in vivo* in promoting rapid turnover of actin patches and cables (Okada *et al.* 2006; Okreglak and Drubin 2010). Purified Aip1 binds weakly to F-actin unless it is decorated with cofilin (Okada *et al.* 1999; Rodal *et al.* 1999; Mohri *et al.* 2004; Okada *et al.* 2006), and Aip1–GFP recruitment to actin patches *in vivo* depends on cofilin (Lin *et al.* 2010). Recent biochemical, structural, and cell biological evidence has suggested that Aip1 may enhance severing by competing with Cof1 for its second binding site on F-actin, leading to filament destabilization. Indeed, in support of this model, mutants in *cof1* that disrupt its second F-actin binding site were found to be hyperactive for filament severing (Aggeli *et al.* 2014).

Crn1: Crn1, the yeast homolog of coronin, binds with high affinity to ATP-F-actin and much lower affinity to ADP-F-actin (Goode *et al.* 1999; Cai *et al.* 2007; Gandhi *et al.* 2009). It contains an N-terminal β-propeller domain (Appleton *et al.* 2006), a central unique sequence (highly distinct across distant species) and a C-terminal coiled-coil domain, through which most coronins trimerize (De Hostos 1999) (Figure 7). Crn1 has two separate actin binding sites, one in its β-propeller domain (Gandhi *et al.* 2010a) and another in its coiled-coil domain (Gandhi *et al.* 2009). A *crn1Δ* mutation alone causes no obvious defects in actin organization or endocytosis (Heil-Chapdelaine *et al.* 1998; Goode *et al.* 1999), but exhibits synthetic genetic interactions with *cof1-22* and *act1-159* alleles, suggesting a role in actin turnover (Goode *et al.* 1999; Gandhi *et al.* 2009). Consistent with this view, purified Crn1 promotes severing of ADP–actin filaments by cofilin *in vitro* (Gandhi *et al.* 2009), and in mammalian systems, coronin, cofilin, and Aip1 have been coisolated from cell extracts as a three-component system that promotes the rapid disassembly of ADP-filaments (Brieher *et al.* 2006; Kueh *et al.* 2008). The mechanisms underlying these effects for either yeast or mammalian coronins are not yet defined and require further investigation. Adding to the potential complexity of coronin's roles in actin turnover are the interactions of its unique sequence and C-terminal coiled-coil domain with Arp2/3 complex (Humphries *et al.* 2002; Liu *et al.* 2011).

Srv2: Srv2, also called CAP (cyclase-associated protein), was discovered in yeast as a suppressor of a hyperactivated *ras* allele and as a binding partner of membrane-associated adenylate cyclase (Fedor-Chaiken *et al.* 1990; Field *et al.* 1990). Srv2/CAP is a bifunctional protein. Its N-terminal half (N-Srv2) hexamerizes into shuriken-like structures that associate with cofilin-decorated F-actin and improve the efficiency of severing (Normoyle and Brieher 2012; Chaudhry *et al.* 2013) (Figure 4 and Figure 7). Point mutations in N-Srv2 that abolish the enhanced severing activity *in vitro* also cause striking defects in actin organization *in vivo*. In contrast to these functions, the C-terminal half of the protein (C-Srv2) binds to G-actin, and has high affinity for ADP-G-actin (K_d 18 nM) but 100-fold lower affinity for ATP-G-actin

(Mattila *et al.* 2004). This enables C-Srv2 to potently catalyze recycling of cofilin from ADP-actin monomers and nucleotide exchange on actin (ATP for ADP). These activities depend on the coordinated effects of two separate actin-binding sites in C-Srv2, and mutations that disrupt these G-actin interactions *in vitro* also cause defects in actin organization *in vivo* (Mattila *et al.* 2004; Chaudhry *et al.* 2010). C-Srv2 also has a binding site for profilin, which may enable Srv2 and profilin to work together in monomer recycling (Bertling *et al.* 2007). One recent study showed that the functions of the N and C termini of Srv2 are autonomous, such that the two physically separated halves can function in *trans* both *in vitro* and *in vivo* (Chaudhry *et al.* 2014). However, it was suggested that linkage of the two halves in a single polypeptide may be important for coordinating the two activities *in vivo*. Finally, the structure, activities, and mechanism of this protein were recently shown to be strikingly similar between the yeast and mammalian homologs of Srv2/CAP (Normoyle and Brieher 2012; Jansen *et al.* 2014), demonstrating that its roles in actin regulation have been conserved in evolution.

Profilin: Profilin is a conserved 15-kDa protein that binds preferentially to ATP-actin monomers and also catalyzes nucleotide exchange on actin (ATP for ADP) (Blanchoin and Pollard 1998; Eads *et al.* 1998; Balcer *et al.* 2003). Profilin suppresses spontaneous nucleation of actin and blocks monomer addition to pointed ends while freely allowing addition to barbed ends. As a result, profilin helps to restrict new actin growth *in vivo* to barbed ends generated by nucleation mechanisms. Profilin also binds to specific polyproline-rich tracts in other actin regulatory proteins, such as formins and Srv2 (Chang 1999; Bertling *et al.* 2007) (Figure 4 and Figure 7). The best understood interaction of profilin is with formins, where it plays a critical role in delivering actin monomers to accelerate barbed end elongation. (Kovar *et al.* 2006; Paul and Pollard 2008, 2009). The actin- and polyproline-binding surfaces on profilin are well defined (Schutt *et al.* 1993; Eads *et al.* 1998), and mutations at these sites in yeast profilin (*PFY1*) cause severe depolarization and misorganization of actin structures (Haarer *et al.* 1993; Wolven *et al.* 2000; Lu and Pollard 2001), including a striking loss of actin cables, consistent with its role in formin-mediated actin assembly (Sagot *et al.* 2002; Kovar *et al.* 2006). Further, at least one of the actin-binding-deficient *pfy1* mutants displays defects in fluid-phase endocytosis (Wolven *et al.* 2000). Since there is no evidence supporting a role for profilin in promoting Arp2/3-dependent assembly of branched actin-filament networks at sites of endocytosis, the potential connections between *PFY1* and endocytosis have remained elusive. However, one possibility is that the loss of actin cables resulting from mutations in profilin indirectly compromises endocytosis. Finally, it should be noted that profilin binds PtdIns(4,5)P$_2$ and that this interaction competes with actin binding, raising the possibility that local changes in membrane composition influence profilin's effects on the actin cytoskeleton (Lassing and Lindberg 1985; Ostrander *et al.* 1995).

Twf1: Twf1 (or twinfilin) was identified in yeast both on F-actin affinity columns and in database searches for proteins with homology to Cof1 (Goode *et al.* 1998). Twinfilin is composed of two ADFH domains connected by a short linker sequence and has a short C-terminal tail region (Figure 7). Studies on yeast and mouse twinfilin have shown that the N-terminal ADFH domain binds G-actin, whereas the C-terminal domain binds G- and F-actin (Paavilainen *et al.* 2002; Hellman *et al.* 2006; Paavilainen *et al.* 2007; Paavilainen *et al.* 2008). In addition, the C-terminal tail of yeast twinfilin binds to CP, and this interaction is critical for Twf1 localization to actin patches and Twf1's shared genetic functions with Cof1 (Palmgren *et al.* 2001; Falck *et al.* 2004). However, Twf1 has no detectable effect on CP activity *in vitro*, nor does CP have any detectable effects on Twf1 interactions with G-actin, which has left the functional role of the Twf1–CP interaction unclear. Yeast and mammalian twinfilin proteins bind with high affinity to ADP-G-actin and suppress monomer addition to filament ends (Goode *et al.* 1998; Vartiainen *et al.* 2000), which led to early models suggesting that twinfilin serves primarily as an actin monomer sequestering protein similar to thymosin-β4 (Palmgren *et al.* 2002; Poukkula *et al.* 2011). However, other lines of evidence have pointed to twinfilin-promoting actin-filament disassembly or turnover, including: (i) twinfilin genetically interacts with cofilin in yeast and flies (Goode *et al.* 1998; Wahlstrom *et al.* 2001); (ii) genetic perturbation of twinfilin in yeast cells slows actin turnover, and in mammalian cells slows cell motility (Moseley *et al.* 2006; Meacham *et al.* 2009; Wang *et al.* 2010); (iii) twinfilin localizes to sites of dynamic actin turnover in these systems: patches in yeast, and the leading edge or endosomal actin tails in mammalian cells (Palmgren *et al.* 2001; Helfer *et al.* 2006); (iv) the C-terminal ADFH domain in twinfilin binds F-actin in addition to G-actin and can interact with the barbed end of the filament (Paavilainen *et al.* 2007); and (v) Twf1 associates with the actin turnover-promoting protein Srv2 *in vivo* (Tarassov *et al.* 2008; Breitkreutz *et al.* 2010). Thus, we have included twinfilin here, with the other actin turnover machinery; however, the specific role and mechanism of twinfilin in promoting turnover remains a mystery.

Coordinated mechanisms of actin-filament disassembly and turnover: Actin patches are highly dynamic structures, in which filaments are being rapidly assembled and disassembled concurrently, and the entire network turns over as rapidly as every 3-4 sec (see above). Here, we discuss the process of branched actin-filament network disassembly, which mechanistically can be broken down into three steps, discussed below: debranching, severing and depolymerization, and monomer recycling.

Debranching: Quantitative studies in *S. cerevisiae* and *S. pombe* suggest that each actin patch contains approximately 5000–7000 actin subunits and 100–300 Arp2/3 complexes and capping proteins (Lin *et al.* 2010; Sirotkin *et al.* 2010). Based on these numbers, the actin networks at endocytic sites are predicted to consist of very short filaments with

frequent branch points (one branch per 35–50 actin subunits), consistent with EM studies (Young *et al.* 2004; Rodal *et al.* 2005a). The Arp2/3 complex–actin filament junction is a specialized structure with a unique F-actin conformation (Rouiller *et al.* 2008) and thus may require specialized mechanisms for inducing its destabilization. The branches generated by purified actin and yeast Arp2/3 complex are extremely stable for at least 10 min (Gandhi *et al.* 2010b), whereas *in vivo* they may be turned over every 3–4 sec. How is this achieved? One factor that influences debranching is the nucleotide state of the Arp2 and Arp3 subunits in Arp2/3 complex. ATP binding (but not hydrolysis) on Arp2 and Arp3 is required for nucleation, and then hydrolysis is thought to occur right after nucleation, which influences debranching (Martin *et al.* 2005, 2006; Ingerman *et al.* 2013). Importantly, hydrolysis is not sufficient to promote debranching; additional factors are required. As mentioned above, Gmf1 is a dedicated debranching factor that targets ADP-Arp2/3–actin junctions (Gandhi *et al.* 2010b; Boczkowska *et al.* 2013; Ydenberg *et al.* 2013), and cofilin catalyzes debranching through its ability to bind the sides of ADP-F-actin (Chan *et al.* 2009). Whether Gmf1 and Cof1 biochemically cooperate in debranching has not yet been tested, but would be consistent with their genetic interactions (Gandhi *et al.* 2010b; Nakano *et al.* 2010). A third factor that may contribute to debranching is Crn1, which like Gmf1 binds Arp2/3 complex (Figure 7) and shifts the conformational distribution of the Arp2/3 complex toward the open (inactive) state (Humphries *et al.* 2002; Rodal *et al.* 2005b; Ydenberg *et al.* 2013). These similarities between Crn1 and Gmf1, along with the suggestion that mammalian Coronin-1B promotes debranching (Cai *et al.* 2008), make Crn1 a promising candidate for participating in pruning daughter filaments.

Filament severing and disassembly: How is the filamentous content of an actin patch disassembled and turned over in seconds? As mentioned above, the nucleotide state of actin is paramount in controlling turnover, and the conversion of newer (ATP or ADP + P_i) actin to aged (ADP) actin determines what regions of a network are targeted for demolition. At the center of the filament disassembly process is cofilin. *COF1* is an essential gene and is *required in vivo* for the rapid turnover of actin patches (Lappalainen and Drubin 1997). However, an important but often overlooked fact is that cofilin, while essential, is not *sufficient* to support rates of actin turnover necessary for cell viability. This is evident from genetic observations defining lethal pairs of other actin disassembly factor mutants, including *srv2Δ* and *aip1Δ* (Balcer *et al.* 2003). As described above, there is now strong evidence for at least four other highly conserved actin-binding proteins (coronin, Srv2, Aip1, and Gmf1), and possibly Twf1, functioning in concert with cofilin to catalyze severing and disassembly through a complex multicomponent mechanism (Figure 7). Thus, cofilin should be viewed as one essential component of a larger ensemble that decorates filaments and induces rapid severing and disassembly. At actin patches, the products of enhanced severing are envisioned to be very short oligomers of F-actin,

blocked at their barbed ends from further monomer addition either by physical obstruction of the end or conformational changes in F-actin mediated by disassembly factors. These short F-actin fragments would depolymerize into ADP-monomers (many of which should be cofilin bound) as a result of pointed end dissociation. This step is inherently slow in purified F-actin, so it will be important to determine whether it can be accelerated by one or more of the disassembly factors (Figure 7).

Monomer recycling: The process of actin-filament disassembly produces a rapid and continuous flow of cofilin-bound ADP-actin monomers, and possibly free ADP-actin monomers, which must be rapidly converted back to ATP-actin to enable new rounds of filament growth. The free ADP-actin monomers can be readily recycled due to the inherently fast rate of nucleotide exchange of G-actin, and this step can be accelerated further by binding of profilin or Srv2 (Balcer *et al.* 2003; Chaudhry *et al.* 2010). However, the cofilin-bound ADP-actin monomers pose a much greater barrier to recycling for multiple reasons. First, cofilin strongly suppresses nucleotide exchange. Second, cofilin and profilin competitively bind G-actin. Third, cofilin binds with at least 20-fold higher affinity than profilin to ADP-G-actin (Eads *et al.* 1998; Blanchoin and Pollard 1999). Indeed, profilin has minimal effects in promoting nucleotide exchange on cofilin-bound ADP-actin *in vitro* (Chaudhry *et al.* 2010). In addition, plant profilins fail to catalyze nucleotide exchange on actin even in the absence of cofilin (Chaudhry *et al.* 2007), and yeast profilin has much weaker nucleotide exchange activity than mammalian profilin. In contrast, Srv2 exhibits a very high affinity for ADP-G-actin (20 nM) and readily displaces cofilin from ADP-actin monomers to efficiently catalyze the conversion of cofilin-bound ADP-actin to ATP-actin. Further, it has a binding site for profilin, which may enable profilin and Srv2 to work together in promoting nucleotide exchange (Figure 4) and/or facilitate loading of profilin onto ATP-actin monomers to more rapidly replenish the assembly-competent monomer pool. More work is needed to determine the molecular mechanisms by which these, and possibly other factors, work in concert to control rapid recycling of actin monomers.

Vesicle Scission and Uncoating

The process of remodeling lipid membranes for invagination and scission at endocytic sites is intimately tied to lipid composition and dynamic lipid modifications. Changes in membrane shape rely on force generation by the actin cytoskeleton and are mediated by physical connections between the membrane, coat, and actin network. The role of the membrane and the functions of interacting machinery during endocytosis is an area of intense current investigation, likely to yield many new insights in the coming years. Below we summarize what is known about the roles played by specific lipids in this process. The interested reader is referred to recent literature that provides a more in-depth

discussion of the currently understood roles of lipids and lipid-binding proteins that are hypothesized to mediate vesicle scission (Kishimoto *et al.* 2011; Kukulski *et al.* 2012a; Smaczynska-De *et al.* 2012).

The uncoating process is dominated by protein kinases that phosphorylate the coat proteins, disrupt their interactions, and thus promote disassembly. As summarized below, uncoating is necessary to recycle the coat proteins for subsequent rounds of endocytosis, as well as to create coat-free zones on the vesicle surface and thus allow contact and fusion with endosomes.

Roles for lipids and vesicle scission in endocytosis

Sphingolipids and sterols: The first link between endocytosis and sphingolipids in yeast came from the discovery that a mutation in the long-chain base biosynthetic enzyme Lcb1 caused endocytic defects (Munn and Riezman 1994). Subsequently, it was shown that Pkh1/2 (sphingolipid-stimulated protein kinases) and Ypk1/2 (Pkh1/2-activated protein kinases) also contribute to the regulation of endocytosis (Friant *et al.* 2001; Dehart *et al.* 2002). The Sur7 family of proteins is not only implicated in sphingolipid metabolism (Young *et al.* 2002), but Sur7 was initially found as a suppressor of *rvs167* (Sivadon *et al.* 1997). Interestingly, Rvs167 is the yeast endophilin/amphiphysin homolog and has been directly linked to the scission of invaginated membrane tubules to generate endocytic vesicles (see below). More recently, several endocytic proteins including Rvs167 were shown to bind sphingolipids in a high-throughput lipid-binding screen (Gallego *et al.* 2010).

