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Clathrin, adaptors and disease: Insights from the yeast *Saccharomyces cerevisiae*

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1. ABSTRACT

Since the identification of clathrin as a vesicular coat protein, numerous studies have contributed to our understanding of the role of clathrin and clathrin-mediated trafficking pathways in cell function. The budding yeast, *Saccharomyces cerevisiae*, offers a wealth of highly developed approaches that have been applied to study clathrin-mediated trafficking events, most of which are conserved in mammalian cells. Here we review the function of clathrin and clathrin adaptors in yeast. We also discuss the role of these proteins in human disease and how certain pathogens have co-opted trafficking pathways for their own use. These studies highlight the advantages of studying complex trafficking events using yeast as a model.

2. INTRODUCTION

Eukaryotic cells are organized into structurally and functionally distinct membrane-delineated compartments. In the endocytic and secretory pathways, distinct compartments are arranged as routes for transporting proteins and lipids into or out of cells. Such pathways present a fundamental traffic problem: how do integral membrane and luminal proteins in one organelle of a pathway move to the next organelle in the pathway? In most cases, traffic between pathway organelles is mediated by tubular/vesicular carriers that bud from one compartment, move through the cytoplasm, and fuse with the appropriate target compartment.

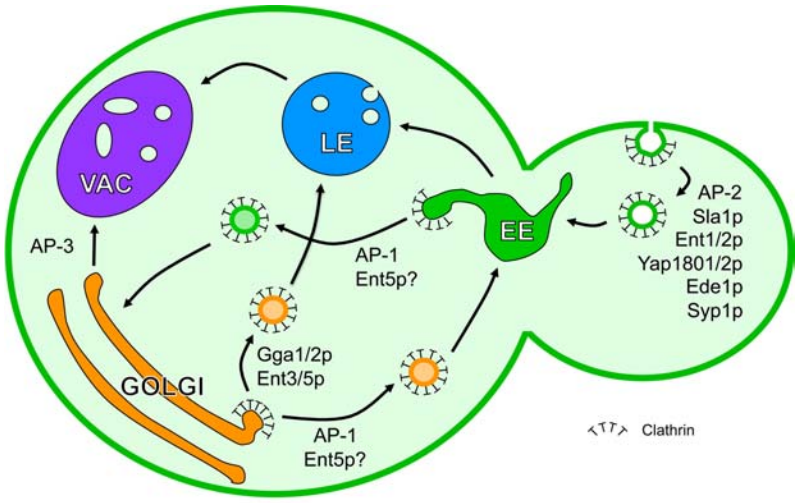

Figure 1. Summary of the major clathrin-mediated trafficking pathways in yeast. VAC: vacuole; EE: early endosome; LE: late endosome. Question marks indicate undertainty about whether Ent5p acts in only one or both AP-1 mediated pathways.

Formation of most carriers requires assembly of a protein coat as a patch on the cytoplasmic face of the donor compartment membrane. The coat drives membrane invagination while concomitantly collecting protein cargo. Three types of evolutionarily conserved coats have been extensively characterized (1). COPI coats function in transport from the Golgi to the endoplasmic reticulum (ER) and in transport between Golgi subcompartments. COPII coats carry out transport from the ER to the *cis* face of the Golgi complex. Clathrin coats mediate endocytosis and transport between the *trans* face of the Golgi complex (*trans* Golgi network or TGN) and endosomes (Figure 1).

All three coats share a common 2-layer architecture: an outer scaffold shell and an inner layer that directs coat assembly to the appropriate membrane and collects cargo (1, 2). Clathrin coats were the first to be identified (3). Clathrin, a hexamer of three heavy and three light chains, forms a polyhedral lattice that constitutes the outer coat scaffold. The inner layer of the coat is primarily comprised of adaptors that simultaneously bind to clathrin, membrane lipids, and sorting signals in the cytoplasmic domains of cargo proteins, thereby bridging clathrin to the membrane. Clathrin is common to all types of clathrin-coated vesicles (CCV) but there are multiple adaptors (Figures 2-4).

Two basic classes of clathrin adaptors have been identified (4, 5). Heterotetrameric adaptor (originally termed AP for assembly polypeptide) complexes were first recognized. Yeast express three AP complexes, AP-1, AP-2, and AP-3 (6-8). Each adaptor complex consists of two large subunits, one medium subunit, and one small subunit (Figure 2). Studies of mammalian and yeast AP complexes indicate that each subunit provides binding surfaces for various interaction partners including clathrin, lipids, cargo, and other coat proteins (4, 9-11). As such, AP complexes are considered to be major hubs in the clathrin coat interaction network, coordinating coat formation with cargo collection (1, 2). The second class of adaptors consists of monomeric adaptors that combine different functions of AP complex subunits into single polypeptides (Figures 3-4).

Here we review the roles of clathrin and adaptor proteins in the budding yeast *Saccharomyces cerevisiae*. The major transport pathways and molecular components of the vesicular transport machinery, including components of clathrin coats, have been conserved between yeast and mammals. The advanced genomic and proteomic databases, combined with the ease of genetic manipulation, make yeast an effective system to address clathrin coat function and regulation. Studies of yeast clathrin coats inform our understanding of the fundamental mechanisms responsible for clathrin-mediated transport, providing a foundation for understanding how defects in these processes can contribute to disease.

3. CLATHRIN

Clathrin has a distinctive three-pronged structure known as a triskelion (12-14). A triskelion consists of three clathrin heavy chains (CHC) and three clathrin light chains (CLC) (15, 16). The CHC oligomerize through their C-terminal "hub" regions to form the triskelion vertex. CLC binds to the CHC primarily along the CHC arm proximal to the hub. The N-terminal region of each CHC forms a seven-bladed beta propeller at the end of each triskelion leg (17). This "terminal domain" serves as a major binding site for adaptors and other clathrin-associated proteins (18). *In vitro*, clathrin can self-assemble into empty polyhedral cages analogous to the polyhedral lattices that distinguish CCV (12, 19, 20). Cryo-electron microscopy of mammalian clathrin cages has provided a 7.9Å resolution structure, revealing the hub regions pointing inwards at each lattice vertex and three TDs also arrayed beneath each vertex (21).

Molecular cloning of the gene encoding yeast clathrin heavy chain (*CHC1*) allowed the first genetic test of clathrin function by taking advantage of homologous

recombination in yeast to delete *CHC1* (22). Considering the relatively strong evolutionary conservation of CHC (yeast and rat CHC are 50% identical and 70% similar, (23), it was surprising that cells harboring the deletion (*chc1*Δ) were viable, although they grew slowly (22) and displayed multiple trafficking defects (discussed below). This result was the first indication that clathrin is not absolutely required for cell viability, a conclusion extended to vertebrate cells by the analysis of a clathrin-deficient chicken B cell line, DT40 (24). In terms of multicellular organisms, clathrin deficiency is lethal in the one reported case, *Drosophila melanogaster* (25), and is likely to be lethal in other organisms given the multitude of functions impacted by clathrin-mediated trafficking. Thus, mutations that severely affect clathrin function are expected to be embryonic lethal.

Subsequent analysis in yeast revealed that in some strain backgrounds *chc1*Δ was lethal (26, 27). The different outcomes of deleting *CHC1* were due to variations in other genes in the wild-type strains used by different investigators. Whether these variations occurred in genes whose products participate in clathrin-dependent trafficking or in unrelated genes has not been resolved. An inviable *chc1*Δ strain has been used to isolate genes capable of rescuing growth when overexpressed from a multicopy plasmid (28). This approach identified genes encoding CLC, protein phosphatase type I subunit Scd5p, a member of the regulatory 14-3-3 protein family Bmh2p, and ubiquitin (28-31). Although the molecular basis of suppression has not been defined, Scd5p has clear links to clathrin through a role in endocytosis (32) and ubiquitin is also associated with protein trafficking (33). How Bmh2p relates to clathrin-mediated trafficking remains unclear. None of these proteins represents the product of the gene responsible for inviability in the parent *chc1*Δ strain.

3.1. Endocytosis

Viable *chc1*Δ cells provided a genetic means to test for clathrin function in specific protein transport pathways. Direct measurements of internalization established that endocytosis is defective in clathrin-deficient cells (34, 35). Uptake of the peptide pheromone alpha-factor by the G-protein-coupled alpha-factor receptor was reduced to 30-50% of wild-type levels in mutant cells. In cells carrying a temperature-sensitive allele of *CHC1* (*chc1-521*), shift to the non-permissive temperature led to an immediate (within 2 minutes) decrease in alpha-factor uptake to a level similar to that observed in *chc1*Δ cells, providing evidence that the effects of clathrin inactivation were direct (35). Internalization of another GPCR, the receptor for the mating pheromone a-factor was also reduced in *chc1-521* cells but subsequent transport to the lysosome-like vacuole was not slowed, suggesting that, after endocytic vesicle formation, trafficking through the endocytic pathway to the lysosome-like vacuole does not require clathrin. Although distinct clathrin-independent pathways could account for the significant level of endocytosis in clathrin-deficient cells, it seems more likely that clathrin acts to facilitate formation of the major class of endocytic vesicles in yeast but is not absolutely required for this process (see below).

In contrast to deletion of *CHC1*, mutational or pharmacological inactivation of actin blocks receptor-mediated endocytosis in yeast, suggesting that actin is a more integral component of the endocytic machinery than clathrin (36-38). The stringent requirement for actin appears to be due to the high turgor pressure in yeast; providing osmotic support to decrease turgor pressure reduces the requirement for actin in endocytosis (39). Whether there is a stronger dependence on clathrin under these conditions was not addressed. In mammalian cells endocytosis is more generally dependent on clathrin than actin. However, recent studies demonstrate that increased membrane tension in mammalian cells induces a requirement for actin to promote endocytosis (40). A parsimonious view based on these data is that the same fundamental machinery operates in both yeast and mammals but the relative contributions of actin versus clathrin vary depending on the cell type and conditions.

Live cell imaging of fluorescently tagged endocytic proteins has defined the temporal sequence of assembly of the actin-based endocytic machinery at sites of endocytosis in yeast (41). In cells expressing fluorescent protein fusions to CLC encoded at the endogenous gene, clathrin is one of the first proteins to appear at endocytic sites, accumulates as the vesicle invaginates, and then rapidly disappears upon vesicle release (42, 43). Clathrin appears at virtually all actin-containing endocytic events, indicating that clathrin and actin are part of the same endocytic process.

The role of clathrin in the dynamics of endocytic vesicle formation was tested by analyzing other endocytic proteins in clathrin-deficient cells. Sla1p is an endocytic adaptor that assembles at endocytic sites after clathrin and disappears coincident with vesicle release (44). Abp1p is an actin binding protein that is recruited at a later phase of endocytic vesicle formation and remains associated with the endocytic vesicle after release (44). These two proteins are commonly used as early and late markers for endocytic vesicle formation. In *chc1*Δ cells, cortical patches of Sla1p were present at only 20-30% of wild-type levels (42, 43). At these Sla1p patches, Abp1p dynamics were comparable to wild-type cells. Thus, clathrin appears to promote or stabilize the initial recruitment of endocytic coat proteins such as Sla1p, a conclusion supported by more recent studies (45). However, based on these results, endocytic coats can still form at reduced levels without clathrin and in such situations endocytic vesicle formation can proceed. These findings are consistent with the partial defect in endocytosis in *chc1*Δ cells. Immuno-electron microscopy localized clathrin to the tips of tubular invaginations at the plasma membrane whereas actin was more uniformly localized along the invagination (46), supporting the view that clathrin participates in organizing the endocytic coat whereas actin polymerization contributes to membrane invagination and scission.

Yeast lacking clathrin light chain (*clc1*Δ) display slow growth and endocytosis defects comparable to *chc1*Δ cells (30, 47). In *clc1*Δ cells, CHC levels are reduced to 10-25% of wild-type levels and the residual CHC is

predominantly monomeric, revealing a role for CLC in CHC trimerization (30, 48). Overexpression of CHC increased levels of CHC trimers and partially improved growth but had no effect on the endocytic defect. Thus, while CHC can provide some clathrin function in the absence of CLC, both subunits are required for clathrin-dependent endocytosis.