Sphingolipids and ergosterol interact to form subdomains in the plasma membrane of yeast and are genetically and functionally linked (Guan *et al.* 2009). Ergosterol is the yeast version of cholesterol. The plasma membrane has a 3:1 ratio of ergosterol to phospholipids (Zinser *et al.* 1991); this high level of ergosterol leads to low fluidity/high rigidity of the plasma membrane, restricting the diffusion of transmembrane proteins (Valdez-Taubas and Pelham 2003). A combination of endocytosis and recycling leads to the polarized localization of some transmembrane proteins, such as the v-SNARE Snc1 (Valdez-Taubas and Pelham 2003). Recently, it has become clear that ergosterol and its biosynthetic enzymes are clustered in plasma membrane subdomains referred to as membrane compartment of Can1 (MCCs). When these membrane domains are disrupted, there are global consequences on the structure and organization of the plasma membrane; however, it is currently disputed whether this leads to elevated rates of endocytosis of some cargos or has no effect (Grossmann *et al.* 2008; Brach *et al.* 2011).

Ergosterol was first linked to endocytic functions by the Riezman lab's identification of a mutation in *ERG2/END11* in a genetic screen for endocytosis mutants (Munn and Riezman 1994). Ergosterol biosynthetic enzymes that generate a specific sterol ring structure are required for hyperphosphorylation and internalization of Ste2, presumably via the Yck1/2 protein kinases (Munn *et al.* 1999). However, the deletion of several other *ERG* genes had no effect on internalization of fluid phase or membrane markers and instead slowed their rate of transport through the intracellular endosome/vacuole system (Heese-Peck *et al.* 2002).

Phosphoinositides: Phosphoinositides are low abundance, highly bioactive lipids in the cytosolic leaflet of cellular membranes. Different hydroxyl groups on the inositol head group of phosphatidylinositol (PtdIns) are phosphorylated by specific lipid kinases at particular organelles. This differential phosphorylation can convey information that, along with sorting signals in cargo, recruits adaptors to initiate formation of a transport vesicle at the appropriate compartment. Lipid phosphatases counteract the kinase activities and promote dissociation of adaptors.

PtdIns(4,5)P$_2$ is the most abundant phosphoinositide in the plasma membrane, and early clues that its metabolism might regulate the endocytic machinery came from studies of mammalian systems (Beck and Keen 1991; McPherson *et al.* 1996). Subsequently, lipid kinases and phosphatases required for the biosynthesis and metabolism of PtdIns(4,5)P$_2$ were also found to contribute to endocytosis in yeast cells (Singer-Kruger *et al.* 1998; Audhya *et al.* 2000). In endocytosis, PtdIns(4,5)P$_2$ is thought to help localize endocytic machinery to the initiating endocytic site at the plasma membrane. PtdIns(4,5)P$_2$ degradation by the yeast synaptojanin-like Inp51/52/53 enzymes in turn mediates the release of that machinery after vesicle scission. However, the altered dynamic behavior of endocytic components in *inp51Δ inp52Δ* cells suggests that PtdIns(4,5)P$_2$ turnover regulates multiple stages of endocytosis and not just the disassembly stage (Sun *et al.* 2007). As mentioned above, PtdIns(4,5)P$_2$ disrupts yeast profilin-actin interactions (Lassing and Lindberg 1985; Goldschmidt-Clermont *et al.* 1991; Ostrander *et al.* 1995) and in other systems weakens interaction between CP and actin-filament ends (Schafer *et al.* 1996). In addition, recent evidence suggests that PtdIns(4,5)P$_2$ can relieve Myo5 autoinhibition, leading to Arp2/3-mediated actin nucleation (Fernández-Golbano *et al.* 2014). Thus, phosphoinositides in the membrane may directly govern the activities of multiple components of the actin cytoskeleton during endocytosis.

The subcellular localization of specific phosphoinositide species such as PtdIns(4,5)P$_2$ is studied using tools such as GFP–PH–PLCδ (Varnai and Balla 2008). Interestingly, the distribution of this reporter in the plasma membrane originally appeared to be rather uniform; however, more recent TIRFM/2D-deconvolution shows that PtdIns(4,5)P$_2$ is actually found in subdomains of the plasma membrane (Stefan *et al.* 2002; Spira *et al.* 2012), suggesting that specific mechanisms govern the synthesis, degradation, or sequestration of PtdIns(4,5)P$_2$ (Ling *et al.* 2012).

Another intriguing aspect of lipids in promoting endocytosis comes from studies of phospholipid flippases, which facilitate the movement of lipid molecules between the two membrane leaflets (Chen *et al.* 1999; Farge *et al.* 1999). Drs2 and related flippases have been indirectly linked to

endocytosis; the Drs2 chaperone Cdc50 is a high-copy suppressor of a temperature-sensitive *myo3 myo5* double mutant (Gall *et al.* 2002; Misu *et al.* 2003), and Drs2 depends upon Pan1 for its correct subcellular localization (Liu *et al.* 2007). Perhaps most intriguingly, phospholipid flippases may contribute to membrane curvature and budding by promoting bilayer asymmetry (Graham 2004). This is reminiscent of recent discoveries in ER transport vesicle formation, where highly asymmetric cargo was found to promote membrane curvature (Copic *et al.* 2012). Such a mechanism may explain how budding occurs in cells lacking some *bona fide* membrane remodeling proteins, such as Rvs161 and Rvs167 (see below), by providing an alternative force to drive membrane deformation and vesicle scission (Kishimoto *et al.* 2011).

Proteins that promote vesicle scission: A significant open question is the mechanism(s) by which scission of a membrane bilayer occurs, liberating a vesicle into the cytosol. In animal cells, the key factors implicated in scission are the GTPase dynamin and the BAR domain proteins amphiphysin and endophilin (Campelo and Malhotra 2012). The yeast homologs of amphiphysin and endophilin are Rvs161 and Rvs167, which form a heterodimer (Sivadon *et al.* 1995; Friesen *et al.* 2006). As described above for the F-BAR domain of Syp1, BAR domains are dimeric curved structures with a positively charged concave surface that binds to curved membranes (Peter *et al.* 2004). Some BAR domains also have the capacity to induce curvature by inserting either a helix or a wedge of protein into one leaflet of a membrane bilayer. The Rvs161/167 heterodimer was first implicated in scission when it was observed in *rvs* mutants that ~20% of the cortical patches that had begun to move into the cytosol then moved back to the plasma membrane instead of separating from the membrane and being internalized as a vesicle; this was referred to as a retraction phenotype (Kaksonen *et al.* 2005). Further, the Rvs proteins localize to the midpoint of the invagination, consistent with a function at the position where scission occurs.

A second endocytic vesicle scission mechanism that is well studied in animals involves the membrane-binding GTPase dynamin. Dynamin has an oligomerization-stimulated GTPase activity, a membrane-targeting pleckstrin homology domain, and an SH3 domain that recruits a wide range of PRD-containing proteins (Ferguson and De Camilli 2012). The dynamin oligomer undergoes nucleotide-dependent conformational changes that lead to the scission of the oligomer-wrapped membrane tubule. The closest yeast homolog of dynamin is Vps1, which is necessary for vesicle formation at the Golgi (Vater *et al.* 1992), binds to Rvs167 and is reported to have a retraction phenotype, suggesting that it facilitates vesicle scission at the plasma membrane during endocytosis (Yu and Cai 2004; Smaczynska-De *et al.* 2010, 2012).

Other recent work has suggested a requirement for actin forces and lipid modifications in scission, since the myosin I proteins Myo3/5 and the PtdIns(4,5)-5-phosphatase Inp52/ Sjl2 support the scission function of the Rvs proteins (Kishimoto *et al.* 2011). Sla2, interacting with F-actin, also cooperates with the scission machinery (Boettner *et al.* 2011). Thus, while the magnitudes of the contributions of actin, dynamin, and other conserved components to endocytosis may differ between yeast and metazoans, fundamentally the same machinery and mechanisms are utilized.

Uncoating: Regulation of endocytic proteins through phosphorylation

The final stage of endocytosis is the disassembly of the coat; this allows the components to be reused in new rounds of endocytosis and exposes the membrane of the vesicle for fusion with an endosome. The conserved Ark1/Prk1 kinases have been linked to this disassembly activity (Sekiya-Kawasaki *et al.* 2003), and indeed they appear at endocytic sites during the later stages, when Abp1 and F-actin appear (Zeng *et al.* 2007; Toret *et al.* 2008). Intriguingly, mammalian cells express related protein kinases that also phosphorylate endocytic components to regulate their activities (Greener *et al.* 2000; Umeda *et al.* 2000; Conner and Schmid 2002).

Prk1 was first discovered as a spontaneous suppressor of a *pan1* allele, and Ark1 was identified as a binding partner of Sla2 (Cope *et al.* 1999; Zeng and Cai 1999). The Ark1 and Prk1 kinases appear to be largely redundant in their functions, although there is evidence that they also fulfill some unique roles (Cope *et al.* 1999; Watson *et al.* 2001; Aghamohammadzadeh *et al.* 2014). A third related protein kinase, Akl1, is much less well studied. Many endocytic proteins contain multiple copies of the Prk1 consensus phosphorylation sequence [L/I/V/M]xx[Q/N/T/S]x**T**G, where there is an absolute requirement for the bolded threonine as the acceptor residue (Zeng and Cai 1999; Zeng *et al.* 2001; Huang *et al.* 2003). In *ark1Δ prk1Δ* double-mutant cells, or in cells where the kinase activity is acutely inhibited, large aggregates of membrane, endocytic proteins, and F-actin rapidly accumulate in the cytoplasm (Watson *et al.* 2001; Sekiya-Kawasaki *et al.* 2003). These protein kinases disrupt the activities of and/ or interactions of specific endocytic components (Watson *et al.* 2001; Zeng *et al.* 2001; Chang *et al.* 2002), *e.g.*, interactions between Pan1 and Sla1, but not Pan1 and End3 (Zeng *et al.* 2001; Toshima *et al.* 2007; Chi *et al.* 2012). Phosphorylation of Pan1 is also suggested to influence its ability to interact productively with F-actin and Arp2/3 complex (Toshima *et al.* 2005). Two other factors that affect disassembly of the Pan1 complex are the Arf3 GTPase-activating proteins Gts1 and Lsb5, both of which arrive late to the endocytic coat (Toret *et al.* 2008). Finally, phosphorylation may play a role in uncoating clathrin, since the yeast auxilin homolog Swa2 implicated in this process is heavily phosphorylated (Gall *et al.* 2000; Krantz *et al.* 2013).

Phosphorylation of endocytic proteins is counteracted by protein phosphatases, which allow complexes to reassociate and thus participate in new rounds of endocytosis. There is substantial evidence that dephosphorylation of many endocytic proteins is mediated by Scd5, which serves as an adaptor for

the PP1 protein phosphatase Glc7 (Chang *et al.* 2002; Zeng *et al.* 2007). Sla1 contains a Glc7 binding site that is required for efficient endocytic function and turnover of phosphorylated Sla1. Pan1 and Ent1 also have predicted Glc7 binding sites (Gardiner *et al.* 2007). Recent work shows that Pan1 dephosphorylation and disappearance from patches depends on Pan1 interactions with End3 and recruitment of Scd5 (Whitworth *et al.* 2014). Thus, there is a critical role for the Scd5–Glc7 complex in regulating the dynamics and behavior of the endocytic machinery at cortical patches (Chi *et al.* 2012).

The results above highlight the importance of phosphoregulation in endocytosis, particularly in the final uncoating stage (Sekiya-Kawasaki *et al.* 2003). Such regulation also facilitates crosstalk between the dynamic protein machinery and the underlying lipid bilayer, *e.g.*, through the PtdIns(4,5)-5-phosphatase Inp52/Sjl2 and the membrane-binding endocytic factors Abp1 and Rvs167 by the kinase Tor2 (Kishimoto *et al.* 2011; Tenay *et al.* 2013). Further studies are needed to map the phosphorylation states of other components of the endocytic machinery and thus understand the full extent of phosphoregulation in endocytosis.

Latest Findings, Emerging Views, and Open Questions

Temporal control of endocytosis

Ultrastructure of endocytic sites over time: As with many cellular structures that are below the resolution limit of standard light microscopy, it has been challenging to assign precise locations to proteins at endocytic sites and to determine how their spatial patterns change during the rapid steps of membrane invagination and vesicle scission. The two most fruitful approaches have been quantitative immuno-EM and correlative light and electron microscopy (CLEM) (Idrissi *et al.* 2008; Kukulski *et al.* 2011; Idrissi *et al.* 2012). One recent CLEM study concluded that membrane bending begins with actin polymerization (Kukulski *et al.* 2012a); this seems reasonable given that substantial membrane bending requires forces from actin polymerization, especially given the combination of high turgor pressure and a stiff plasma membrane rich in ergosterol (Basu *et al.* 2014; Carlsson and Bayly 2014). However, a different study using immuno-EM reported that membrane bending preceded the arrival of actin (Idrissi *et al.* 2012). These differences highlight a need for continued efforts on this problem.

Another unresolved issue is the orientation of the actin filaments in the developing patch, which requires having ultrastructural information. To date, it has been extremely challenging to perform myosin S1 decoration coupled with EM to define the orientation of filaments in yeast actin structures. However, Mabuchi and colleagues managed to obtain ultrastructures of *S. pombe* actin cables and cytokinetic rings (Kamasaki *et al.* 2007; Mishra *et al.* 2013), suggesting that with more effort it may be possible to do the same for cortical patches. In the absence of such information for actin patches, models of actin-filament architecture assume that the positions of the NPFs and Arp2/3 complex inform the location of the growing barbed ends. One immuno-EM study reported a marked enrichment of Arp2/3 complex compared to other patch components away from the membrane, which was interpreted to suggest that the growing barbed ends of actin filaments face the base of the invagination (Rodal *et al.* 2005b). Consistent with this view, Las17–GFP was initially reported to localize to the base of the invagination and not move inward with other patch components (Kaksonen *et al.* 2003). However, more recent studies, one using a Las17–GFP fusion with the tag in an alternative position and one using immuno-EM, concluded that Las17 does move in with the membrane invagination (Galletta *et al.* 2008; Idrissi *et al.* 2008). Thus, the field has not yet reached consensus on the locations of NPFs and hence the orientation of F-actin in the cortical patch. More definitive answers may come when higher-resolution instruments are developed and applied to a carefully vetted set of reagents. Further, we anticipate the need to consider whether some of the reported phenotypes of patch mutants have been partially misinterpreted because deletion or tagging of that component caused (then unrealized) compensatory changes, such as premature arrival or departure of another component.

Do checkpoints control transitions Between endocytic coats?: Multicolor live imaging of patch components in yeast cells has become an incredibly powerful assay for defining the roles and dynamics of different endocytic proteins and the consequences of mutations or perturbations. And from such approaches, it is now clear that dozens of endocytic proteins are recruited in an ordered sequence, organized as coats. However, what remain unclear are the rules governing the recruitment and progression through each step in this coordinated program. The transition times between some stages are quite regular. For instance, in wild-type cells the gap between the arrival of the middle and late coat is typically very short, although in certain mutants the gap is prolonged and more variable (*e.g.*, Newpher and Lemmon 2006; Chi *et al.* 2012). In contrast, the early-to-middle coat transition is highly variable even in wild-type cells. The source of this variability is not currently known, but may result from a dependence on an increasing local concentration of transmembrane cargo and/or PtdIns(4,5)P$_2$. If so, how concentrated must the cargo or lipid become, and how is this sensed? Since the length of time between initiation and the arrival of the middle coat is the most variable of all the endocytic stages, it seems likely to involve some kind of checkpoint. What mechanism could be at work is pure speculation at this point, but thoughts turn to there being a critical level of cargo, or a critical level of cargo interactions with early coat proteins leading to formation of an organized structure with multiple copies of several different proteins.

Unresolved issues in actin network formation and dissolution

There are also a number of unresolved questions about how actin networks assemble at sites of endocytosis, how the

filaments are organized, and how the networks facilitate membrane remodeling. While we know that Arp2/3 complex is the actin nucleator and generates short, branched filaments, we do not know whether all, or even most filaments in a patch are branched. Branches could be rapidly pruned by GMF and cofilin, such that only a fraction of filaments in a patch would be branched at any given point. Further, debranching may occur more robustly in specific regions of a patch, or at specific phases of patch maturation. This remodeling may in turn permit crosslinking proteins such as Sac6 and Scp1 to organize filaments into parallel or orthogonal arrays, establishing heterogenous filament geometries within a patch. Whatever form the F-actin takes, another open question is whether proteins are free to diffuse within the network, or is the internal environment too dense, making only the outermost shell accessible to cytosolic factors?

Another key question is what restricts the size of the actin network. There is a large enough supply of building blocks (actin, Arp2/3 complex, etc.) in the cytosol to build many actin patches at once, so what factors limit the growth of an individual endocytic site? Is there limiting access to membrane or to active NPFs? A valuable clue in further investigating this process is the observation that *end3* and *sla1* mutants accumulate fewer but aberrantly large patches (Holtzman *et al.* 1993; Benedetti *et al.* 1994). A related question is what controls the orientation of polymerization to direct the forces that drive membrane invagination and vesicle scission. Critical to this issue may be the precise locations where each NPF is anchored and/or activated. In this regard, superresolution light microscopy may be very helpful in determining if patches have subdomains enriched or depleted of specific proteins.