A likely explanation for the dependence of endocytosis on CLC is a role for CLC in coupling the clathrin coat to the actin machinery. CLC binds to the F-actin-binding protein Sla2p (49), and cells lacking Sla2p or expressing a CLC-binding-defective Sla2p mutant exhibit reduced endocytosis (49, 50). Furthermore, overexpression of the Sla2p-binding region of CLC suppresses the endocytic defects of *chc1*Δ cells (51). Although deletion of the Sla2p-binding region of CLC does not alter endocytosis, the mutant does suppress endocytic defects of several late-acting actin nucleation promoting factors (52). Based on these results it has been proposed that CLC binding inhibits Sla2p interaction with actin, restricting interaction between the incipient endocytic vesicle and F-actin to the neck region where it promotes invagination and scission (52).

Unlike yeast, knockdown of CLCs in cultured mammalian cells does not alter endocytosis (53). In part this difference can be attributed to the ability of mammalian CHC to trimerize in the absence of CLC (54). However, an additional explanation is likely related to the role of CLC in coupling clathrin coats to actin. In the cell lines tested, actin is probably not a major factor in clathrin-mediated endocytosis (55, 56). It will be worthwhile to test whether CLC knockdown in mammalian cells has effects on endocytosis in cell lines or under conditions where actin is required for endocytosis (40).

3.2. TGN-endosome traffic

Cells lacking CHC or CLC display additional phenotypes indicative of defective protein transport in the secretory and endocytic pathways. Secretion of invertase is slowed approximately 50%, suggesting a minor defect in the secretory pathway (57). There are two classes of secretory vesicles in yeast that can be distinguished by density (58). The more dense population that normally carries invertase is absent in clathrin-deficient cells, leading to the suggestion that CCV mediated transport from the TGN to endosomes represents an intermediate stage of one branch of the secretory pathway (59, 60).

Consistent with a role for clathrin in TGN-endosome traffic, a distinctive phenotype of *chc1*Δ or *clc1*Δ cells is secretion of incompletely matured alpha-factor mating pheromone (30, 48, 61). In wild-type cells alpha-factor is synthesized as part of a larger precursor which is glycosylated during transport through the secretory pathway (62). In the TGN, the precursor is subject to proteolytic maturation by several proteases including the furin-like Kex2p protease and dipeptidyl aminopeptidase A (DPAP-A). Kex2p and DPAP-A normally cycle intracellularly between the TGN and endosomes, a process that is essential for their localization to the TGN (63, 64). Inactivation of clathrin causes mislocalization of Kex2p and DPAP-A from the Golgi to the cell surface, leading to inefficient alpha-factor precursor maturation and secretion of the precursor and intermediate cleavage forms (61, 65). These results provided the first direct evidence that clathrin plays an important role in localization of TGN proteins by directing the proteins into a pathway that allows cycling between the TGN and endosomes.

Unexpectedly, maturation of newly-synthesized vacuolar proteins such as carboxypeptidase Y (CPY) appeared normal in *chc1*Δ and *clc1*Δ cells, suggesting that transport of these proteins from the secretory pathway to vacuoles was unaffected (34, 48). However, in *chc1-521* cells analyzed immediately after shift to the non-permissive temperature, CPY maturation was blocked and precursor CPY was secreted, indicative of defective sorting from the TGN (66). CPY sorting and maturation returned to normal after extended incubation at the nonpermissive temperature. Thus, the vacuolar protein sorting pathway can adapt to the absence of clathrin. Analysis of the CPY sorting receptor, Vps10p, which cycles between the TGN and endosomes, provided evidence that Vps10p was missorted to the plasma membrane in *chc1*Δ cells but could recycle back to the TGN by endocytosis and transport from endosomes (67). This pathway could allow continued sorting of CPY, accounting for the lack of a clear sorting defect in clathrin-deficient cells. Together with data indicating that Vps10p and CPY are sorted into CCV (68, 69), these results support a model in which CCV play a major role in sorting proteins between the TGN and endosomes, a role conserved in mammalian cells(4).

4. MULTIMERIC ADAPTORS

Adaptor (AP) complexes were first identified as major structural components of brain-derived CCV (20). To date, three distinct AP complexes have been identified in yeast (AP1-3) whereas mammals express two additional AP complexes, AP-4 and AP-5 that appear to function independently of clathrin (5, 70)(Figure 2). Each AP complex is a heterotetramer comprising two large subunits (gamma and beta1 in AP-1, alpha and beta2 in AP-2, and delta and beta3 in AP-3), one medium (mu1-3) and one small (sigma1-3) subunit (Figure 2). The structure of mammalian AP-2 has been most extensively characterized and serves as a paradigm for the other APs (71). The N-terminal regions of the two large subunits together with the medium and small subunits are arranged as a core complex (Figure 2). The core domain binds to lipids and sorting signals in the cytoplasmic domains of cargo proteins. Two consensus sorting signals have been well characterized: a tyrosine based motif, YxxΦ (single amino acid code, where x indicates any amino acid and Φ indicates a bulky hydrophobic residue), which binds to the mu subunit; and a dileucine-based motif, [D/E]xxxL[L/I] that binds a hemicomplex of alpha/gamma/delta and the corresponding sigma subunit (72).

A flexible hinge region on each large subunit extends from the core to C-terminal appendage domains (often referred to as “ears”). The appendage domains of

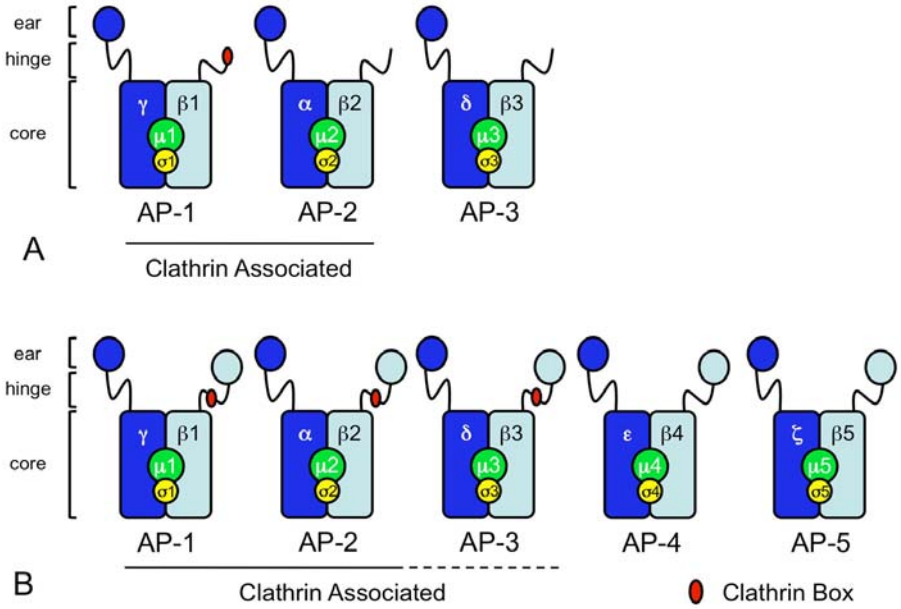

Figure 2. Domain representation of yeast (A) and mammalian (B) AP complexes. Homology between yeast and mammalian beta subunits is restricted to the core domain. Furthermore, the yeast beta subunits are shorter than the mammalian subunits by a length corresponding to the appendage domain of the mammalian subunits. Secondary structure prediction programs suggest that the C-terminal sequences in the yeast beta subunits are likely to be mostly unstructured. These properties suggest that the yeast beta subunits do not contain C-terminal appendage domains but end after a region corresponding to the hinge region of the mammalian beta subunits. Dashed line in B indicates uncertainty of the requirement for clathrin binding by mammalian AP-3. Yeast AP-3 and mammalian AP-4 and AP-5 are not clathrin associated and appear to function independently of clathrin.

the large subunits constitute binding platforms for clathrin and a large number of accessory proteins that participate in coat assembly and vesicle formation, including other adaptors (73). The hinge region of the beta subunit contains clathrin binding motifs (clathrin box motifs) and less well-defined motifs that interact with the CHC terminal domain (71, 73, 74).

Subunits of the three AP complexes in yeast exhibit 25-54% maximum sequence identity over corresponding regions of the human homologues (Table 1). In AP-1 and AP-2, sequence homology extends throughout the length of the alpha/gamma, mu, and sigma subunits. The beta1 and beta2 subunits exhibit sequence similarity only to the core regions of human beta1 and beta2 and are considerably shorter than the human proteins, implying significant structural and perhaps functional differences between yeast and mammalian beta appendage domains. Another difference is that yeast beta1 and beta2 subunits share only 26% identity whereas the human beta1 and beta2 subunits are 83% identical, suggesting that the beta subunit functions have diverged in yeast AP-1 and AP-2. Indeed, yeast beta1, like mammalian beta1, binds directly to clathrin but yeast beta2, unlike its mammalian homologue, does not bind clathrin (8). Although there are substantial sequence differences between yeast and mammalian AP1-3 complexes, each yeast AP appears to be functionally analogous to its mammalian homologue: AP-1 functions in TGN-endosome transport, AP-2 in endocytosis, and AP-3 in traffic to vacuoles/lysosomes.

4.1. AP-1

The four standard subunits of yeast AP-1 are Aps1p (sigma1), Apm1p (mu1), Apl2p (beta1), and Apl4p (gamma). AP-1 interacts with clathrin and is a component of purified CCV (8,11, 75). In addition, yeast encodes a second mu1-related protein, Apm2p, which is larger than other mu proteins due to a ≈100aa insertion in the amino terminal region of the protein. Apm2p appears to associate with other AP-1 subunits, suggesting that it may define a second AP-1 complex (8, 76). However, no function has been ascribed to Apm2p. There also has been diversification of AP-1 composition (and function) in mammals where an alternative mu subunit, mu1B, is expressed specifically in epithelial cells (77). This subunit together with ubiquitously expressed large and small AP-1 subunits forms a cell-specific AP-1 complex involved in transport to the basolateral plasma membrane.

Despite the evolutionary conservation of AP-1 subunits revealed by sequence analysis, individual deletions of subunit genes or combined deletion of all four standard AP-1 subunits in yeast resulted in no overt phenotypes in growth, endocytosis, or alpha-factor maturation (8, 75, 78-80). However, in combination with *chc1-521* or *chc1*Δ, deletion of individual AP-1 subunit genes (with the exception of *apm2*Δ) accentuates slow growth and alpha-factor maturation defects of the clathrin mutants but does not affect endocytosis. These results suggest that AP-1 functions with clathrin in TGN protein localization.

Table 1. Comparison of mammalian and yeast AP subunits

Yeast AP subunit (accession number)	AP complex	Length (aa)	Human homologue (accession number)	Length (aa)	Identity (region in yeast subunit)
Aps1 (NP_013271.1)	AP-1	156	AP-1 sigma1B (NP_003907.3)	157	54% (aa4-153)
Apm1 (NP_015064.1)	AP-1	475	AP-1 mu1 (NP_115882.1)	423	51% (aa2-473)
Apm2 (NP_011844.1)	AP-1	605	AP-1 mu1 (NP_115882.1)	423	29% (aa253-595)
Apl2 (NP_012787.1)	AP-1	726	AP-1 beta (NP_001159491)	919	40% (aa25-600)
Apl4 (NP_015354.1)	AP-1	832	Ap-1 gamma (NP_001119.3)	822	31% (aa5-817)
Aps2 (NP_012592.1)	AP-2	147	AP-2 sigma (NP_004060)	142	49% (aa3-147)
Apm4 (NP_014579.1)	AP-2	491	AP-2 mu (NP_004059.2)	435	32% (aa1-490)
Apl1 (NP_012538.1)	AP-2	700	AP-2 beta (NP_001273.1)	937	32% (aa1-596)
Apl3 (NP_009516.1)	AP-2	1025	AP-2 alpha (NP_001229766.1)	940	26% (aa19-992)
Aps3 (NP_012510.1)	AP-3	194	AP-3 sigma (NP_001275.1)	193	46% (aa1-162)
Apm3 (NP_009847.1)	AP-3	483	AP-3 mu (NP_006794.1)	418	25% (aa198-482)
Apl6 (NP_011777.1)	AP-3	809	AP-3 beta (NP_003655.3)	1094	27% (aa21-748)
Apl5 (NP_015129.1)	AP-3	932	AP-3 delta (NP_003929.4)	1153	32% (aa23-732)

The innocuous consequences of deleting AP-1 subunit genes on growth or alpha-factor maturation of cells expressing wild-type clathrin are not unusual; similar observations have been made with other clathrin adaptors and clathrin-associated proteins (9, 81-84). The likely explanation is that there are alternative pathways and/or redundant proteins that allow cells to circumvent the effects of single protein inactivation. These findings and the adaptation of CPY sorting in *chc1-521* cells suggest that eukaryotic secretory and endocytic pathways have evolved robust traffic networks that allow alternative routes in response to transport perturbations. Accordingly, much genetic analysis of adaptors in yeast has involved using strains carrying mutations such as the *chc1-521* allele that sensitize cells to inhibition of individual clathrin-mediated pathways. In this review, defects that are only apparent or are exacerbated when two mutations are combined will be referred to as synthetic, and the mutant combination as a synthetic interaction.