There are also important questions left to answer concerning the mechanisms regulating the onset of actin patch assembly, as well as transport and subsequent disassembly of the internalized vesicle/patch. As discussed earlier, it is still unresolved how the first actin filaments are nucleated at an actin patch, *i.e.*, whether there is machinery that activates the Arp2/3 complex for nucleation in the absence of a mother filament, or instead a short mother filament is captured, or if specialized machinery assembles a short "starter" seed (as depicted in Figure 6). Further, how is Las17/WASP activated at the onset of actin network formation? Is Bzz1 the switch, or is additional machinery involved? How is Bzz1 recruited to patches at this time, and does it require its F-BAR domain and/or recognition of specific lipids or specific curvature in the membrane? Once vesicles are internalized, how many of them are transported on actin cables, and how are they attached to cables? If a patch moving inward on a cable does not encounter an endosome, does it travel to the back of the mother cell, and if so, what is its fate? Finally, by what mechanisms do internalized vesicles shed their actin coat, and is this a prerequisite for fusion with endosomes?

Alternative pathways for endocytic internalization

Exciting recent evidence demonstrates the existence of an endocytic route that is independent of the canonical clathrin- and Arp2/3-dependent pathway, and instead depends upon Rho1, the formin Bni1, and tropomyosin (Tpm1), critical factors in actin cable assembly (Prosser *et al.* 2011). Consistent with this finding, evidence for an Arp2/3-independent endocytic route has also been obtained in the pathogenic yeast *Candida albicans* (Epp *et al.* 2010). Still another recent study has described an alternative endocytic pathway that is evident even after pharmacological interference with actin polymerization by Latrunculin A (Aghamohammadzadeh *et al.* 2014). This pathway was found to be dependent on Abp1 and its binding partner Ark1, but not the related Prk1 kinase, or several other proteins involved in "canonical" CME. Given the low dose of Latrunculin A used in this study though, and the finding that the Arp2/3-binding A region of Abp1 was required, actin dynamics may still play a role in this pathway. Many questions about these two noncanonical endocytic pathways remain unanswered, including whether they are harnessed to specific cargo or activated by specific environmental conditions. Applying many of the same techniques that have shed so much light on the CME pathway (*e.g.*, live cell imaging of GFP-tagged components and electron microscopy) should allow for a more mechanistic understanding of these alternate pathways.

Comparisons between yeast and mammalian endocytic mechanisms

A fundamental distinction between CME in yeast *vs.* mammalian cells has been the relative functional importance of clathrin and actin in these two systems. Internalization of CME sites in yeast proceeds even in the absence of clathrin, albeit with significantly reduced efficiency (Kaksonen *et al.* 2005; Newpher and Lemmon 2006), whereas clathrin depletion in mammalian cells arrests CME completely (Motley *et al.* 2003; Huang *et al.* 2004). Hence, the current view is that clathrin fulfills a primarily scaffolding/organizational role in yeast, while it mediates a more structural role (in shaping the vesicle) in mammalian cells. In yeast, the requirement for actin polymerization is essential and is believed to contribute the structural role by providing the force for membrane remodeling that opposes the high turgor pressure that exists across the plasma membrane (Aghamohammadzadeh and Ayscough 2009; Basu *et al.* 2014; Carlsson and Bayly 2014). Further, it has traditionally been thought that there is only a minimal requirement for actin during CME in animal cells. However, recent findings show that the requirement for actin polymerization in animal cell CME can depend on the surrounding environment (Basu *et al.* 2014). Two types of clathrin structures have been observed in mammalian cells. One is the classic clathrin-coated pit, which forms a spherical clathrin cage structure with an enclosed membrane, leading to internalization even when actin polymerization is blocked (Ehrlich *et al.* 2004; Loerke *et al.* 2009). The second is referred to as a plaque and is flat and larger than a traditional clathrin pit; these plaques become associated with F-actin as they are internalized (Saffarian *et al.* 2009). Actin appears to convert these clathrin scaffolding plaques into internalizing

structures, often at locations in cells of high transmembrane pressure or tight contact with other cells or extracellular matrix (Saffarian *et al.* 2009; Collins *et al.* 2011). The extracellular matrix is reminiscent of the yeast cell wall, as is the fact that clathrin is not predicted to form a full spherical cage at yeast endocytic sites based on quantification of clathrin abundance per actin patch in *S. pombe* (Berro *et al.* 2010; Sirotkin *et al.* 2010). These observations raise interesting possibilities that require further investigation, including whether there is an analogy to draw between the features of clathrin structures in yeast and the clathrin plaques in mammalian cells. It will be interesting to learn whether the loss of clathrin can be partially tolerated at these sites in mammalian cells as it can be in yeast.

Homeostatic regulation of endocytosis

Response to changing environmental conditions: Localization and trafficking of many nutrient permeases and other transmembrane proteins in the plasma membrane are regulated by the concentrations of particular compounds in the media. For example, there tend to be both high- and low-affinity permeases for specific molecules (*e.g.,* methionine, tryptophan, inositol, or uracil), and typically the high-affinity permease is rapidly internalized and degraded when the cells are exposed to high concentrations of the substance (Levy *et al.* 2011). Similarly, when the metabolic state of the cell changes and cytosolic concentrations of certain compounds are affected (*e.g.,* amino acids or sugars), this can again lead to changes in the residence time and trafficking of particular permeases or transporters (Rubio-Texeira and Kaiser 2006). The general concept of a plasma membrane quality-control system has been proposed, *i.e.,* that there is machinery that detects and removes damaged or misfolded proteins from the plasma membrane, although there is some disagreement on the subject (Lewis and Pelham 2009; Zhao *et al.* 2013). This suggested plasma membrane quality-control system appears to require the activities of the E3 Ub-ligase Rsp5, much like nutrient-triggered transporter internalization (*Cargo sorting signals and adaptors*), raising the possibility that an intermediate conformation in transporter endocytosis resembles a misfolded protein (Keener and Babst 2013). However, there are some differences in terms of what components work together with Rsp5 to mediate recognition and modification of the substrate, including a recently discovered N-terminal domain in many permeases that directs an apparently novel Rsp5-dependent modification (Keener and Babst 2013).

Response to physiological stress: The normal polarized localization of endocytic actin patches to the bud is also sensitive to stress, *e.g.,* elevated temperature or high salt. In stressed wild-type cells, the polarization is temporarily lost; patches become distributed over the mother and bud surface within 30 min of the shock, but they regain a polarized distribution within 90–120 min (Desrivières *et al.* 1998). A common feature of cells with endocytic defects and depolarized actin patches is hyperactivity of cell-wall stress pathways, leading to overly thick cell walls (Tang *et al.* 2000; Dehart *et al.* 2003; Ge *et al.* 2005); this supports the view that the endocytic machinery might internalize enzymes involved in cell-wall remodeling (Chapa-Y-Lazo *et al.* 2014). A number of factors have been implicated in regulating patch redistribution or depolarization, including the cell-wall stress PKC1–MAPK pathway and perturbed sphingolipid metabolism (Delley and Hall 1999; Balguerie *et al.* 2002). For detailed insights into the causes of this depolarization, readers are referred to the article by Howell and Lew in this collection of reviews (Howell and Lew 2012).

Cell cycle and polarity: Cortical actin/endocytic patches have a characteristic distribution pattern in yeast cells, which changes in different stages of mitotic growth (Adams and Pringle 1984; Amberg 1998), suggesting that it is cell-cycle regulated. These distribution patterns are under intense study and are likely to be governed by a combination of inputs that include bud site landmarks, regulatory GTPases that read the landmarks, and other factors such as plasma membrane phospholipids, delivery of secretory cargo by fusion of exocytic vesicles, and feedback loops of signaling pathways (Li *et al.* 1995; Kuo *et al.* 2014). Interestingly, this may also involve the morphogenetic checkpoint mediated by Swe1 kinase, which arrests mitosis in response to defects in actin organization and bud growth (McMillan *et al.* 1998). Recent work in both *C. albicans* and *S. cerevisiae* found evidence for Swe1-dependent phenotypes in endocytosis mutants; combining deletion of *SWE1* with a *sla1* or *sla2* mutation led to an increase in multinucleated cells, indicative of a Swe1-dependent checkpoint acting on (and regulating?) components of the endocytic machinery (Gale *et al.* 2009). Finally, Tor2, a member of the cell-cycle-dependent kinase complex TORC2, appears to play a role in controlling the recruitment of endocytic factors Abp1 and Rvs167 to facilitate internalization of α-factor receptor (Dehart *et al.* 2003; Tenay *et al.* 2013).

Nuclear functions of endocytic proteins

It has long been known that several endocytic proteins in mammalian systems undergo nucleocytoplasmic shuttling and can influence transcription (Vecchi *et al.* 2001). Thus, it was intriguing to find that several yeast endocytic proteins (Scd5, Pan1, Rsp5, and Sla1) likewise spend part of their time in the nucleus (Chang *et al.* 2006). These studies suggest that endocytic proteins may have other important roles in regulating the assembly and activity of multisubunit complexes in the nucleus and have pointed to possible roles for the karyopherins Crm1 and Kap120 in fluid-phase endocytosis. Additionally, the PtdIns(4)P-5-kinase Mss4 is found in both the cytosol and the nucleus, but it must localize to the plasma membrane to generate $PtdIns(4,5)P_2$ and support viability (Audhya and Emr 2003). Bcp1 is a nuclear protein that facilitates Mss4 export from the nucleus to cytosol and restores $PtdIns(4,5)P_2$ synthesis, suggesting a possible regulatory cycle between the nuclear and cytosolic/plasma membrane

compartments. Given the nucleocytoplasmic shuttling by other endocytic proteins, this regulatory cycle is likely to control more than just the levels of PtdIns(4,5)P$_2$.

New approaches for studying yeast endocytosis and actin dynamics

The relatively small size of its genome and the availability of extremely powerful genetic tools have made yeast a pioneering model organism for genome-wide high-throughput analyses and have formed the basis for many systems biology approaches. For example, automated techniques allowed for systematic combination of nonlethal mutants into every possible pairwise double mutant followed by phenotypic analysis (Costanzo *et al.* 2010). This has expanded to analyzing more subtle phenotypes by combining mutations with strains expressing GFP-tagged proteins and determining effects on protein localization or abundance (Breker *et al.* 2013). The small number of proteins encoded by the yeast genome have also made it a fertile testing ground for identifying new protein complexes through genome-wide purification of TAP-tagged proteins coupled to mass spectrometry or genome-wide yeast two-hybrid screens and other protein–protein interaction strategies. The strength of analyzing the effects of one or a few mutations on the whole organism carries over into the ability to test the predictions of sophisticated computational modeling.

The innovations described above played an instrumental role in bringing about key discoveries over the past 17 years and will undoubtedly continue to serve as critical tools in addressing open questions about actin and endocytosis over the coming decades. However, it is anticipated that the next wave of advances will also rely on development and application of new tools and instrumentation, as well as new strategies and approaches. Super-resolution light microscopy may enable one to more precisely define the locations of patch proteins and subdomains at endocytic sites. The development of new fast-acting chemical inhibitors that target specific endocytic patch proteins may enable one to acutely arrest patches in specific stages of their maturation and provide new mechanistic insights into the functional roles of each patch component. Moreover, it may be possible to reconstitute some of the steps in endocytosis *in vitro* and to develop single molecule assays to define the activities and interactions of early patch components. Arguably, many of the important advances made in understanding the mechanisms of actin network formation in endocytosis have come from sophisticated biochemical assays and mathematical modeling, which have provided rigorous, quantitative information about each actin regulatory protein. Future advances are likely to require similar assays for the functions of earlier components in the pathway and will enable us to define their order of assembly, affinities, and specific contributions to the process. Ultimately, this anticipated increase in knowledge of the activities, interactions, and regulation of each component of the endocytic patch may enable us to build a complete model for endocytosis and to test experimentally its design principles and gain unprecedented understanding of this biological process.

Literature Cited

Adams, A. E., and J. R. Pringle, 1984   Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. J. Cell Biol. 98: 934–945.

Adams, A. E., D. Botstein, and D. G. Drubin, 1989   A yeast actin-binding protein is encoded by SAC6, a gene found by suppression of an actin mutation. Science 243: 231–233.

Aggeli, D., E. Kish-Trier, M. C. Lin, B. Haarer, G. Cingolani *et al.*, 2014   Coordination of the filament stabilizing *vs.* destabilizing activities of cofilin through its secondary binding site on actin. Cytoskeleton 71: 361–379.

Aghamohammadzadeh, S., and K. R. Ayscough, 2009   Differential requirements for actin during yeast and mammalian endocytosis. Nat. Cell Biol. 11: 1039–1042.

Aghamohammadzadeh, S., I. I. Smaczynska-de Rooij, and K. R. Ayscough, 2014   An Abp1-dependent route of endocytosis functions when the classical endocytic pathway in yeast is inhibited. PLoS ONE 9: e103311.

Aguilar, R. C., H. A. Watson, and B. Wendland, 2003   The yeast Epsin Ent1 is recruited to membranes through multiple independent interactions. J. Biol. Chem. 278: 10737–10743.

Aguilar, R. C., S. A. Longhi, J. D. Shaw, L. Y. Yeh, S. Kim *et al.*, 2006   Epsin N-terminal homology domains perform an essential function regulating Cdc42 through binding Cdc42 GTPase-activating proteins. Proc. Natl. Acad. Sci. USA 103: 4116–4121.

Albuquerque, C. P., M. B. Smolka, S. H. Payne, V. Bafna, J. Eng *et al.*, 2008   A multidimensional chromatography technology for in-depth phosphoproteome analysis. Mol. Cell. proteomics. 7: 1389–1396.

Amatruda, J. F., J. F. Cannon, K. Tatchell, C. Hug, and J. A. Cooper, 1990   Disruption of the actin cytoskeleton in yeast capping protein mutants. Nature 344: 352–354.

Amberg, D. C., 1998   Three-dimensional imaging of the yeast actin cytoskeleton through the budding cell cycle. Mol. Biol. Cell 9: 3259–3262.

Amberg, D. C., E. Basart, and D. Botstein, 1995   Defining protein interactions with yeast actin in vivo. Nat. Struct. Biol. 2: 28–35.

Anderson, R. G., J. L. Goldstein, and M. S. Brown, 1977   A mutation that impairs the ability of lipoprotein receptors to localise in coated pits on the cell surface of human fibroblasts. Nature 270: 695–699.

Appleton, B. A., P. Wu, and C. Wiesmann, 2006   The crystal structure of murine coronin-1: a regulator of actin cytoskeletal dynamics in lymphocytes. Structure 14: 87–96.

Arasada, R., and T. D. Pollard, 2011   Distinct roles for F-BAR proteins Cdc15p and Bzz1p in actin polymerization at sites of endocytosis in fission yeast. Curr. Biol. 21: 1450–1459.

Asakura, T., T. Sasaki, F. Nagano, A. Satoh, H. Obaishi *et al.*, 1998   Isolation and characterization of a novel actin filament-binding protein from *Saccharomyces cerevisiae*. Oncogene 16: 121–130.

Audhya, A., and S. D. Emr, 2003   Regulation of PI4,5P2 synthesis by nuclear-cytoplasmic shuttling of the Mss4 lipid kinase. EMBO J. 22: 4223–4236.

Audhya, A., M. Foti, and S. D. Emr, 2000   Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. Mol. Biol. Cell 11: 2673–2689.

Baggett, J. J., and B. Wendland, 2001   Clathrin function in yeast endocytosis. Traffic 2: 297–302.

Baggett, J. J., K. E. D'Aquino, and B. Wendland, 2003   The Sla2p talin domain plays a role in endocytosis in *Saccharomyces cerevisiae*. Genetics 165: 1661–1674.

Balcer, H. I., A. L. Goodman, A. A. Rodal, E. Smith, J. Kugler *et al.*, 2003   Coordinated regulation of actin filament turnover by a high-molecular-weight Srv2/CAP complex, cofilin, profilin, and Aip1. Curr. Biol. 13: 2159–2169.

Balcer, H. I., K. Daugherty-Clarke, and B. L. Goode, 2010   The p40/ARPC1 subunit of Arp2/3 complex performs multiple essential roles in WASp-regulated actin nucleation. J. Biol. Chem. 285: 8481–8491.

Balguerie, A., M. Bagnat, M. Bonneu, M. Aigle, and A. M. Breton, 2002   Rvs161p and sphingolipids are required for actin repolarization following salt stress. Eukaryot. Cell 1: 1021–1031.

Barker, S. L., L. Lee, B. D. Pierce, L. Maldonado-Baez, D. G. Drubin *et al.*, 2007   Interaction of the endocytic scaffold protein Pan1 with the type I myosins contributes to the late stages of endocytosis. Mol. Biol. Cell 18: 2893–2903.

Bartles, J. R., 2000   Parallel actin bundles and their multiple actin-bundling proteins. Curr. Opin. Cell Biol. 12: 72–78.

Basu, R., and F. Chang, 2011   Characterization of dip1p reveals a switch in Arp2/3-dependent actin assembly for fission yeast endocytosis. Curr. Biol. 21: 905–916.

Basu, R., E. L. Munteanu, and F. Chang, 2014   Role of turgor pressure in endocytosis in fission yeast. Mol. Biol. Cell 25: 679–687.

Beck, K. A., and J. H. Keen, 1991   Interaction of phosphoinositide cycle intermediates with the plasma membrane-associated clathrin assembly protein AP-2. J. Biol. Chem. 266: 4442–4447.

Beltzner, C. C., and T. D. Pollard, 2004   Identification of functionally important residues of Arp2/3 complex by analysis of homology models from diverse species. J. Mol. Biol. 336: 551–565.

Beltzner, C. C., and T. D. Pollard, 2008   Pathway of actin filament branch formation by Arp2/3 complex. J. Biol. Chem. 283: 7135–7144.