In addition to Kex2p trafficking, AP-1 has been implicated in transport of DPAP-A, the P4-ATPase Drs2p, and the chitin synthase Chs3p between the TGN and endosomes (10, 85, 86). The only AP-1-interacting sorting signal that has been clearly established in yeast is located in the N-terminal 12 amino acids of the cytoplasmic domain of DPAP-A, consisting of the sequences $MSASTHSHKRKN_{12}$ (10). This sequence directly interacts with the mu1 subunit but is unrelated to the tyrosine-based and dileucine-based sorting signals recognized by mammalian APs. It remains to be determined which specific amino acids in the DPAP-A sorting signal are required for mu1 binding and whether similar sorting signals function in other AP-1-dependent cargo in yeast. A conventional tyrosine-based motif has been characterized in the vacuolar membrane protein Sna2p that appears to provide sorting into an AP-1 mediated pathway to the vacuole, but whether this involves direct binding has not been reported (87). Sorting of Sna2p appears to be complicated, involving two different tyrosine-based motifs, one for AP-1- and one for AP-3-dependent sorting.

Like mammalian AP-1, yeast AP-1 is associated with bidirectional traffic between the TGN and early endosomes (Figure 1), acting in transport of Drs2p from the TGN to early endosomes (85) and of DPAP-A and Chs3p from early endosomes to the TGN (10, 86). These studies imply that AP-1 can assemble at distinct organelles (TGN or endosome) to sort specific cargo into CCV targeted to different destinations (endosomes or TGN). An alternative model consistent with current data is that AP-1 vesicles assemble and bud from both the TGN and endosomes but act in retrieval to a common target in the Golgi, either the TGN or an earlier compartment (85). Recruitment of cargo into CCV at different sites could depend on the affinity of sorting signals for AP-1 or participation of different auxiliary adaptors at the TGN and endosomes.

Knockout of either mu1A or gamma subunit genes in mice results in embryonic lethality (88, 89). Fibroblasts from mu1A-/- mice are viable and exhibit defects in endosome to TGN traffic of the mannose-6-phosphate receptor, implicating AP-1 in retrieval from early endosomes to the TGN. Fibroblasts could not be cultured from gamma-/- mice, which die at an earlier embryonic stage (E4.5 versus E13.5 for mu1A-/- mice).

4.2. AP-2

Yeast AP-2 contains Apl1p (beta2), Apl3p (alpha), Apm4p (mu2), and Aps2p (sigma2). Although AP-2 does not appear to directly bind clathrin, it is associated with clathrin coated vesicles (75). Additionally, AP-2 localizes to the plasma membrane at virtually all forming endocytic vesicles (42, 43), suggesting that yeast AP-2 functions, like its mammalian counterpart, as a major clathrin adaptor for endocytosis (Figure 1). However, deletion of the yeast beta2 gene alone or in combination with deletion of the beta1 gene had no observable effect on uptake of alpha-factor and did not exacerbate the endocytic defect in *chc1-521* cells (8, 79). Moreover, cells lacking all four AP mu subunits or all six AP large subunits exhibited no defects in the initial rate of alpha-factor uptake (78). Thus, AP-2 does not appear to function in the clathrin-dependent receptor-mediated endocytosis of alpha-factor.

A role for AP-2 in endocytosis was revealed through a screen of deletion mutants for resistance to the K28 killer toxin (90). K28 is a toxin produced from a double-stranded RNA virus present in some strains of yeast. Cell killing by secreted K28 depends on endocytosis by the target cell (91). Cells deficient in AP-2 are defective in K28 internalization and therefore resistant to K28 (90).

This result suggests that AP-2 may function as a cargo-specific clathrin adaptor in yeast. Although there are examples of cargo in mammalian cells that rely on other endocytic adaptors (92), knockdown experiments provide evidence that AP-2 is required for formation of most endocytic clathrin coats in mammalian cells (93-95). The different consequences of AP-2 inactivation in yeast and mammalian cells favors a view that AP-2 has adopted a more cargo-selective function in the actin-based endocytic process in yeast.

AP-2 is required for the viability of mice. Mu2-/- mice die at E3.5 (96), and mu2-/- embryonic stem cells could not be generated (97) suggesting that AP-2 may be required for viability of mammalian cells. However, depletion of AP-2 in cell culture has no apparent effects on cellular viability, suggesting that either the residual levels of AP-2 in depleted cells allows for cell survival, or that AP-2 is not required for viability of at least some cell types (94, 95).

4.3. AP-3

Mammalian AP-3 subunit genes were originally uncovered in studies of neurons but subsequent studies revealed the existence of both neuronal and ubiquitously-expressed forms of AP-3 (98). In yeast, all four subunits of AP-3 were identified as targets for inactivating mutations that suppressed growth defects of cells deficient in the activities of plasma membrane casein kinases Yck1p and Yck2p (7). The basis for suppression was not evident at the time (see below). Yeast AP-3 contains Apl5p (delta), Apl6p (beta3), Apm3p (mu3) and Aps3 (sigma3).

A sorting function for AP-3, in either mammals or yeast, was first revealed through a screen for genes required for transport of vacuolar membrane alkaline phosphatase (ALP) to the vacuole (6). The motivation for the screen came from studies indicating that ALP reached the vacuole by a route that was independent of clathrin and transport through late endosomes (66, 99-103). Deletion of any AP-3 subunit gene impairs transport of ALP to the vacuole without affecting delivery of proteins such as CPY that travel through late endosomes. In cells expressing a temperature-sensitive mutant of Vam3p, a t-SNARE on the vacuolar membrane, shift to the restrictive temperature caused accumulation of vesicles containing AP-3 and ALP but not clathrin or CPY (104). Generation of ALP-containing vesicles was dependent on AP-3, indicating that AP-3 is necessary for vesicle formation. Together these results provided evidence for two distinct vesicle-mediated pathways from the Golgi to the yeast vacuole, one involving clathrin and proceeding by way of late endosomes, and the other dependent on AP-3 but independent of late endosomes (Figure 1).