Benedetti, H., S. Raths, F. Crausaz, and H. Riezman, 1994   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: 1023–1037.

Berro, J., and T. D. Pollard, 2014   Synergies between Aip1p and capping protein subunits (Acp1p and Acp2p) in clathrin-mediated endocytosis and cell polarization in fission yeast. Mol. Biol. Cell 25: 3515–3527.

Berro, J., V. Sirotkin, and T. D. Pollard, 2010   Mathematical modeling of endocytic actin patch kinetics in fission yeast: disassembly requires release of actin filament fragments. Mol. Biol. Cell 21: 2905–2915.

Bertling, E., O. Quintero-Monzon, P. K. Mattila, B. L. Goode, and P. Lappalainen, 2007   Mechanism and biological role of profilin-Srv2/CAP interaction. J. Cell Sci. 120: 1225–1234.

Blanchoin, L., and T. D. Pollard, 1998   Interaction of actin monomers with *Acanthamoeba actophorin* (ADF/cofilin) and profilin. J. Biol. Chem. 273: 25106–25111.

Blanchoin, L., and T. D. Pollard, 1999   Mechanism of interaction of *Acanthamoeba actophorin* (ADF/Cofilin) with actin filaments. J. Biol. Chem. 274: 15538–15546.

Blanchoin, L., K. J. Amann, H. N. Higgs, J. B. Marchand, D. A. Kaiser *et al.*, 2000   Direct observation of dendritic actin filament networks nucleated by Arp2/3 complex and WASP/Scar proteins. Nature 404: 1007–1011.

Boczkowska, M., G. Rebowski and R. Dominguez, 2013   GMF interacts with Arp2/3 complex in a nucleotide state-dependent manner. J. Biol. Chem. 288: 25683–25688.

Boeck, R., S. Tarun Jr., M. Rieger, J. A. Deardorff, S. Muller-Auer *et al.*, 1996   The yeast Pan2 protein is required for poly(A)-binding protein-stimulated poly(A)-nuclease activity. J. Biol. Chem. 271: 432–438.

Boettner, D. R., J. L. D'Agostino, O. T. Torres, K. Daugherty-Clarke, A. Uygur *et al.*, 2009   The F-BAR protein Syp1 negatively regulates WASp-Arp2/3 complex activity during endocytic patch formation. Curr. Biol. 19: 1979–1987.

Boettner, D. R., H. Friesen, B. Andrews, and S. K. Lemmon, 2011   Clathrin light chain directs endocytosis by influencing the binding of the yeast Hip1R homologue, Sla2, to F-actin. Mol. Biol. Cell 22: 3699–3714.

Boettner, D. R., R. J. Chi, and S. K. Lemmon, 2012   Lessons from yeast for clathrin-mediated endocytosis. Nat. Cell Biol. 14: 2–10.

Botstein, D., D. C. Amberg, J. Mulholland, T. C. Huffaker, A. E. Adams *et al.*, 1997   The yeast cytoskeleton, pp. 1–90 in *The Molecular and Cellular Biology of the Yeast Saccharomyces,Volume 3: Cell Cycle and Cell Biology*, edited by J. R. Pringle, J. R. Broach, and E. W. Jones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Brach, T., T. Specht, and M. Kaksonen, 2011   Reassessment of the role of plasma membrane domains in the regulation of vesicular traffic in yeast. J. Cell Sci. 124: 328–337.

Brady, R. J., C. K. Damer, J. E. Heuser, and T. J. O'Halloran, 2010   Regulation of Hip1r by epsin controls the temporal and spatial coupling of actin filaments to clathrin-coated pits. J. Cell Sci. 123: 3652–3661.

Breitkreutz, A., H. Choi, J. R. Sharom, L. Boucher, V. Neduva *et al.*, 2010   A global protein kinase and phosphatase interaction network in yeast. Science 328: 1043–1046.

Breker, M., M. Gymrek, and M. Schuldiner, 2013   A novel single-cell screening platform reveals proteome plasticity during yeast stress responses. J. Cell Biol. 200: 839–850.

Bretscher, A., 1981   Fimbrin is a cytoskeletal protein that cross-links F-actin in vitro. Proc. Natl. Acad. Sci. USA 78: 6849–6853.

Brieher, W. M., H. Y. Kueh, B. A. Ballif, and T. J. Mitchison, 2006   Rapid actin monomer-insensitive depolymerization of Listeria actin comet tails by cofilin, coronin, and Aip1. J. Cell Biol. 175: 315–324.

Bryan, K. E., and P. A. Rubenstein, 2005   An intermediate form of ADP-F-actin. J. Biol. Chem. 280: 1696–1703.

Burke, T. A., J. R. Christensen, E. Barone, C. Suarez, V. Sirotkin *et al.*, 2014   Homeostatic actin cytoskeleton networks are regulated by assembly factor competition for monomers. Curr. Biol. 24: 579–585.

Burston, H. E., L. Maldonado-Baez, M. Davey, B. Montpetit, C. Schluter *et al.*, 2009   Regulators of yeast endocytosis identified by systematic quantitative analysis. J. Cell Biol. 185: 1097–1110.

Cai, L., A. M. Makhov, and J. E. Bear, 2007   F-actin binding is essential for coronin 1B function in vivo. J. Cell Sci. 120: 1779–1790.

Cai, L., A. M. Makhov, D. A. Schafer, and J. E. Bear, 2008   Coronin 1B antagonizes cortactin and remodels Arp2/3-containing actin branches in lamellipodia. Cell 134: 828–842.

Campellone, K. G., and M. D. Welch, 2010   A nucleator arms race: cellular control of actin assembly. Nat. Rev. Mol. Cell Biol. 11: 237–251.

Campelo, F., and V. Malhotra, 2012   Membrane fission: the biogenesis of transport carriers. Annu. Rev. Biochem. 81: 407–427.

Carlsson, A. E., and P. V. Bayly, 2014   Force generation by endocytic actin patches in budding yeast. Biophys. J. 106: 1596–1606.

Carroll, S. Y., P. C. Stirling, H. E. Stimpson, E. Giesselmann, M. J. Schmitt *et al.*, 2009   A yeast killer toxin screen provides insights into a/b toxin entry, trafficking, and killing mechanisms. Dev. Cell 17: 552–560.

Carroll, S. Y., H. E. Stimpson, J. Weinberg, C. P. Toret, Y. Sun *et al.*, 2012   Analysis of yeast endocytic site formation and maturation

through a regulatory transition point. Mol. Biol. Cell 23: 657–668.

Chan, C., C. C. Beltzner, and T. D. Pollard, 2009 Cofilin dissociates Arp2/3 complex and branches from actin filaments. Curr. Biol. 19: 537–545.

Chang, F., 1999 Movement of a cytokinesis factor cdc12p to the site of cell division. Curr. Biol. 9: 849–852.

Chang, J. S., K. Henry, B. L. Wolf, M. Geli, and S. K. Lemmon, 2002 Protein phosphatase-1 binding to scd5p is important for regulation of actin organization and endocytosis in yeast. J. Biol. Chem. 277: 48002–48008.

Chang, J. S., K. Henry, M. I. Geli, and S. K. Lemmon, 2006 Cortical recruitment and nuclear-cytoplasmic shuttling of Scd5p, a protein phosphatase-1-targeting protein involved in actin organization and endocytosis. Mol. Biol. Cell 17: 251–262.

Chapa-y-Lazo, B., E. G. Allwood, I. I. Smaczynska-de Rooij, M. L. Snape, and K. R. Ayscough, 2014 Yeast endocytic adaptor AP-2 binds the stress sensor Mid2 and functions in polarized cell responses. Traffic 15: 546–557.

Chaudhry, F., C. Guerin, M. von Witsch, L. Blanchoin, and C. J. Staiger, 2007 Identification of Arabidopsis cyclase-associated protein 1 as the first nucleotide exchange factor for plant actin. Mol. Biol. Cell 18: 3002–3014.

Chaudhry, F., K. Little, L. Talarico, O. Quintero-Monzon, and B. L. Goode, 2010 A central role for the WH2 domain of Srv2/CAP in recharging actin monomers to drive actin turnover in vitro and in vivo. Cytoskeleton 67: 120–133.

Chaudhry, F., D. Breitsprecher, K. Little, G. Sharov, O. Sokolova et al., 2013 Srv2/cyclase-associated protein forms hexameric shurikens that directly catalyze actin filament severing by cofilin. Mol. Biol. Cell 24: 31–41.

Chaudhry, F., S. Jansen, K. Little, C. Suarez, R. Boujemaa-Paterski et al., 2014 Autonomous and in trans functions for the two halves of Srv2/CAP in promoting actin turnover. Cytoskeleton 71: 351–360.

Chen, C. Y., M. F. Ingram, P. H. Rosal, and T. R. Graham, 1999 Role for Drs2p, a P-type ATPase and potential aminophospholipid translocase, in yeast late Golgi function. J. Cell Biol. 147: 1223–1236.

Chen, Q., and T. D. Pollard, 2013 Actin filament severing by cofilin dismantles actin patches and produces mother filaments for new patches. Curr. Biol. 23: 1154–1162.

Chen, Z., D. Borek, S. B. Padrick, T. S. Gomez, Z. Metlagel et al., 2010 Structure and control of the actin regulatory WAVE complex. Nature 468: 533–538.

Chesarone, M. A., and B. L. Goode, 2009 Actin nucleation and elongation factors: mechanisms and interplay. Curr. Opin. Cell Biol. 21: 28–37.

Chi, R. J., O. T. Torres, V. A. Segarra, T. Lansley, J. S. Chang et al., 2012 Role of Scd5, a protein phosphatase-1 targeting protein, in phosphoregulation of Sla1 during endocytosis. J. Cell Sci. 125: 4728–4739.

Chvatchko, Y., I. Howald, and H. Riezman, 1986 Two yeast mutants defective in endocytosis are defective in pheromone response. Cell 46: 355–364.

Clark, M. G., J. Teply, B. K. Haarer, S. C. Viggiano, D. Sept et al., 2006 A genetic dissection of Aip1p's interactions leads to a model for Aip1p-cofilin cooperative activities. Mol. Biol. Cell 17: 1971–1984.

Cocucci, E., F. Aguet, S. Boulant, and T. Kirchhausen, 2012 The first five seconds in the life of a clathrin-coated pit. Cell 150: 495–507.

Collins, A., A. Warrington, K. A. Taylor, and T. Svitkina, 2011 Structural organization of the actin cytoskeleton at sites of clathrin-mediated endocytosis. Curr. Biol. 21: 1167–1175.

Condeelis, J., 2001 How is actin polymerization nucleated in vivo? Trends Cell Biol. 11: 288–293.

Conner, S. D., and S. L. Schmid, 2002 Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis. J. Cell Biol. 156: 921–929.

Cooper, J. A., and D. Sept, 2008 New insights into mechanism and regulation of actin capping protein. Intl. Rev. Cell Mol. Biol. 267: 183–206.

Cope, M. J., S. Yang, C. Shang, and D. G. Drubin, 1999 Novel protein kinases Ark1p and Prk1p associate with and regulate the cortical actin cytoskeleton in budding yeast. J. Cell Biol. 144: 1203–1218.

Copic, A., C. F. Latham, M. A. Horlbeck, J. G. D'Arcangelo, and E. A. Miller, 2012 ER cargo properties specify a requirement for COPII coat rigidity mediated by Sec13p. Science 335: 1359–1362.

Costanzo, M., A. Baryshnikova, J. Bellay, Y. Kim, E. D. Spear et al., 2010 The genetic landscape of a cell. Science 327: 425–431.

D'Agostino, J. L., and B. L. Goode, 2005 Dissection of Arp2/3 complex actin nucleation mechanism and distinct roles for its nucleation-promoting factors in Saccharomyces cerevisiae. Genetics 171: 35–47.

Daugherty, K. M., and B. L. Goode, 2008 Functional surfaces on the p35/ARPC2 subunit of Arp2/3 complex required for cell growth, actin nucleation, and endocytosis. J. Biol. Chem. 283: 16950–16959.

De Craene, J. O., O. Soetens, and B. Andre, 2001 The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease. J. Biol. Chem. 276: 43939–43948.

de Hostos, E. L., 1999 The coronin family of actin-associated proteins. Trends Cell Biol. 9: 345–350.

deHart, A. K., J. D. Schnell, D. A. Allen, and L. Hicke, 2002 The conserved Pkh-Ypk kinase cascade is required for endocytosis in yeast. J. Cell Biol. 156: 241–248.

deHart, A. K., J. D. Schnell, D. A. Allen, J. Y. Tsai, and L. Hicke, 2003 Receptor internalization in yeast requires the Tor2-Rho1 signaling pathway. Mol. Biol. Cell 14: 4676–4684.

Delley, P. A., and M. N. Hall, 1999 Cell wall stress depolarizes cell growth via hyperactivation of RHO1. J. Cell Biol. 147: 163–174.

Desrivières, S., F. T. Cooke, P. J. Parker, and M. N. Hall, 1998 MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in Saccharomyces cerevisiae. J. Biol. Chem. 273: 15787–15793.

Di Pietro, S. M., D. Cascio, D. Feliciano, J. U. Bowie, and G. S. Payne, 2010 Regulation of clathrin adaptor function in endocytosis: novel role for the SAM domain. EMBO J. 29: 1033–1044.

Dominguez, R., and K. C. Holmes, 2011 Actin structure and function. Ann. Rev. Biophys. 40: 169–186.

Dores, M. R., J. D. Schnell, L. Maldonado-Baez, B. Wendland, and L. Hicke, 2010 The function of yeast epsin and Ede1 ubiquitin-binding domains during receptor internalization. Traffic 11: 151–160.

Doyle, T., and D. Botstein, 1996 Movement of yeast cortical actin cytoskeleton visualized in vivo. Proc. Natl. Acad. Sci. USA 93: 3886–3891.

Dreyling, M. H., J. A. Martinez-Climent, M. Zheng, J. Mao, J. D. Rowley et al., 1996 The t(10;11)(p13;q14) in the U937 cell line results in the fusion of the AF10 gene and CALM, encoding a new member of the AP-3 clathrin assembly protein family. Proc. Natl. Acad. Sci. USA 93: 4804–4809.

Drubin, D. G., K. G. Miller, and D. Botstein, 1988 Yeast actin-binding proteins: evidence for a role in morphogenesis. J. Cell Biol. 107: 2551–2561.

D'Silva, S., S. J. Haider, and E. M. Phizicky, 2011 A domain of the actin binding protein Abp140 is the yeast methyltransferase responsible for 3-methylcytidine modification in the tRNA anticodon loop. RNA 17: 1100–1110.

Dulic, V., and H. Riezman, 1989 Characterization of the END1 gene required for vacuole biogenesis and gluconeogenic growth of budding yeast. EMBO J. 8: 1349–1359.

Duncan, M. C., M. J. Cope, B. L. Goode, B. Wendland, and D. G. Drubin, 2001   Yeast Eps15-like endocytic protein, Pan1p, activates the Arp2/3 complex. Nat. Cell Biol. 3: 687–690.

Eads, J. C., N. M. Mahoney, S. Vorobiev, A. R. Bresnick, K. K. Wen et al., 1998   Structure determination and characterization of *Saccharomyces cerevisiae* profilin. Biochemistry 37: 11171–11181.

Eden, S., R. Rohatgi, A. V. Podtelejnikov, M. Mann, and M. W. Kirschner, 2002   Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. Nature 418: 790–793.

Ehrlich, M., W. Boll, A. Van Oijen, R. Hariharan, K. Chandran et al., 2004   Endocytosis by random initiation and stabilization of clathrin-coated pits. Cell 118: 591–605.

Ensenat, D., Z. Yao, X. S. Wang, R. Kori, G. Zhou et al., 1999   A novel src homology 3 domain-containing adaptor protein, HIP-55, that interacts with hematopoietic progenitor kinase 1. J. Biol. Chem. 274: 33945–33950.

Epp, E., A. Walther, G. Lepine, Z. Leon, A. Mullick et al., 2010   Forward genetics in *Candida albicans* that reveals the Arp2/3 complex is required for hyphal formation, but not endocytosis. Mol. Microbiol. 75: 1182–1198.

Erpapazoglou, Z., M. Dhaoui, M. Pantazopoulou, F. Giordano, M. Mari et al., 2012   A dual role for K63-linked ubiquitin chains in multivesicular body biogenesis and cargo sorting. Mol. Biol. Cell 23: 2170–2183.

Evangelista, M., B. M. Klebl, A. H. Tong, B. A. Webb, T. Leeuw et al., 2000   A role for myosin-I in actin assembly through interactions with Vrp1p, Bee1p, and the Arp2/3 complex. J. Cell Biol. 148: 353–362.

Falck, S., V. O. Paavilainen, M. A. Wear, J. G. Grossmann, J. A. Cooper et al., 2004   Biological role and structural mechanism of twinfilin-capping protein interaction. EMBO J. 23: 3010–3019.

Farge, E., D. M. Ojcius, A. Subtil, and A. Dautry-Varsat, 1999   Enhancement of endocytosis due to aminophospholipid transport across the plasma membrane of living cells. Am. J. Physiol. 276: C725–C733.

Fazi, B., M. J. Cope, A. Douangamath, S. Ferracuti, K. Schirwitz et al., 2002   Unusual binding properties of the SH3 domain of the yeast actin-binding protein Abp1: structural and functional analysis. J. Biol. Chem. 277: 5290–5298.

Fedor-Chaiken, M., R. J. Deschenes, and J. R. Broach, 1990   SRV2, a gene required for RAS activation of adenylate cyclase in yeast. Cell 61: 329–340.

Feliciano, D., and S. M. Di Pietro, 2012   SLAC, a complex between Sla1 and Las17, regulates actin polymerization during clathrin-mediated endocytosis. Mol. Biol. Cell 23: 4256–4272.

Ferguson, S. M., and P. De Camilli, 2012   Dynamin, a membrane-remodelling GTPase. Nat. Rev. Mol. Cell Biol. 13: 75–88.

Fernández-Golbano, I. M., F.-Z. Idrissi, J. P. Giblin, B. L. Grosshans, V. Robles et al., 2014   Crosstalk between PI(4,5)P2 and CK2 modulates actin polymerization during endocytic uptake. Dev. Cell 30: 746–758.

Field, J., A. Vojtek, R. Ballester, G. Bolger, J. Colicelli et al., 1990   Cloning and characterization of CAP, the *S. cerevisiae* gene encoding the 70 kd adenylyl cyclase-associated protein. Cell 61: 319–327.

Freeman, N. L., T. Lila, K. A. Mintzer, Z. Chen, A. J. Pahk et al., 1996   A conserved proline-rich region of the *Saccharomyces cerevisiae* cyclase-associated protein binds SH3 domains and modulates cytoskeletal localization. Mol. Cell. Biol. 16: 548–556.

Friant, S., R. Lombardi, T. Schmelzle, M. N. Hall, and H. Riezman, 2001   Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. EMBO J. 20: 6783–6792.

Friesen, H., C. Humphries, Y. Ho, O. Schub, K. Colwill et al., 2006   Characterization of the yeast amphiphysins Rvs161p and Rvs167p reveals roles for the Rvs heterodimer in vivo. Mol. Biol. Cell 17: 1306–1321.

Fujimoto, L. M., R. Roth, J. E. Heuser, and S. L. Schmid, 2000   Actin assembly plays a variable, but not obligatory role

in receptor-mediated endocytosis in mammalian cells. Traffic 1: 161–171.

Gagny, B., A. Wiederkehr, P. Dumoulin, B. Winsor, H. Riezman et al., 2000   A novel EH domain protein of *Saccharomyces cerevisiae*, Ede1p, involved in endocytosis. J. Cell Sci. 113(18): 3309–3319.

Gaidarov, I., F. Santini, R. A. Warren, and J. H. Keen, 1999   Spatial control of coated-pit dynamics in living cells. Nat. Cell Biol. 1: 1–7.

Gale, C. A., M. D. Leonard, K. R. Finley, L. Christensen, M. McClellan et al., 2009   SLA2 mutations cause SWE1-mediated cell cycle phenotypes in *Candida albicans* and *Saccharomyces cerevisiae*. Microbiology 155: 3847–3859.

Galkin, V. E., A. Orlova, D. S. Kudryashov, A. Solodukhin, E. Reisler et al., 2011   Remodeling of actin filaments by ADF/cofilin proteins. Proc. Natl. Acad. Sci. USA 108: 20568–20572.

Gall, W. E., M. A. Higginbotham, C. Chen, M. F. Ingram, D. M. Cyr et al., 2000   The auxilin-like phosphoprotein Swa2p is required for clathrin function in yeast. Curr. Biol. 10: 1349–1358.

Gall, W. E., N. C. Geething, Z. Hua, M. F. Ingram, K. Liu et al., 2002   Drs2p-dependent formation of exocytic clathrin-coated vesicles in vivo. Curr. Biol. 12: 1623–1627.

Gallego, O., M. J. Betts, J. Gvozdenovic-Jeremic, K. Maeda, C. Matetzki et al., 2010   A systematic screen for protein-lipid interactions in *Saccharomyces cerevisiae*. Mol. Syst. Biol. 6: 430.

Galletta, B. J., D. Y. Chuang, and J. A. Cooper, 2008   Distinct roles for Arp2/3 regulators in actin assembly and endocytosis. PLoS Biol. 6: e1.

Gandhi, M., V. Achard, L. Blanchoin, and B. L. Goode, 2009   Coronin switches roles in actin disassembly depending on the nucleotide state of actin. Mol. Cell 34: 364–374.

Gandhi, M., M. Jangi, and B. L. Goode, 2010a   Functional surfaces on the actin-binding protein coronin revealed by systematic mutagenesis. J. Biol. Chem. 285: 34899–34908.

Gandhi, M., B. A. Smith, M. Bovellan, V. Paavilainen, K. Daugherty-Clarke et al., 2010b   GMF is a cofilin homolog that binds Arp2/3 complex to stimulate filament debranching and inhibit actin nucleation. Curr. Biol. 20: 861–867.

Gao, L., and A. Bretscher, 2008   Analysis of unregulated formin activity reveals how yeast can balance F-actin assembly between different microfilament-based organizations. Mol. Biol. Cell 19: 1474–1484.

Gardiner, F. C., R. Costa, and K. R. Ayscough, 2007   Nucleocytoplasmic trafficking is required for functioning of the adaptor protein Sla1p in endocytosis. Traffic 8: 347–358.

Gautreau, A., H. Y. Ho, J. Li, H. Steen, S. P. Gygi et al., 2004   Purification and architecture of the ubiquitous Wave complex. Proc. Natl. Acad. Sci. USA 101: 4379–4383.

Gavin, A. C., M. Bosche, R. Krause, P. Grandi, M. Marzioch et al., 2002   Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415: 141–147.

Ge, W., T. G. Chew, V. Wachtler, S. N. Naqvi, and M. K. Balasubramanian, 2005   The novel fission yeast protein Pal1p interacts with Hip1-related Sla2p/End4p and is involved in cellular morphogenesis. Mol. Biol. Cell 16: 4124–4138.

Geli, M. I., and H. Riezman, 1996   Role of type I myosins in receptor-mediated endocytosis in yeast. Science 272: 533–535.

Gheorghe, D. M., S. Aghamohammadzadeh, R. Smaczynska-de II. E. G. Allwood, S. J. Winder et al., 2008   Interactions between the yeast SM22 homologue Scp1 and actin demonstrate the importance of actin bundling in endocytosis. J. Biol. Chem. 283: 15037–15046.

Glenney, Jr., J. R., P. Kaulfus, P. Matsudaira, and K. Weber, 1981   F-actin binding and bundling properties of fimbrin, a major cytoskeletal protein of microvillus core filaments. J. Biol. Chem. 256: 9283–9288.

Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon et al., 1996   Life with 6000 genes. Science 274(546): 546–567.

Goldschmidt-Clermont, P. J., L. M. Machesky, S. K. Doberstein, and T. D. Pollard, 1991   Mechanism of the interaction of human platelet profilin with actin. J. Cell Biol. 113: 1081–1089.

Goley, E. D., S. E. Rodenbusch, A. C. Martin, and M. D. Welch, 2004 Critical conformational changes in the Arp2/3 complex are induced by nucleotide and nucleation promoting factor. Mol. Cell 16: 269–279.

Goley, E. D., A. Rammohan, E. A. Znameroski, E. N. Firat-Karalar, D. Sept et al., 2010 An actin-filament-binding interface on the Arp2/3 complex is critical for nucleation and branch stability. Proc. Natl. Acad. Sci. USA 107: 8159–8164.

Goode, B. L., D. G. Drubin, and P. Lappalainen, 1998 Regulation of the cortical actin cytoskeleton in budding yeast by twinfilin, a ubiquitous actin monomer-sequestering protein. J. Cell Biol. 142: 723–733.

Goode, B. L., J. J. Wong, A. C. Butty, M. Peter, A. L. McCormack et al., 1999 Coronin promotes the rapid assembly and cross-linking of actin filaments and may link the actin and microtubule cytoskeletons in yeast. J. Cell Biol. 144: 83–98.

Goode, B. L., A. A. Rodal, G. Barnes, and D. G. Drubin, 2001 Activation of the Arp2/3 complex by the actin filament binding protein Abp1p. J. Cell Biol. 153: 627–634.

Goodman, A., B. L. Goode, P. Matsudaira, and G. R. Fink, 2003 The Saccharomyces cerevisiae calponin/transgelin homolog Scp1 functions with fimbrin to regulate stability and organization of the actin cytoskeleton. Mol. Biol. Cell 14: 2617–2629.

Goroncy, A. K., S. Koshiba, N. Tochio, T. Tomizawa, M. Sato et al., 2009 NMR solution structures of actin depolymerizing factor homology domains. Protein Sci. 18: 2384–2392.

Gottlieb, T. A., I. E. Ivanov, M. Adesnik, and D. D. Sabatini, 1993 Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells. J. Cell Biol. 120: 695–710.

Graham, T. R., 2004 Flippases and vesicle-mediated protein transport. Trends Cell Biol. 14: 670–677.

Greener, T., X. Zhao, H. Nojima, E. Eisenberg, and L. E. Greene, 2000 Role of cyclin G-associated kinase in uncoating clathrin-coated vesicles from non-neuronal cells. J. Biol. Chem. 275: 1365–1370.

Gross, S. R., and T. G. Kinzy, 2005 Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. Nat. Struct. Mol. Biol. 12: 772–778.

Grossmann, G., J. Malinsky, W. Stahlschmidt, M. Loibl, I. Weig-Meckl et al., 2008 Plasma membrane microdomains regulate turnover of transport proteins in yeast. J. Cell Biol. 183: 1075–1088.

Guan, X. L., C. M. Souza, H. Pichler, G. Dewhurst, O. Schaad et al., 2009 Functional interactions between sphingolipids and sterols in biological membranes regulating cell physiology. Mol. Biol. Cell 20: 2083–2095.

Gurunathan, S., D. Chapman-Shimshoni, S. Trajkovic, and J. E. Gerst, 2000 Yeast exocytic v-SNAREs confer endocytosis. Mol. Biol. Cell 11: 3629–3643.

Haarer, B. K., S. H. Lillie, A. E. Adams, V. Magdolen, W. Bandlow et al., 1990 Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells. J. Cell Biol. 110: 105–114.

Haarer, B. K., A. S. Petzold, and S. S. Brown, 1993 Mutational analysis of yeast profilin. Mol. Cell. Biol. 13: 7864–7873.

Hamburger, D., M. Egerton, and H. Riezman, 1995 Yeast Gaa1p is required for attachment of a completed GPI anchor onto proteins. J. Cell Biol. 129: 629–639.

Hanein, D., N. Volkmann, S. Goldsmith, A. M. Michon, W. Lehman et al., 1998 An atomic model of fimbrin binding to F-actin and its implications for filament crosslinking and regulation. Nat. Struct. Biol. 5: 787–792.

Haynes, J., B. Garcia, E. J. Stollar, A. Rath, B. J. Andrews et al., 2007 The biologically relevant targets and binding affinity requirements for the function of the yeast actin-binding protein 1 Src-homology 3 domain vary with genetic context. Genetics 176: 193–208.

Heath, R. J., and R. H. Insall, 2008 F-BAR domains: multifunctional regulators of membrane curvature. J. Cell Sci. 121: 1951–1954.

Heese-Peck, A., H. Pichler, B. Zanolari, R. Watanabe, G. Daum et al., 2002 Multiple functions of sterols in yeast endocytosis. Mol. Biol. Cell 13: 2664–2680.

Heil-Chapdelaine, R. A., N. K. Tran and J. A. Cooper, 1998 The role of Saccharomyces cerevisiae coronin in the actin and microtubule cytoskeletons. Curr. Biol. 8: 1281–1284.

Hein, C., J. Y. Springael, C. Volland, R. Haguenauer-Tsapis, and B. Andre, 1995 NPl1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Mol. Microbiol. 18: 77–87.

Helfer, E., E. M. Nevalainen, P. Naumanen, S. Romero, D. Didry et al., 2006 Mammalian twinfilin sequesters ADP-G-actin and caps filament barbed ends: implications in motility. EMBO J. 25: 1184–1195.

Hellman, M., V. O. Paavilainen, A. Annila, P. Lappalainen, and P. Permi, 2006 NMR assignment of the C-terminal ADF-H domain of an actin monomer binding protein, twinfilin. J. Biomol. NMR 36(Suppl. 1): 66.

Henne, W. M., E. Boucrot, M. Meinecke, E. Evergren, Y. Vallis et al., 2010 FCHo proteins are nucleators of clathrin-mediated endocytosis. Science 328: 1281–1284.

Henry, K. R., K. D'Hondt, J. Chang, T. Newpher, K. Huang et al., 2002 Scd5p and clathrin function are important for cortical actin organization, endocytosis, and localization of sla2p in yeast. Mol. Biol. Cell 13: 2607–2625.

Hesketh, J., 1994 Translation and the cytoskeleton: a mechanism for targeted protein synthesis. Mol. Biol. Rep. 19: 233–243.

Hicke, L., and H. Riezman, 1996 Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. Cell 84: 277–287.

Higgs, H. N., L. Blanchoin, and T. D. Pollard, 1999 Influence of the C terminus of Wiskott–Aldrich syndrome protein (WASp) and the Arp2/3 complex on actin polymerization. Biochemistry 38: 15212–15222.

Hofmann, K., and L. Falquet, 2001 A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. Trends Biochem. Sci. 26: 347–350.

Holtzman, D. A., S. Yang, and D. G. Drubin, 1993 Synthetic-lethal interactions identify two novel genes, SLA1 and SLA2, that control membrane cytoskeleton assembly in Saccharomyces cerevisiae. J. Cell Biol. 122: 635–644.

Howard, J. P., J. L. Hutton, J. M. Olson, and G. S. Payne, 2002 Sla1p serves as the targeting signal recognition factor for NPFX(1,2) D-mediated endocytosis. J. Cell Biol. 157: 315–326.

Howell, A. S., and D. J. Lew, 2012 Morphogenesis and the cell cycle. Genetics 190: 51–77.

Huang, B., G. Zeng, A. Y. Ng, and M. Cai, 2003 Identification of novel recognition motifs and regulatory targets for the yeast actin-regulating kinase Prk1p. Mol. Biol. Cell 14: 4871–4884.

Huang, F., A. Khvorova, W. Marshall, and A. Sorkin, 2004 Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. J. Biol. Chem. 279: 16657–16661.

Huang, K. M., K. D'Hondt, H. Riezman, and S. K. Lemmon, 1999 Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast. EMBO J. 18: 3897–3908.

Huh, W. K., J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson et al., 2003 Global analysis of protein localization in budding yeast. Nature 425: 686–691.

Humphries, C. L., H. I. Balcer, J. L. D'Agostino, B. Winsor, D. G. Drubin et al., 2002 Direct regulation of Arp2/3 complex activity and function by the actin binding protein coronin. J. Cell Biol. 159: 993–1004.

Idrissi, F. Z., H. Grotsch, I. M. Fernandez-Golbano, C. Presciatto-Baschong, H. Riezman *et al.*, 2008 Distinct acto/myosin-I structures associate with endocytic profiles at the plasma membrane. J. Cell Biol. 180: 1219–1232.

Idrissi, F. Z., A. Blasco, A. Espinal, and M. I. Geli, 2012 Ultrastructural dynamics of proteins involved in endocytic budding. Proc. Natl. Acad. Sci. USA 109: E2587–E2594.

Idrissi, F.-Z., and M. I. Geli, 2014 Zooming in on the molecular mechanisms of endocytic budding by time-resolved electron microscopy. Cell. Mol. Life Sci. 71: 641–657.

Ikeda, K., R. K. Kundu, S. Ikeda, M. Kobara, H. Matsubara *et al.*, 2006 Glia maturation factor-gamma is preferentially expressed in microvascular endothelial and inflammatory cells and modulates actin cytoskeleton reorganization. Circ. Res. 99: 424–433.

Ingerman, E., J. Y. Hsiao, and R. D. Mullins, 2013 Arp2/3 complex ATP hydrolysis promotes lamellipodial actin network disassembly but is dispensable for assembly. J. Cell Biol. 200: 619–633.

Jansen, S., A. Collins, L. Golden, O. Sokolova and B. L. Goode, 2014 Structure and mechanism of mouse cyclase-associated protein (CAP1) in regulating actin dynamics. J. Biol. Chem. 289: 30732–42.

Jegou, A., T. Niedermayer, J. Orban, D. Didry, R. Lipowsky *et al.*, 2011 Individual actin filaments in a microfluidic flow reveal the mechanism of ATP hydrolysis and give insight into the properties of profilin. PLoS Biol. 9: e1001161.

Jonsdottir, G. A., and R. Li, 2004 Dynamics of yeast Myosin I: evidence for a possible role in scission of endocytic vesicles. Curr. Biol. 14: 1604–1609.

Jung, N., M. Wienisch, M. Gu, J. B. Rand, S. L. Muller *et al.*, 2007 Molecular basis of synaptic vesicle cargo recognition by the endocytic sorting adaptor stonin 2. J. Cell Biol. 179: 1497–1510.

Kaiser, C. A., R. E. Gimeno, and D. A. Shaywitz, 1997 Protein secretion, membrane biogenesis, and endocytosis, pp. 91–228 in *The Molecular and Cellular Biology of the Yeast Saccharomyces, Volume 3: Cell Cycle and Cell Biology*, edited by J. R. Pringle, J. R. Broach, and E. W. Jones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kaksonen, M., Y. Sun, and D. G. Drubin, 2003 A pathway for association of receptors, adaptors, and actin during endocytic internalization. Cell 115: 475–487.