Several AP-3-specific cargo have been characterized, all of which are localized to the limiting membrane of the vacuole [Nvy1p, Vam3p, ALP, Yck3p, Sna2p, Sna4p;(6, 87, 105-109)]. Misrouting of the vacuolar casein kinase Yck3p to the plasma membrane in AP-3 mutants allows Yck3p to substitute for Yck1/2p, accounting for the suppression of *yck1/2* mutant growth defects by deletions of AP-3 subunits (106). Tyrosine- or dileucine-based signals are required for sorting into the AP-3 pathway, and in the case of the tyrosine signal of the SNARE protein Nvy1p, interaction with AP-3 appears to be mediated by the mu3 subunit (108), as would be expected from characterization of tyrosine-based signals in mammalian cells.

Yeast AP-3 functions independently of clathrin by a number of criteria. In addition to the results mentioned above, ALP transport to the vacuole is not perturbed in *chc1-521* cells at restrictive temperatures where CPY transport is blocked (66, 107). Additionally, AP-3 does not directly bind clathrin and is not present in CCV (8, 69). In contrast, the original clathrin binding motif was defined in mammalian beta3 and AP-3 colocalizes with clathrin on endosomes (110). However, the significance of this association is uncertain because a clathrin-binding defective form of beta3 rescues AP-3-dependent sorting when introduced into beta3-deficient fibroblasts (111).

AP-3 plays a more complex role in mammalian cells (112). Unlike yeast AP-3, which most likely acts at the TGN, mammalian AP-3 localizes primarily to early endosomes (113, 114). Nevertheless, in both organisms, AP-3 appears to provide a common function, sorting proteins away from later endosomal compartments that undergo intraluminal vesicle formation. In non-specialized mammalian cells such as fibroblasts, AP-3 directs proteins to the limiting membrane of lysosomes much like the case in yeast. In specialized cells such as melanocytes, AP-3 is important for sorting proteins away from the lysosomal pathway and into pathways to specialized lysosome-related organelles such as melanosomes. There is also a brain-specific AP-3 isoform that functions in synaptic vesicle formation (115, 116).

Unlike loss of AP-1 or AP-2, loss of AP-3 in multicellular organisms does not lead to embryonic lethality, indicating that AP-3 function in multicellular organisms is not essential. Prominent phenotypes of AP-3 deficient mammals are loss of function of lysosome-related organelles such as melanosomes and platelet dense granules (112). Consequently, mice and humans lacking AP-3 function have pigmentation and bleeding defects (117-119). Also, mutation of neuron-specific subunits or subunits common to both ubiquitous and neuron-specific AP-3 isoforms can result in neuronal disorders (119-121).

5. MONOMERIC ENDOCYTIC ADAPTORS

5.1. Sla1p

The endocytic adaptor Sla1p was initially identified in a screen for proteins required for yeast cell viability in the absence of the actin binding protein Abp1p (122). Sla1p consists of three N-terminal polyproline binding SH3 (Src Homology 3) domains, two central SHD (Sla1 Homology Domains) domains unrelated to each other followed by a variant clathrin box and a C-terminus predicted to be unstructured (Figure 3a). There is not a clear mammalian homologue of Sla1p although similarities

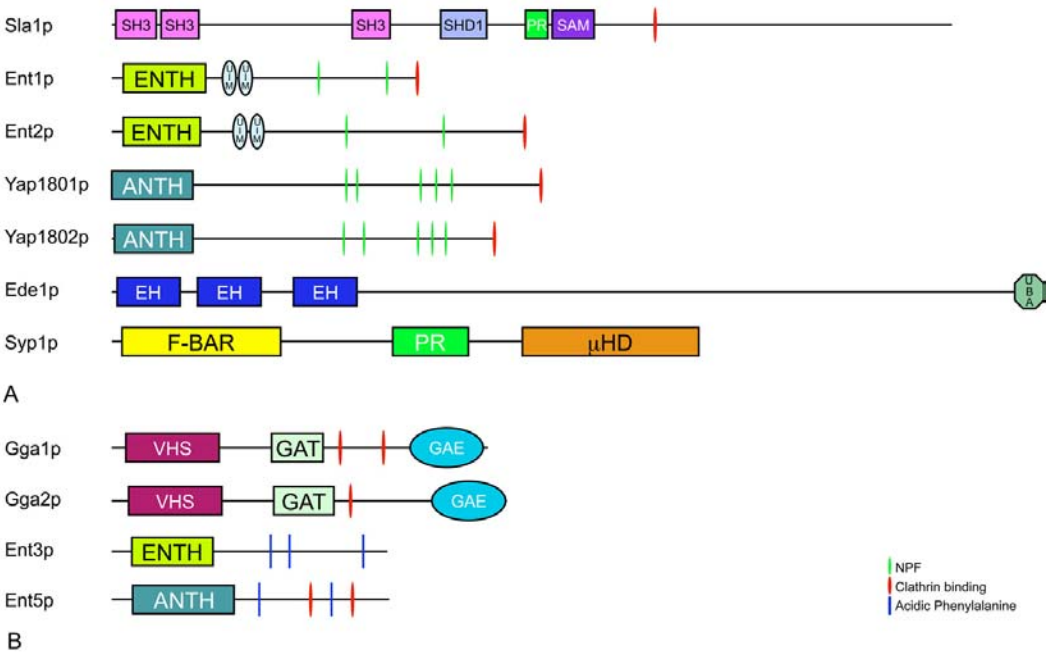

Figure 3. Domain representation of yeast monomeric adaptors. A) Endocytic adaptors. SH3: Src homology 3; SHD1: Sla1p-homology domain 1; SAM: Sterile alpha motif; ENTH domain: Epsin N-terminal homology domain; ANTH: AP180 N-terminal homology; EH: Eps15 homology; F-BAR: Fer/CIP4 homology Bin-Amphiphysin-Rvs; PR: proline-rich; muHD: mu-homology domain. B) TGN-endosome adaptors. VHS: Vps27, Hrs, STAM; GAT: GGA and TOM; GAE: gamma adaptin ear.

in organization have been noted with intersectin (123), an endocytic scaffold protein (124), CIN85 and CD2AP (CD2-associated protein) (125), multifunctional proteins with roles in cytoskeleton remodeling, cell survival, and endocytosis (126).