Kaksonen, M., C. P. Toret, and D. G. Drubin, 2005 A modular design for the clathrin- and actin-mediated endocytosis machinery. Cell 123: 305–320.

Kaksonen, M., C. P. Toret, and D. G. Drubin, 2006 Harnessing actin dynamics for clathrin-mediated endocytosis. Nat. Rev. Mol. Cell Biol. 7: 404–414.

Kalthoff, C., J. Alves, C. Urbanke, R. Knorr, and E. J. Ungewickell, 2002 Unusual structural organization of the endocytic proteins AP180 and epsin 1. J. Biol. Chem. 277: 8209–8216.

Kamasaki, T., M. Osumi, and I. Mabuchi, 2007 Three-dimensional arrangement of F-actin in the contractile ring of fission yeast. J. Cell Biol. 178: 765–771.

Kaminska, J., M. Spiess, M. Stawiecka-Mirota, R. Monkaityte, R. Haguenauer-Tsapis *et al.*, 2011 Yeast Rsp5 ubiquitin ligase affects the actin cytoskeleton in vivo and in vitro. Eur. J. Cell Biol. 90: 1016–1028.

Kaouass, M., I. Gamache, D. Ramotar, M. Audette, and R. Poulin, 1998 The spermidine transport system is regulated by ligand inactivation, endocytosis, and by the Npr1p Ser/Thr protein kinase in *Saccharomyces cerevisiae*. J. Biol. Chem. 273: 2109–2117.

Kasanov, J., G. Pirozzi, A. J. Uveges, and B. K. Kay, 2001 Characterizing Class I WW domains defines key specificity determinants and generates mutant domains with novel specificities. Chem. Biol. 8: 231–241.

Keener, J. M., and M. Babst, 2013 Quality control and substrate-dependent downregulation of the nutrient transporter Fur4. Traffic 14: 412–427.

Kessels, M. M., A. E. Engqvist-Goldstein, and D. G. Drubin, 2000 Association of mouse actin-binding protein 1 (mAbp1/SH3P7), an Src kinase target, with dynamic regions of the cortical actin cytoskeleton in response to Rac1 activation. Mol. Biol. Cell 11: 393–412.

Kilchert, C., and A. Spang, 2011 Cotranslational transport of ABP140 mRNA to the distal pole of *S. cerevisiae*. EMBO J. 30: 3567–3580.

Kim, K., B. J. Galletta, K. O. Schmidt, F. S. Chang, K. J. Blumer *et al.*, 2006 Actin-based motility during endocytosis in budding yeast. Mol. Biol. Cell 17: 1354–1363.

Kishimoto, T., Y. Sun, C. Buser, J. Liu, A. Michelot *et al.*, 2011 Determinants of endocytic membrane geometry, stability, and scission. Proc. Natl. Acad. Sci. USA 108: E979–E988.

Klein, M. G., W. Shi, U. Ramagopal, Y. Tseng, D. Wirtz *et al.*, 2004 Structure of the actin crosslinking core of fimbrin. Structure 12: 999–1013.

Kolling, R., and C. P. Hollenberg, 1994 The ABC-transporter Ste6 accumulates in the plasma membrane in a ubiquitinated form in endocytosis mutants. EMBO J. 13: 3261–3271.

Kovar, D. R., E. S. Harris, R. Mahaffy, H. N. Higgs, and T. D. Pollard, 2006 Control of the assembly of ATP- and ADP-actin by formins and profilin. Cell 124: 423–435.

Krantz, K. C., J. Puchalla, R. Thapa, C. Kobayashi, M. Bisher *et al.*, 2013 Clathrin coat disassembly by the yeast Hsc70/Ssa1p and auxilin/Swa2p proteins observed by single-particle burst analysis spectroscopy. J. Biol. Chem. 288: 26721–26730.

Kubler, E., and H. Riezman, 1993 Actin and fimbrin are required for the internalization step of endocytosis in yeast. EMBO J. 12: 2855–2862.

Kueh, H. Y., G. T. Charras, T. J. Mitchison, and W. M. Brieher, 2008 Actin disassembly by cofilin, coronin, and Aip1 occurs in bursts and is inhibited by barbed-end cappers. J. Cell Biol. 182: 341–353.

Kukulski, W., M. Schorb, S. Welsch, A. Picco, M. Kaksonen *et al.*, 2011 Correlated fluorescence and 3D electron microscopy with high sensitivity and spatial precision. J. Cell Biol. 192: 111–119.

Kukulski, W., M. Schorb, M. Kaksonen, and J. A. Briggs, 2012a Plasma membrane reshaping during endocytosis is revealed by time-resolved electron tomography. Cell 150: 508–520.

Kukulski, W., M. Schorb, S. Welsch, A. Picco, M. Kaksonen *et al.*, 2012b Precise, correlated fluorescence microscopy and electron tomography of lowicryl sections using fluorescent fiducial markers. Methods Cell Biol. 111: 235–257.

Kuo, C.-C., N. S. Savage, H. Chen, C.-F. Wu, T. R. Zyla *et al.*, 2014 Inhibitory GEF phosphorylation provides negative feedback in the yeast polarity circuit. Curr. Biol. 24: 753–759.

Lappalainen, P., and D. G. Drubin, 1997 Cofilin promotes rapid actin filament turnover in vivo. Nature 388: 78–82.

Lappalainen, P., E. V. Fedorov, A. A. Fedorov, S. C. Almo, and D. G. Drubin, 1997 Essential functions and actin-binding surfaces of yeast cofilin revealed by systematic mutagenesis. EMBO J. 16: 5520–5530.

Larbolette, O., B. Wollscheid, J. Schweikert, P. J. Nielsen, and J. Wienands, 1999 SH3P7 is a cytoskeleton adapter protein and is coupled to signal transduction from lymphocyte antigen receptors. Mol. Cell. Biol. 19: 1539–1546.

Lassing, I., and U. Lindberg, 1985 Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. Nature 314: 472–474.

Layton, A. T., N. S. Savage, A. S. Howell, S. Y. Carroll, D. G. Drubin *et al.*, 2011 Modeling vesicle traffic reveals unexpected consequences for Cdc42p-mediated polarity establishment. Curr. Biol. **21**: 184–194.

Lechler, T., A. Shevchenko, and R. Li, 2000 Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. J. Cell Biol. 148: 363–373.

Lemmon, S. K., and E. W. Jones, 1987   Clathrin requirement for normal growth of yeast. Science 238: 504–509.

Levy, S., M. Kafri, M. Carmi, and N. Barkai, 2011   The competitive advantage of a dual-transporter system. Science 334: 1408–1412.

Lewis, M. J., and H. R. Pelham, 2009   Inefficient quality control of thermosensitive proteins on the plasma membrane. PLoS ONE 4: e5038.

Li, R., 1997   Bee1, a yeast protein with homology to Wiscott–Aldrich syndrome protein, is critical for the assembly of cortical actin cytoskeleton. J. Cell Biol. 136: 649–658.

Li, R., Y. Zheng, and D. G. Drubin, 1995   Regulation of cortical actin cytoskeleton assembly during polarized cell growth in budding yeast. J. Cell Biol. 128: 599–615.

Lila, T., and D. G. Drubin, 1997   Evidence for physical and functional interactions among two Saccharomyces cerevisiae SH3 domain proteins, an adenylyl cyclase-associated protein and the actin cytoskeleton. Mol. Biol. Cell 8: 367–385.

Lin, C. H., J. A. MacGurn, T. Chu, C. J. Stefan, and S. D. Emr, 2008   Arrestin-related ubiquitin-ligase adaptors regulate endocytosis and protein turnover at the cell surface. Cell 135: 714–725.

Lin, M. C., B. J. Galletta, D. Sept, and J. A. Cooper, 2010   Overlapping and distinct functions for cofilin, coronin and Aip1 in actin dynamics in vivo. J. Cell Sci. 123: 1329–1342.

Ling, Y., C. J. Stefan, J. A. Macgurn, A. Audhya, and S. D. Emr, 2012   The dual PH domain protein Opy1 functions as a sensor and modulator of PtdIns(4,5)P(2) synthesis. EMBO J. 31: 2882–2894.

Liu, K., Z. Hua, J. A. Nepute, and T. R. Graham, 2007   Yeast P4-ATPases Drs2p and Dnf1p are essential cargos of the NPFXD/Sla1p endocytic pathway. Mol. Biol. Cell 18: 487–500.

Liu, S.-L., K. M. Needham, J. R. May, and B. J. Nolen, 2011   Mechanism of a concentration-dependent switch between activation and inhibition of Arp2/3 complex by coronin. J. Biol. Chem. 286: 17039–17046.

Loerke, D., M. Mettlen, D. Yarar, K. Jaqaman, H. Jaqaman et al., 2009   Cargo and dynamin regulate clathrin-coated pit maturation. PLoS Biol. 7: e57.

Loisel, T. P., R. Boujemaa, D. Pantaloni, and M. F. Carlier, 1999   Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. Nature 401: 613–616.

Lu, J., and T. D. Pollard, 2001   Profilin binding to poly-l-proline and actin monomers along with ability to catalyze actin nucleotide exchange is required for viability of fission yeast. Mol. Biol. Cell 12: 1161–1175.

Luan, Q., and B. J. Nolen, 2013   Structural basis for regulation of Arp2/3 complex by GMF. Nat. Struct. Mol. Biol. 20: 1062–1068.

MacGurn, J. A., P. C. Hsu, M. B. Smolka, and S. D. Emr, 2011   TORC1 regulates endocytosis via Npr1-mediated phosphoinhibition of a ubiquitin ligase adaptor. Cell 147: 1104–1117.

Machesky, L. M., S. J. Atkinson, C. Ampe, J. Vandekerckhove, and T. D. Pollard, 1994   Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. J. Cell Biol. 127: 107–115.

Machesky, L. M., R. D. Mullins, H. N. Higgs, D. A. Kaiser, L. Blanchoin et al., 1999   Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. Proc. Natl. Acad. Sci. USA 96: 3739–3744.

Mahadev, R. K., S. M. Di Pietro, J. M. Olson, H. L. Piao, G. S. Payne et al., 2007   Structure of Sla1p homology domain 1 and interaction with the NPFxD endocytic internalization motif. EMBO J. 26: 1963–1971.

Maldonado-Baez, L., M. R. Dores, E. M. Perkins, T. G. Drivas, L. Hicke et al., 2008   Interaction between Epsin/Yap180 adaptors and the scaffolds Ede1/Pan1 is required for endocytosis. Mol. Biol. Cell 19: 2936–2948.

Maritzen, T., S. J. Koo and V. Haucke, 2012   Turning CALM into excitement: AP180 and CALM in endocytosis and disease. Biol. Cell **104:** 588–602.

Martin, A. C., M. D. Welch, and D. G. Drubin, 2006   Arp2/3 ATP hydrolysis-catalysed branch dissociation is critical for endocytic force generation. Nat. Cell Biol. 8: 826–833.

Martin, A. C., X. P. Xu, I. Rouiller, M. Kaksonen, Y. Sun et al., 2005   Effects of Arp2 and Arp3 nucleotide-binding pocket mutations on Arp2/3 complex function. J. Cell Biol. 168: 315–328.

Mattila, P. K., O. Quintero-Monzon, J. Kugler, J. B. Moseley, S. C. Almo et al., 2004   A high-affinity interaction with ADP-actin monomers underlies the mechanism and in vivo function of Srv2/cyclase-associated protein. Mol. Biol. Cell 15: 5158–5171.

McGough, A., and W. Chiu, 1999   ADF/cofilin weakens lateral contacts in the actin filament. J. Mol. Biol. 291: 513–519.

McMahon, H. T., and E. Boucrot, 2011   Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat. Rev. Mol. Cell Biol. 12: 517–533.

McMillan, J. N., R. A. Sia, and D. J. Lew, 1998   A morphogenesis checkpoint monitors the actin cytoskeleton in yeast. J. Cell Biol. 142: 1487–1499.

McPherson, P. S., E. P. Garcia, V. I. Slepnev, C. David, X. Zhang et al., 1996   A presynaptic inositol-5-phosphatase. Nature 379: 353–357.

Meacham, C. E., E. E. Ho, E. Dubrovsky, F. B. Gertler, and M. T. Hemann, 2009   In vivo RNAi screening identifies regulators of actin dynamics as key determinants of lymphoma progression. Nat. Genet. 41: 1133–1137.

Merhi, A., and B. Andre, 2012   Internal amino acids promote Gap1 permease ubiquitylation via TORC1/Npr1/14–3-3-dependent control of the Bul arrestin-like adaptors. Mol. Cell. Biol. 32: 4510–4522.

Merrifield, C. J., M. E. Feldman, L. Wan, and W. Almers, 2002   Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. Nat. Cell Biol. 4: 691–698.

Michelot, A., and D. G. Drubin, 2011   Building distinct actin filament networks in a common cytoplasm. Curr. Biol. **21:** R560–569.

Michelot, A., M. Costanzo, A. Sarkeshik, C. Boone, J. R. Yates, 3rd et al., 2010   Reconstitution and protein composition analysis of endocytic actin patches. Curr. Biol. **20:** 1890–1899.

Michelot, A., A. Grassart, V. Okreglak, M. Costanzo, C. Boone et al., 2013   Actin filament elongation in Arp2/3-derived networks is controlled by three distinct mechanisms. Dev. Cell 24: 182–195.

Michelsen, K., V. Schmid, J. Metz, K. Heusser, U. Liebel et al., 2007   Novel cargo-binding site in the beta and delta subunits of coatomer. J. Cell Biol. 179: 209–217.

Miliaras, N. B., and B. Wendland, 2004   EH proteins: multivalent regulators of endocytosis (and other pathways). Cell Biochem. Biophys. 41: 295–318.

Mishra, M., J. Kashiwazaki, T. Takagi, R. Srinivasan, Y. Huang et al., 2013   In vitro contraction of cytokinetic ring depends on myosin II but not on actin dynamics. Nat. Cell Biol. 15: 853–859.

Misu, K., K. Fujimura-Kamada, T. Ueda, A. Nakano, H. Katoh et al., 2003   Cdc50p, a conserved endosomal membrane protein, controls polarized growth in Saccharomyces cerevisiae. Mol. Biol. Cell 14: 730–747.

Mohri, K., S. Vorobiev, A. A. Fedorov, S. C. Almo, and S. Ono, 2004   Identification of functional residues on Caenorhabditis elegans actin-interacting protein 1 (UNC-78) for disassembly of actin depolymerizing factor/cofilin-bound actin filaments. J. Biol. Chem. 279: 31697–31707.

Moon, A. L., P. A. Janmey, K. A. Louie, and D. G. Drubin, 1993   Cofilin is an essential component of the yeast cortical cytoskeleton. J. Cell Biol. 120: 421–435.

Mooren, O. L., B. J. Galletta, and J. A. Cooper, 2012   Roles for actin assembly in endocytosis. Annu. Rev. Biochem. 81: 661–686.

Moseley, J. B., and B. L. Goode, 2006   The yeast actin cytoskeleton: from cellular function to biochemical mechanism. Microbiol. Mol. Biol. Rev. 70: 605–645.

Moseley, J. B., K. Okada, H. I. Balcer, D. R. Kovar, T. D. Pollard *et al.*, 2006   Twinfilin is an actin-filament-severing protein and promotes rapid turnover of actin structures in vivo. J. Cell Sci. 119: 1547–1557.

Motley, A., N. A. Bright, M. N. Seaman, and M. S. Robinson, 2003   Clathrin-mediated endocytosis in AP-2-depleted cells. J. Cell Biol. 162: 909–918.

Mulholland, J., D. Preuss, A. Moon, A. Wong, D. Drubin *et al.*, 1994   Ultrastructure of the yeast actin cytoskeleton and its association with the plasma membrane. J. Cell Biol. 125: 381–391.

Mullins, R. D., J. F. Kelleher, and T. D. Pollard, 1996   Actin' like actin? Trends Cell Biol. 6: 208–212.

Mullins, R. D., J. A. Heuser, and T. D. Pollard, 1998   The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proc. Natl. Acad. Sci. USA 95: 6181–6186.

Munn, A. L., and H. Riezman, 1994   Endocytosis is required for the growth of vacuolar H(+)-ATPase-defective yeast: identification of six new END genes. J. Cell Biol. 127: 373–386.

Munn, A. L., B. J. Stevenson, M. I. Geli, and H. Riezman, 1995   end5, end6, and end7: mutations that cause actin delocalization and block the internalization step of endocytosis in *Saccharomyces cerevisiae*. Mol. Biol. Cell 6: 1721–1742.

Munn, A. L., A. Heese-Peck, B. J. Stevenson, H. Pichler, and H. Riezman, 1999   Specific sterols required for the internalization step of endocytosis in yeast. Mol. Biol. Cell 10: 3943–3957.

Munshi, R., K. A. Kandl, A. Carr-Schmid, J. L. Whitacre, A. E. Adams *et al.*, 2001   Overexpression of translation elongation factor 1A affects the organization and function of the actin cytoskeleton in yeast. Genetics 157: 1425–1436.

Murray, J. W., B. T. Edmonds, G. Liu, and J. Condeelis, 1996   Bundling of actin filaments by elongation factor 1 alpha inhibits polymerization at filament ends. J. Cell Biol. 135: 1309–1321.