A role for Sla1p in endocytosis was first suggested by studies showing that hyperphosphorylation of Sla1p correlated with defects in endocytosis (127). Subsequently, it was discovered that Sla1p is an endocytic adaptor for an NPxFD endocytic sorting motif present in the cytoplasmic domain of the alpha-factor receptor (123, 128). NPxFD binding requires the SHD1 domain of Sla1p, which is structurally related to SH3 domains (129). Cells lacking Sla1p or expressing Sla1ΔSHD are impaired in NPxFD-, but not ubiquitin-mediated endocytosis of the alpha-factor receptor (123). Subsequently, additional NPFxD containing cargo have been identified for Sla1p, including Wsc1p, Dnf1p and Drs2p (130, 131). All Sla1p-specific cargo cycle between the plasma membrane, endosomes and the TGN, leading to the proposal that the NPFxD/Sla1p system is specialized for proteins that constitutively recycle between the cell surface and internal compartments (131).

Sla1p binds to clathrin through a variant of the consensus clathrin box motif, an interaction that is required for optimal endocytosis of the NPFxD-dependent cargo Wsc1p (132). Sla1p localizes to sites of endocytosis in yeast and colocalizes with clathrin (42, 43). Ultrastructural analyses of Sla1p at sites of endocytosis have demonstrated that Sla1p colocalizes with clathrin at the tip of the growing invagination (46). As the pit grows, Sla1p continues to localize to the tip of the invagination, suggesting that Sla1p concentrates cargo in the region of the invagination pinching off from the plasma membrane. Sla1p localization to sites of endocytosis occurs after recruitment of clathrin, indicating that the initial recruitment of clathrin to endocytic structures may be mediated by other adaptors (42, 43). Nevertheless, Sla1p is recruited early in the lifetime of the endocytic patch and precedes clustering of alpha-factor at sites of endocytosis (42, 133).

Structural analysis of the SHD2 domain of Sla1p revealed that it is a sterile alpha motif (SAM) domain (132). SAM domains constitute a large domain family, members of which can mediate protein oligomerization and serve as protein interaction platforms (134). SHD2 binds to the variant clathrin box, providing intramolecular inhibition of clathrin binding. Such inhibition was proposed to prevent premature clathrin binding in the cytoplasm, thereby restraining the clathrin box until Sla1p assembles at endocytic patches where the high concentration of clathrin could displace SHD2. SHD2 also mediates Sla1p oligomerization through an interface that contains the clathrin box-binding site; mutations in the SAM domain predicted to disrupt Sla1p oligomerization without affecting the clathrin box binding site decrease Wsc1p endocytosis. This observation suggests that oligomerization of adaptors may be an important feature of cargo clustering and coated vesicle formation.

Although Sla1p exhibits a similar domain organization to mammalian CIN85, CD2AP, and intersectin

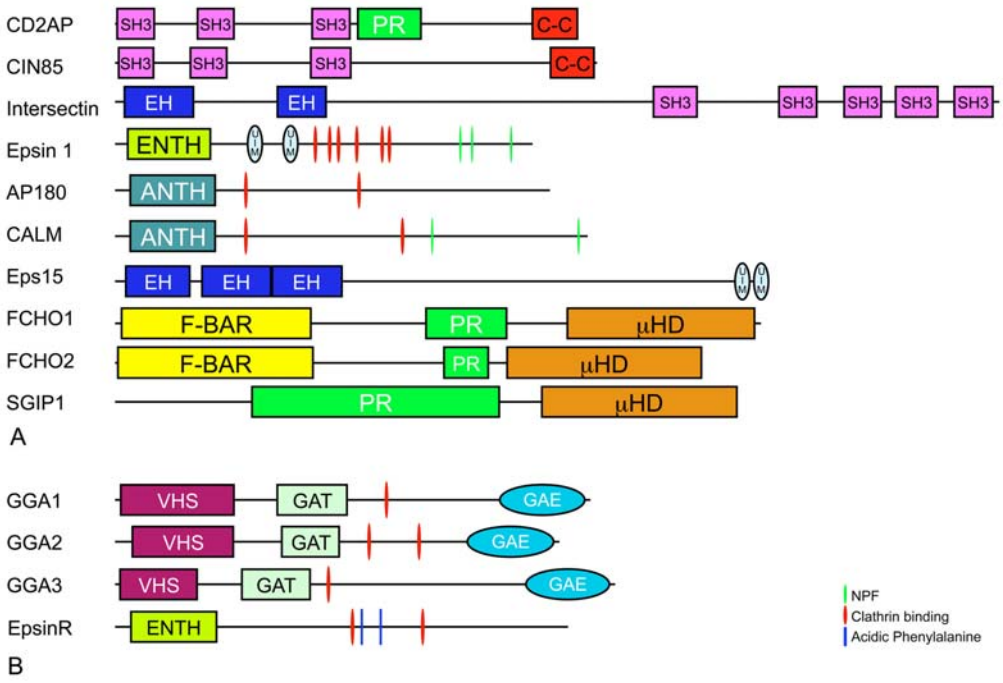

Figure 4. Domain representation of human monomeric adaptors. A) Endocytic adaptors. Domains labeled as in legend to Figure 3A. C-C: coiled-coil. B) TGN-endosome adaptors. Domains labeled as in legend to Figure 3B.

(Figure 4a), it is not clear how analogous the functions of these proteins are. All are associated with clathrin-mediated endocytosis and interact with both clathrin- and actin based machineries CIN85 and CD2AP can link specific receptors to clathrin coats (124). Clathrin adaptor function for specific cargo has not been defined for intersectin. Mice deficient in CD2AP exhibit kidney and immune cell malfunction, but these symptoms can be attributed to defects in actin-based clustering of surface proteins in podocytes and T cells rather than endocytosis (135). Mice deficient in brain-specific isoforms of CIN85 display defects in dopamine receptor endocytosis in neurons and hyperactive behaviors (136). Intersectin knockout mice do not have overt phenotypes but neuronal endocytosis is reduced (137).