Nakano, K., H. Kuwayama, M. Kawasaki, O. Numata, and M. Takaine, 2010   GMF is an evolutionarily developed Adf/cofilin-super family protein involved in the Arp2/3 complex-mediated organization of the actin cytoskeleton. Cytoskeleton 67: 373–382.

Naqvi, S. N., R. Zahn, D. A. Mitchell, B. J. Stevenson, and A. L. Munn, 1998   The WASp homologue Las17p functions with the WIP homologue End5p/verprolin and is essential for endocytosis in yeast. Curr. Biol. 8: 959–962.

Nelson, K. K., and S. K. Lemmon, 1993   Suppressors of clathrin deficiency: overexpression of ubiquitin rescues lethal strains of clathrin-deficient *Saccharomyces cerevisiae*. Mol. Cell. Biol. 13: 521–532.

Nelson, H., and N. Nelson, 1990   Disruption of genes encoding subunits of yeast vacuolar H(+)-ATPase causes conditional lethality. Proc. Natl. Acad. Sci. USA 87: 3503–3507.

Newpher, T. M., and S. K. Lemmon, 2006   Clathrin is important for normal actin dynamics and progression of Sla2p-containing patches during endocytosis in yeast. Traffic 7: 574–588.

Newpher, T. M., R. P. Smith, V. Lemmon, and S. K. Lemmon, 2005   In vivo dynamics of clathrin and its adaptor-dependent recruitment to the actin-based endocytic machinery in yeast. Dev. Cell 9: 87–98.

Nikko, E., and H. R. Pelham, 2009   Arrestin-mediated endocytosis of yeast plasma membrane transporters. Traffic 10: 1856–1867.

Noma, A., S. Yi, T. Katoh, Y. Takai, and T. Suzuki, 2011   Actin-binding protein ABP140 is a methyltransferase for 3-methylcytidine at position 32 of tRNAs in *Saccharomyces cerevisiae*. RNA 17: 1111–1119.

Normoyle, K. P., and W. M. Brieher, 2012   Cyclase-associated protein (CAP) acts directly on F-actin to accelerate cofilin-mediated actin severing across the range of physiological pH. J. Biol. Chem. 287: 35722–35732.

Okada, K., T. Obinata, and H. Abe, 1999   XAIP1: a Xenopus homologue of yeast actin interacting protein 1 (AIP1), which induces disassembly of actin filaments cooperatively with ADF/cofilin family proteins. J. Cell Sci. 112(10): 1553–1565.

Okada, K., L. Blanchoin, H. Abe, H. Chen, T. D. Pollard *et al.*, 2002   Xenopus actin-interacting protein 1 (XAip1) enhances cofilin fragmentation of filaments by capping filament ends. J. Biol. Chem. 277: 43011–43016.

Okada, K., H. Ravi, E. M. Smith, and B. L. Goode, 2006   Aip1 and cofilin promote rapid turnover of yeast actin patches and cables: a coordinated mechanism for severing and capping filaments. Mol. Biol. Cell 17: 2855–2868.

Okreglak, V., and D. G. Drubin, 2007   Cofilin recruitment and function during actin-mediated endocytosis dictated by actin nucleotide state. J. Cell Biol. 178: 1251–1264.

Okreglak, V., and D. G. Drubin, 2010   Loss of Aip1 reveals a role in maintaining the actin monomer pool and an in vivo oligomer assembly pathway. J. Cell Biol. 188: 769–777.

Ono, S., 2007   Mechanism of depolymerization and severing of actin filaments and its significance in cytoskeletal dynamics. Int. Rev. Cytol. 258: 1–82.

Ono, S., K. Mohri, and K. Ono, 2004   Microscopic evidence that actin-interacting protein 1 actively disassembles actin-depolymerizing factor/Cofilin-bound actin filaments. J. Biol. Chem. 279: 14207–14212.

Ostrander, D. B., J. A. Gorman, and G. M. Carman, 1995   Regulation of profilin localization in *Saccharomyces cerevisiae* by phosphoinositide metabolism. J. Biol. Chem. 270: 27045–27050.

Owen, D. J., and P. R. Evans, 1998   A structural explanation for the recognition of tyrosine-based endocytotic signals. Science 282: 1327–1332.

Paavilainen, V. O., M. C. Merckel, S. Falck, P. J. Ojala, E. Pohl *et al.*, 2002   Structural conservation between the actin monomer-binding sites of twinfilin and actin-depolymerizing factor (ADF)/cofilin. J. Biol. Chem. 277: 43089–43095.

Paavilainen, V. O., M. Hellman, E. Helfer, M. Bovellan, A. Annila *et al.*, 2007   Structural basis and evolutionary origin of actin filament capping by twinfilin. Proc. Natl. Acad. Sci. USA 104: 3113–3118.

Paavilainen, V. O., E. Oksanen, A. Goldman, and P. Lappalainen, 2008   Structure of the actin-depolymerizing factor homology domain in complex with actin. J. Cell Biol. 182: 51–59.

Padrick, S. B., H. C. Cheng, A. M. Ismail, S. C. Panchal, L. K. Doolittle *et al.*, 2008   Hierarchical regulation of WASP/WAVE proteins. Mol. Cell 32: 426–438.

Padrick, S. B., L. K. Doolittle, C. A. Brautigam, D. S. King, and M. K. Rosen, 2011   Arp2/3 complex is bound and activated by two WASP proteins. Proc. Natl. Acad. Sci. USA 108: E472–E479.

Palmgren, S., P. J. Ojala, M. A. Wear, J. A. Cooper, and P. Lappalainen, 2001   Interactions with PIP2, ADP-actin monomers, and capping protein regulate the activity and localization of yeast twinfilin. J. Cell Biol. 155: 251–260.

Palmgren, S., M. Vartiainen, and P. Lappalainen, 2002   Twinfilin, a molecular mailman for actin monomers. J. Cell Sci. 115: 881–886.

Paoluzi, S., L. Castagnoli, I. Lauro, A. E. Salcini, L. Coda *et al.*, 1998   Recognition specificity of individual EH domains of mammals and yeast. EMBO J. 17: 6541–6550.

Paul, A. S., and T. D. Pollard, 2008   The role of the FH1 domain and profilin in formin-mediated actin-filament elongation and nucleation. Curr. Biol. **18:** 9–19.

Paul, A. S., and T. D. Pollard, 2009   Review of the mechanism of processive actin filament elongation by formins. Cell Motil. Cytoskeleton 66: 606–617.

Payne, G. S., D. Baker, E. van Tuinen, and R. Schekman, 1988   Protein transport to the vacuole and receptor-mediated

endocytosis by clathrin heavy chain-deficient yeast. J. Cell Biol. 106: 1453–1461.

Payne, G. S., T. B. Hasson, M. S. Hasson, and R. Schekman, 1987 Genetic and biochemical characterization of clathrin-deficient *Saccharomyces cerevisiae*. Mol. Cell. Biol. 7: 3888–3898.

Pearse, B. M., 1975 Coated vesicles from pig brain: purification and biochemical characterization. J. Mol. Biol. 97: 93–98.

Peter, B. J., H. M. Kent, I. G. Mills, Y. Vallis, P. J. Butler *et al.*, 2004 BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. Science 303: 495–499.

Piao, H. L., I. M. Machado, and G. S. Payne, 2007 NPFXD-mediated endocytosis is required for polarity and function of a yeast cell wall stress sensor. Mol. Biol. Cell 18: 57–65.

Pishvaee, B., G. Costaguta, B. G. Yeung, S. Ryazantsev, T. Greener *et al.*, 2000 A yeast DNA J protein required for uncoating of clathrin-coated vesicles in vivo. Nat. Cell Biol. 2: 958–963.

Pollard, T. D., 1986 Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. J. Cell Biol. 103: 2747–2754.

Pollard, T. D., and G. G. Borisy, 2003 Cellular motility driven by assembly and disassembly of actin filaments. Cell 112: 453–465.

Pollard, T. D., and J. A. Cooper, 1984 Quantitative analysis of the effect of Acanthamoeba profilin on actin filament nucleation and elongation. Biochemistry 23: 6631–6641.

Pollard, T. D., and J. A. Cooper, 2009 Actin, a central player in cell shape and movement. Science 326: 1208–1212.

Pollard, T. D., L. Blanchoin, and R. D. Mullins, 2000 Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. Annu. Rev. Biophys. Biomol. Struct. 29: 545–576.

Polo, S., S. Confalonieri, A. E. Salcini, and P. P. Di Fiore, 2003 EH and UIM: endocytosis and more. Sci. STKE, 213, re17.

Poukkula, M., E. Kremneva, M. Serlachius, and P. Lappalainen, 2011 Actin-depolymerizing factor homology domain: a conserved fold performing diverse roles in cytoskeletal dynamics. Cytoskeleton 68: 471–490.

Poukkula, M., M. Hakala, N. Pentinmikko, M. O. Sweeney, S. Jansen *et al.*, 2014 GMF promotes leading-edge dynamics and collective cell migration in vivo. Curr. Biol. 24: 2533–2540.

Prosser, D. C., T. G. Drivas, L. Maldonado-Baez, and B. Wendland, 2011 Existence of a novel clathrin-independent endocytic pathway in yeast that depends on Rho1 and formin. J. Cell Biol. 195: 657–671.

Quintero-Monzon, O., A. A. Rodal, B. Strokopytov, S. C. Almo, and B. L. Goode, 2005 Structural and functional dissection of the Abp1 ADFH actin-binding domain reveals versatile in vivo adapter functions. Mol. Biol. Cell 16: 3128–3139.

Raths, S., J. Rohrer, F. Crausaz, and H. Riezman, 1993 end3 and end4: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. J. Cell Biol. 120: 55–65.

Reider, A., and B. Wendland, 2011 Endocytic adaptors: social networking at the plasma membrane. J. Cell Sci. 124: 1613–1622.

Reider, A., S. L. Barker, S. K. Mishra, Y. J. Im, L. Maldonado-Baez *et al.*, 2009 Syp1 is a conserved endocytic adaptor that contains domains involved in cargo selection and membrane tubulation. EMBO J. 28: 3103–3116.

Riedl, J., A. H. Crevenna, K. Kessenbrock, J. H. Yu, D. Neukirchen *et al.*, 2008 Lifeact: a versatile marker to visualize F-actin. Nat. Methods 5: 605–607.

Robertson, A. S., E. G. Allwood, A. P. Smith, F. C. Gardiner, R. Costa *et al.*, 2009 The WASP homologue Las17 activates the novel actin-regulatory activity of Ysc84 to promote endocytosis in yeast. Mol. Biol. Cell 20: 1618–1628.

Robinson, R. C., K. Turbedsky, D. A. Kaiser, J. B. Marchand, H. N. Higgs *et al.*, 2001 Crystal structure of Arp2/3 complex. Science 294: 1679–1684.

Rodal, A. A., J. W. Tetreault, P. Lappalainen, D. G. Drubin, and D. C. Amberg, 1999 Aip1p interacts with cofilin to disassemble actin filaments. J. Cell Biol. 145: 1251–1264.

Rodal, A. A., A. L. Manning, B. L. Goode, and D. G. Drubin, 2003 Negative regulation of yeast WASp by two SH3 domain-containing proteins. Curr. Biol. 13: 1000–1008.

Rodal, A. A., O. Sokolova, D. B. Robins, K. M. Daugherty, S. Hippenmeyer *et al.*, 2005b Conformational changes in the Arp2/3 complex leading to actin nucleation. Nat. Struct. Mol. Biol. 12: 26–31.

Rodal, A. A., L. Kozubowski, B. L. Goode, D. G. Drubin, and J. H. Hartwig, 2005a Actin and septin ultrastructures at the budding yeast cell cortex. Mol. Biol. Cell 16: 372–384.

Rohatgi, R., L. Ma, H. Miki, M. Lopez, T. Kirchhausen *et al.*, 1999 The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. Cell 97: 221–231.

Roth, A. F., and N. G. Davis, 1996 Ubiquitination of the yeast a-factor receptor. J. Cell Biol. 134: 661–674.

Rouiller, I., X. P. Xu, K. J. Amann, C. Egile, S. Nickell *et al.*, 2008 The structural basis of actin filament branching by the Arp2/3 complex. J. Cell Biol. 180: 887–895.

Rozenblum, G. T., and M. Gimona, 2008 Calponins: adaptable modular regulators of the actin cytoskeleton. Int. J. Biochem. Cell Biol. 40: 1990–1995.

Rubio-Texeira, M., and C. A. Kaiser, 2006 Amino acids regulate retrieval of the yeast general amino acid permease from the vacuolar targeting pathway. Mol. Biol. Cell 17: 3031–3050.

Sachs, A. B., and J. A. Deardorff, 1992 Translation initiation requires the PAB-dependent poly(A) ribonuclease in yeast. Cell 70: 961–973.

Saffarian, S., E. Cocucci, and T. Kirchhausen, 2009 Distinct dynamics of endocytic clathrin-coated pits and coated plaques. PLoS Biol. 7: e1000191.

Sagot, I., A. A. Rodal, J. Moseley, B. L. Goode, and D. Pellman, 2002 An actin nucleation mechanism mediated by Bni1 and profilin. Nat. Cell Biol. 4: 626–631.

Schafer, D. A., P. B. Jennings, and J. A. Cooper, 1996 Dynamics of capping protein and actin assembly in vitro: uncapping barbed ends by polyphosphoinositides. J. Cell Biol. 135: 169–179.

Schmid, S. L., A. Sorkin, and M. Zerial, 2014 Endocytosis: past, present, and future. Cold Spring Harb. Perspect. Biol. 6: 1–10.

Schutt, C. E., J. C. Myslik, M. D. Rozycki, N. C. Goonesekere, and U. Lindberg, 1993 The structure of crystalline profilin-beta-actin. Nature 365: 810–816.

Sekiya-Kawasaki, M., A. C. Groen, M. J. Cope, M. Kaksonen, H. A. Watson *et al.*, 2003 Dynamic phosphoregulation of the cortical actin cytoskeleton and endocytic machinery revealed by real-time chemical genetic analysis. J. Cell Biol. 162: 765–772.

Sept, D., and J. A. McCammon, 2001 Thermodynamics and kinetics of actin filament nucleation. Biophys. J. 81: 667–674.

Shaw, J. D., H. Hama, F. Sohrabi, D. B. DeWald, and B. Wendland, 2003 PtdIns(3,5)P2 is required for delivery of endocytic cargo into the multivesicular body. Traffic 4: 479–490.

Singer-Kruger, B., Y. Nemoto, L. Daniell, S. Ferro-Novick, and P. De Camilli, 1998 Synaptojanin family members are implicated in endocytic membrane traffic in yeast. J. Cell Sci. 111(22): 3347–3356.

Sirotkin, V., C. C. Beltzner, J. B. Marchand, and T. D. Pollard, 2005 Interactions of WASp, myosin-I, and verprolin with Arp2/3 complex during actin patch assembly in fission yeast. J. Cell Biol. 170: 637–648.

Sirotkin, V., J. Berro, K. Macmillan, L. Zhao, and T. D. Pollard, 2010 Quantitative analysis of the mechanism of endocytic actin patch assembly and disassembly in fission yeast. Mol. Biol. Cell 21: 2894–2904.

Sivadon, P., F. Bauer, M. Aigle, and M. Crouzet, 1995 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: 485–495.

Sivadon, P., M. F. Peypouquet, F. Doignon, M. Aigle, and M. Crouzet, 1997 Cloning of the multicopy suppressor gene SUR7: evidence for a functional relationship between the yeast actin-binding protein Rvs167 and a putative membranous protein. Yeast 13: 747–761.

Skau, C. T., and D. R. Kovar, 2010 Fimbrin and tropomyosin competition regulates endocytosis and cytokinesis kinetics in fission yeast. Curr. Biol. 20: 1415–1422.

Skau, C. T., D. S. Courson, A. J. Bestul, J. D. Winkelman, R. S. Rock et al., 2011 Actin filament bundling by fimbrin is important for endocytosis, cytokinesis, and polarization in fission yeast. J. Biol. Chem. 286: 26964–26977.

Skruzny, M., T. Brach, R. Ciuffa, S. Rybina, M. Wachsmuth et al., 2012 Molecular basis for coupling the plasma membrane to the actin cytoskeleton during clathrin-mediated endocytosis. Proc. Natl. Acad. Sci. USA 109: E2533–E2542.

Smaczynska-de, II, R., E. G. Allwood, S. Aghamohammadzadeh, E. H. Hettema, M. W. Goldberg et al., 2010 A role for the dynamin-like protein Vps1 during endocytosis in yeast. J. Cell Sci. 123: 3496–3506.

Smaczynska-de, II, R., E. G. Allwood, R. Mishra, W. I. Booth, S. Aghamohammadzadeh et al., 2012 Yeast dynamin Vps1 and amphiphysin Rvs167 function together during endocytosis. Traffic 13: 317–328.

Smith, B. A., K. Daugherty-Clarke, B. L. Goode, and J. Gelles, 2013a Pathway of actin filament branch formation by Arp2/3 complex revealed by single-molecule imaging. Proc. Natl. Acad. Sci. USA 110: 1285–1290.

Smith, B. A., S. B. Padrick, L. K. Doolittle, K. Daugherty-Clarke, I. R. Correa Jr., et al., 2013b Tri-color single-molecule imaging shows WASP release from the nascent branch triggers actin nucleation by Arp2/3 complex. eLife 2: e01008.

Soulard, A., T. Lechler, V. Spiridonov, A. Shevchenko, R. Li et al., 2002 Saccharomyces cerevisiae Bzz1p is implicated with type I myosins in actin patch polarization and is able to recruit actin-polymerizing machinery in vitro. Mol. Cell. Biol. 22: 7889–7906.

Soulard, A., S. Friant, C. Fitterer, C. Orange, G. Kaneva et al., 2005 The WASP/Las17p-interacting protein Bzz1p functions with Myo5p in an early stage of endocytosis. Protoplasma 226: 89–101.

Spiess, M., J. O. de Craene, A. Michelot, B. Rinaldi, A. Huber et al., 2013 Lsb1 is a negative regulator of las17 dependent actin polymerization involved in endocytosis. PLoS ONE 8: e61147.

Spira, F., N. S. Mueller, G. Beck, P. von Olshausen, J. Beig et al., 2012 Patchwork organization of the yeast plasma membrane into numerous coexisting domains. Nat. Cell Biol. 14: 640–648.

Springael, J. Y., J. O. De Craene, and B. Andre, 1999 The yeast Npi1/Rsp5 ubiquitin ligase lacking its N-terminal C2 domain is competent for ubiquitination but not for subsequent endocytosis of the gap1 permease. Biochem. Biophys. Res. Commun. 257: 561–566.

Stamenova, S. D., M. E. French, Y. He, S. A. Francis, Z. B. Kramer et al., 2007 Ubiquitin binds to and regulates a subset of SH3 domains. Mol. Cell 25: 273–284.

Stefan, C. J., A. Audhya, and S. D. Emr, 2002 The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. Mol. Biol. Cell 13: 542–557.

Stimpson, H. E., C. P. Toret, A. T. Cheng, B. S. Pauly, and D. G. Drubin, 2009 Early-arriving Syp1p and Ede1p function in endocytic site placement and formation in budding yeast. Mol. Biol. Cell 20: 4640–4651.

Suarez, C., J. Roland, R. Boujemaa-Paterski, H. Kang, B. R. McCullough et al., 2011 Cofilin tunes the nucleotide state of actin filaments and severs at bare and decorated segment boundaries. Curr. Biol. 21: 862–868.

Sun, Y., A. C. Martin, and D. G. Drubin, 2006 Endocytic internalization in budding yeast requires coordinated actin nucleation and myosin motor activity. Dev. Cell 11: 33–46.

Sun, Y., S. Carroll, M. Kaksonen, J. Y. Toshima, and D. G. Drubin, 2007 PtdIns(4,5)P2 turnover is required for multiple stages during clathrin- and actin-dependent endocytic internalization. J. Cell Biol. 177: 355–367.

Svitkina, T. M., and G. G. Borisy, 1999 Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. J. Cell Biol. 145: 1009–1026.

Szymkiewicz, I., K. Kowanetz, P. Soubeyran, A. Dinarina, S. Lipkowitz et al., 2002 CIN85 participates in Cbl-b-mediated down-regulation of receptor tyrosine kinases. J. Biol. Chem. 277: 39666–39672.

Tan, P. K., N. G. Davis, G. F. Sprague, and G. S. Payne, 1993 Clathrin facilitates the internalization of seven transmembrane segment receptors for mating pheromones in yeast. J. Cell Biol. 123: 1707–1716.

Tan, P. K., J. P. Howard, and G. S. Payne, 1996 The sequence NPFXD defines a new class of endocytosis signal in Saccharomyces cerevisiae. J. Cell Biol. 135: 1789–1800.

Tang, H. Y., A. Munn, and M. Cai, 1997 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: 4294–4304.

Tang, H. Y., J. Xu, and M. Cai, 2000 Pan1p, End3p, and S1a1p, three yeast proteins required for normal cortical actin cytoskeleton organization, associate with each other and play essential roles in cell wall morphogenesis. Mol. Cell. Biol. 20: 12–25.

Tarassov, K., V. Messier, C. R. Landry, S. Radinovic, M. M. Serna Molina et al., 2008 An in vivo map of the yeast protein interactome. Science 320: 1465–1470.

Tenay, B., E. Kimberlin, M. Williams, J. Denise, J. Fakilahyel et al., 2013 Inactivation of Tor proteins affects the dynamics of endocytic proteins in early stage of endocytosis. J. Biosci. 38: 351–361.

Terrell, J., S. Shih, R. Dunn, and L. Hicke, 1998 A function for monoubiquitination in the internalization of a G protein-coupled receptor. Mol. Cell 1: 193–202.

Ti, S. C., C. T. Jurgenson, B. J. Nolen, and T. D. Pollard, 2011 Structural and biochemical characterization of two binding sites for nucleation-promoting factor WASp-VCA on Arp2/3 complex. Proc. Natl. Acad. Sci. USA 108: E463–E471.

Tilney, L. G., and D. A. Portnoy, 1989 Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes. J. Cell Biol. 109: 1597–1608.

Tilney, L. G., E. M. Bonder, L. M. Coluccio, and M. S. Mooseker, 1983 Actin from Thyone sperm assembles on only one end of an actin filament: a behavior regulated by profilin. J. Cell Biol. 97: 112–124.

Tonikian, R., X. Xin, C. P. Toret, D. Gfeller, C. Landgraf et al., 2009 Bayesian modeling of the yeast SH3 domain interactome predicts spatiotemporal dynamics of endocytosis proteins. PLoS Biol. 7: e1000218.

Toret, C. P., L. Lee, M. Sekiya-Kawasaki, and D. G. Drubin, 2008 Multiple pathways regulate endocytic coat disassembly in Saccharomyces cerevisiae for optimal downstream trafficking. Traffic 9: 848–859.

Toshima, J., J. Y. Toshima, A. C. Martin, and D. G. Drubin, 2005 Phosphoregulation of Arp2/3-dependent actin assembly during receptor-mediated endocytosis. Nat. Cell Biol. 7: 246–254.

Toshima, J., J. Y. Toshima, M. C. Duncan, M. J. Cope, Y. Sun et al., 2007 Negative regulation of yeast Eps15-like Arp2/3 complex activator, Pan1p, by the Hip1R-related protein, Sla2p, during endocytosis. Mol. Biol. Cell 18: 658–668.

Tsuji, T., T. Miyoshi, C. Higashida, S. Narumiya, and N. Watanabe, 2009 An order of magnitude faster AIP1-associated actin

disruption than nucleation by the Arp2/3 complex in lamellipodia. PLoS ONE 4: e4921.

Umeda, A., A. Meyerholz, and E. Ungewickell, 2000 Identification of the universal cofactor (auxilin 2) in clathrin coat dissociation. Eur. J. Cell Biol. 79: 336–342.

Umikawa, M., K. Tanaka, T. Kamei, K. Shimizu, H. Imamura *et al.*, 1998 Interaction of Rho1p target Bni1p with F-actin-binding elongation factor 1alpha: implication in Rho1p-regulated reorganization of the actin cytoskeleton in *Saccharomyces cerevisiae*. Oncogene 16: 2011–2016.

Urbanek, A. N., A. P. Smith, E. G. Allwood, W. I. Booth and K. R. Ayscough, 2013 A novel actin-binding motif in Las17/WASP nucleates actin filaments independently of Arp2/3. Curr. Biol. 23: 196–203.

Vaduva, G., N. C. Martin, and A. K. Hopper, 1997 Actin-binding verprolin is a polarity development protein required for the morphogenesis and function of the yeast actin cytoskeleton. J. Cell Biol. 139: 1821–1833.

Valdez-Taubas, J., and H. R. Pelham, 2003 Slow diffusion of proteins in the yeast plasma membrane allows polarity to be maintained by endocytic cycling. Curr. Biol. 13: 1636–1640.

Varnai, P., and T. Balla, 2008 Live cell imaging of phosphoinositides with expressed inositide binding protein domains. Methods 46: 167–176.

Varshavsky, A., 2012 The ubiquitin system, an immense realm. Annu. Rev. Biochem. 81: 167–176.

Vartiainen, M., P. J. Ojala, P. Auvinen, J. Peranen, and P. Lappalainen, 2000 Mouse A6/twinfilin is an actin monomer-binding protein that localizes to the regions of rapid actin dynamics. Mol. Cell. Biol. 20: 1772–1783.

Vater, C. A., C. K. Raymond, K. Ekena, I. Howald-Stevenson, and T. H. Stevens, 1992 The VPS1 protein, a homolog of dynamin required for vacuolar protein sorting in *Saccharomyces cerevisiae*, is a GTPase with two functionally separable domains. J. Cell Biol. 119: 773–786.

Vecchi, M., S. Polo, V. Poupon, J. W. van de Loo, A. Benmerah *et al.*, 2001 Nucleocytoplasmic shuttling of endocytic proteins. J. Cell Biol. 153: 1511–1517.

Vida, T. A., and S. D. Emr, 1995 A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J. Cell Biol. 128: 779–792.

Voegtli, W. C., A. Y. Madrona, and D. K. Wilson, 2003 The structure of Aip1p, a WD repeat protein that regulates Cofilin-mediated actin depolymerization. J. Biol. Chem. 278: 34373–34379.

Volkmann, N., D. DeRosier, P. Matsudaira, and D. Hanein, 2001 An atomic model of actin filaments cross-linked by fimbrin and its implications for bundle assembly and function. J. Cell Biol. 153: 947–956.

Volland, C., J. M. Galan, D. Urban-Grimal, G. Devilliers, and R. Haguenauer-Tsapis, 1994 Endocytose and degradation of the uracil permease of *S. cerevisiae* under stress conditions: possible role of ubiquitin. Folia Microbiol. 39: 554–557.

Waddle, J. A., T. S. Karpova, R. H. Waterston, and J. A. Cooper, 1996 Movement of cortical actin patches in yeast. J. Cell Biol. 132: 861–870.

Wagner, A. R., Q. Luan, S.-L. Liu and B. J. Nolen, 2013 Dip1 defines a class of Arp2/3 complex activators that function without preformed actin filaments. Curr. Biol. 23: 1990–1998.

Wahlstrom, G., M. Vartiainen, L. Yamamoto, P. K. Mattila, P. Lappalainen *et al.*, 2001 Twinfilin is required for actin-dependent developmental processes in Drosophila. J. Cell Biol. 155: 787–796.

Wang, D., L. Zhang, G. Zhao, G. Wahlstrom, T. I. Heino *et al.*, 2010 Drosophila twinfilin is required for cell migration and synaptic endocytosis. J. Cell Sci. 123: 1546–1556.

Warren, D. T., P. D. Andrews, C. W. Gourlay, and K. R. Ayscough, 2002 Sla1p couples the yeast endocytic machinery to proteins regulating actin dynamics. J. Cell Sci. 115: 1703–1715.

Watson, H. A., M. J. Cope, A. C. Groen, D. G. Drubin, and B. Wendland, 2001 In vivo role for actin-regulating kinases in endocytosis and yeast epsin phosphorylation. Mol. Biol. Cell 12: 3668–3679.

Weinberg, J., and D. G. Drubin, 2012 Clathrin-mediated endocytosis in budding yeast. Trends Cell Biol. 22: 1–13.

Welch, M. D., and R. D. Mullins, 2002 Cellular control of actin nucleation. Annu. Rev. Cell Dev. Biol. 18: 247–288.

Welch, M. D., A. H. DePace, S. Verma, A. Iwamatsu, and T. J. Mitchison, 1997a The human Arp2/3 complex is composed of evolutionarily conserved subunits and is localized to cellular regions of dynamic actin filament assembly. J. Cell Biol. 138: 375–384.

Welch, M. D., A. Iwamatsu, and T. J. Mitchison, 1997b Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. Nature 385: 265–269.

Welch, M. D., J. Rosenblatt, J. Skoble, D. A. Portnoy, and T. J. Mitchison, 1998 Interaction of human Arp2/3 complex and the Listeria monocytogenes ActA protein in actin filament nucleation. Science 281: 105–108.

Wen, K. K., and P. A. Rubenstein, 2005 Acceleration of yeast actin polymerization by yeast Arp2/3 complex does not require an Arp2/3-activating protein. J. Biol. Chem. 280: 24168–24174.

Wendland, B., 2002 Epsins: Adaptors in endocytosis? Nat. Rev. Mol. Cell Biol. 3: 971–977.

Wendland, B., and S. D. Emr, 1998 Pan1p, yeast eps15, functions as a multivalent adaptor that coordinates protein-protein interactions essential for endocytosis. J. Cell Biol. 141: 71–84.

Wendland, B., J. M. McCaffery, Q. Xiao, and S. D. Emr, 1996 A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. J. Cell Biol. 135: 1485–1500.

Wendland, B., K. E. Steece, and S. D. Emr, 1999 Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis. EMBO J. 18: 4383–4393.

Wesp, A., L. Hicke, J. Palecek, R. Lombardi, T. Aust *et al.*, 1997 End4p/Sla2p interacts with actin-associated proteins for endocytosis in *Saccharomyces cerevisiae*. Mol. Biol. Cell 8: 2291–2306.

Whitworth, K., M. K. Bradford, N. Camara, and B. Wendland, 2014 Targeted disruption of an EH-domain protein endocytic complex, Pan1-End3. Traffic 15: 43–59.

Wiederkehr, A., S. Avaro, C. Prescianotto-Baschong, R. Haguenauer-Tsapis, and H. Riezman, 2000 The F-box protein Rcy1p is involved in endocytic membrane traffic and recycling out of an early endosome in *Saccharomyces cerevisiae*. J. Cell Biol. 149: 397–410.

Wiederkehr, A., K. D. Meier, and H. Riezman, 2001 Identification and characterization of *Saccharomyces cerevisiae* mutants defective in fluid-phase endocytosis. Yeast 18: 759–773.

Winder, S. J., T. Jess, and K. R. Ayscough, 2003 SCP1 encodes an actin-bundling protein in yeast. Biochem. J. 375: 287–295.

Winter, D., T. Lechler and R. Li, 1999 Activation of the yeast Arp2/3 complex by Bee1p, a WASP-family protein. Curr. Biol. 9: 501–504.

Wolven, A. K., L. D. Belmont, N. M. Mahoney, S. C. Almo, and D. G. Drubin, 2000 In vivo importance of actin nucleotide exchange catalyzed by profilin. J. Cell Biol. 150: 895–904.

Wu, K. C., and J. P. Jin, 2008 Calponin in non-muscle cells. Cell Biochem. Biophys. 52: 139–148.

Yamabhai, M., N. G. Hoffman, N. L. Hardison, P. S. McPherson, L. Castagnoli *et al.*, 1998 Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. J. Biol. Chem. 273: 31401–31407.

Yang, H. C., and L. A. Pon, 2002 Actin cable dynamics in budding yeast. Proc. Natl. Acad. Sci. USA 99: 751–756.

Yang, S., K. R. Ayscough, and D. G. Drubin, 1997 A role for the actin cytoskeleton of *Saccharomyces cerevisiae* in bipolar bud-site selection. J. Cell Biol. 136: 111–123.

Ydenberg, C. A., S. B. Padrick, M. O. Sweeney, M. Gandhi, O. Sokolova *et al.*, 2013 GMF Severs Actin-Arp2/3 Complex Branch Junctions by a Cofilin-like Mechanism. Curr. Biol. 23: 1037–1045.

Yeung, B. G., H. L. Phan, and G. S. Payne, 1999 Adaptor complex-independent clathrin function in yeast. Mol. Biol. Cell 10: 3643–3659.

Young, M. E., T. S. Karpova, B. Brugger, D. M. Moschenross, G. K. Wang *et al.*, 2002 The Sur7p family defines novel cortical domains in *Saccharomyces cerevisiae*, affects sphingolipid metabolism, and is involved in sporulation. Mol. Cell. Biol. 22: 927–934.

Young, M. E., J. A. Cooper, and P. C. Bridgman, 2004 Yeast actin patches are networks of branched actin filaments. J. Cell Biol. 166: 629–635.

Yu, X., and M. Cai, 2004 The yeast dynamin-related GTPase Vps1p functions in the organization of the actin cytoskeleton via interaction with Sla1p. J. Cell Sci. 117: 3839–3853.

Zeng, G., and M. Cai, 1999 Regulation of the actin cytoskeleton organization in yeast by a novel serine/threonine kinase Prk1p. J. Cell Biol. 144: 71–82.

Zeng, G., X. Yu, and M. Cai, 2001 Regulation of yeast actin cytoskeleton-regulatory complex Pan1p/Sla1p/End3p by serine/threonine kinase Prk1p. Mol. Biol. Cell 12: 3759–3772.

Zeng, G., B. Huang, S. P. Neo, J. Wang, and M. Cai, 2007 Scd5p mediates phosphoregulation of actin and endocytosis by the type 1 phosphatase Glc7p in yeast. Mol. Biol. Cell 18: 4885–4898.

Zhao, Y., J. A. Macgurn, M. Liu, and S. Emr, 2013 The ART-Rsp5 ubiquitin ligase network comprises a plasma membrane quality control system that protects yeast cells from proteotoxic stress. eLife 2: e00459.

Zinser, E., C. D. Sperka-Gottlieb, E. V. Fasch, S. D. Kohlwein, F. Paltauf *et al.*, 1991 Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. J. Bacteriol. 173: 2026–2034.

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