5.2. Ent1p, Ent2p, Ede1p, Yap1801p and Yap1802p

In addition to Sla1p and AP-2, there are at least five other adaptor proteins that function during endocytosis in yeast: Ent1p/Ent2p, Yap1801p/Yap1802p and Ede1p (Figures 1, 3a). Ent1/2p and Yap1801/2p colocalize with clathrin at the cell cortex (43), and all four contain C-terminal clathrin box motifs that bind clathrin (138, 139) and function redundantly in clathrin recruitment to endocytic sites (43). In contrast, Ede1p does not contain a clathrin box motif and has not been demonstrated to directly interact with clathrin. These adaptors form a complex physical and functional network that is integral to endocytosis.

5.2.1. Ent1p and Ent2p

Ent1p and Ent2p are homologues of mammalian epsin endocytic adaptors and provide redundant functions that are necessary for endocytosis (139). Like the mammalian epsin (Figure 4a), the N-termini of Ent1p and Ent2p contain an N-terminal ENTH (Epsin N-terminal homology) domain that binds to $PI(4,5)P_2$, which is enriched at the plasma membrane (140-142). Based on studies of mammalian epsin1, ENTH domains contain an amphipathic helix that can insert into the lipid bilayer and induce membrane curvature (141, 143). Downstream of the ENTH domain, Ent1p and Ent2p each contain two ubiquitin interacting motifs (UIMs), two NPF (asparagine, proline, phenylalanine) motifs that bind to EH (Eps15 Homology) domain-containing proteins, and a C-terminal clathrin box that binds to clathrin (139, 140). Unlike mutations in other endocytic proteins, *ent1*Δ *ent2*Δ cells are inviable, a phenotype that is due to a role for the ENTH domain in cell polarity that appears to be independent of endocytosis (144).

The most widely used internalization signal in yeast appears to be ubiquitin (Ub), which is coupled to lysines in the cytoplasmic domains of endocytic cargo proteins (33). Therefore, the presence of UIMs in Ent1p and Ent2p make them candidate clathrin adaptors. Both Ent1p and Ent2p contain two UIMs that are required in part for their recruitment to cellular membranes (140). Ent1p and Ent2p are recruited to sites of endocytosis with dynamics similar to Sla1p, consistent with adaptor function (145). Although ent1Δ ent2Δ cells expressing a temperature-sensitive form of Ent1p are defective for endocytosis (139), ent1Δ ent2Δ cells expressing just the ENTH domain of Ent1p (thus lacking UIMs) are endocytically competent (146), indicating that other adaptor proteins may compensate for absence of Ub binding by Ent1p and Ent2p.

5.2.2. Ede1p

One protein that can compensate for Ub-binding by Ent1p and Ent2p is Ede1p, a homologue of the mammalian endocytic scaffold protein Eps15 (Figure 4a)(147). Ede1p localizes to endocytic sites, and Ede1-deficient yeast display defects in both fluid phase and alpha-factor endocytosis (148). Ede1p binds ubiquitin via a C-terminal ubiquitin-associated (UBA) domain and this interaction contributes to recruitment of Ede1p to membranes (140). Ede1p also contains three N-terminal NPF-binding EH domains, the third of which binds to NPF motifs in Ent1p. Although Ede1p does not contain a recognizable clathrin binding motif, interactions of Ede1p with the beta subunit of the AP-2 complex (149), Ent1p (140), Sla1p (150), and Yap1802p (123, 151) may allow indirect association with clathrin.

Ede1p assembles at endocytic sites concurrently with clathrin and before Ent1/2p, Yap1801/2p, and Sla1p (42, 43, 45, 133, 152). In addition, Ede1-deficient yeast contain fewer endocytic patches (42, 152). Together these findings support a role for Ede1p in initiation of endocytosis. Cells lacking Ede1p also fail to recruit Ent1p to sites of endocytosis, indicating that Ede1p may function in recruiting other adaptor proteins to endocytic sites (45, 152). Consistent with this hypothesis, Ede1p disappears from sites of endocytosis prior to clathrin and does not appear to move inward with the invaginating vesicle (44, 133). Thus, adaptors other than Ede1p may be functioning at later stages of endocytosis.

As expected for redundant function with Ent1/2p in recognition of ubiquitin internalization signals, *ent1*Δ *ent2*Δ *ede1*Δ cells expressing Ent1p lacking UIMs are defective in uptake of Ub-dependent cargo (147). However, a more recent study revealed that these cells are also deficient in uptake of NPFxD-dependent cargo, indicating that ubiquitin binding by these proteins plays a more general role in the function of the endocytic machinery, probably by providing sites for protein-protein interactions (153). Consequently, the identity of Ub receptors for internalization in yeast remains to be established. One possible candidate is Sla1p, as the third SH3 domain of Sla1p has been shown to bind ubiquitin (154, 155).

5.2.3. Yap1801p and Yap1802p

Yap1801p and Yap1802p are yeast homologues of the mammalian monomeric adaptors AP180/CALM (Figures 3a, 4a). Similar in organization to Ent1p and Ent2p, Yap1801p and Yap1802p contain an N-terminal $PI(4,5)P_2$ binding domain and a C-terminal region predicted to be unstructured with a variable number of NPF motifs and a C-terminal clathrin box. Unlike Ent1p and Ent2p however, Yap1801p and Yap1802p do not contain a Ub-binding domain. Also, the N-termini of Yap1801p and Yap1802p contain an ANTH (AP180 N-terminal Homology) domain instead of an ENTH domain. ANTH domains are similar to ENTH domains but lack the N-terminal amphipathic helix that participates in membrane curvature (141, 156).

Considering that Yap1801/2p are evolutionarily conserved, the absence of obvious endocytic defects in cells lacking both AP180 homologs (*yap1801*Δ *yap1802*Δ) was unexpected (78, 139). However, further analysis uncovered evidence for redundant function with Ent1/2p. For example, clathrin recruitment to endocytic sites does not occur in cells expressing a clathrin-binding mutant of Ent1p and lacking Ent2p and Yap180 proteins, but clathrin recruitment can be restored by expression of any individual adaptor (43). Also, in a similar adaptor-deficient background, expression of any one of the four adaptors allows endocytosis (146). Thus, there appears to be extensive redundancy in the general endocytic functions of Ent and Yap180 adaptors. In contrast, it is likely that there is specialization among the adaptors for cargo recognition. In the case of Yap1801/2p, a screen for mutants defective in endocytosis of the v-SNARE Snc1p identified Yap1801/2p (157). Since Yap1801/2-deficient cells are proficient in endocytosis of other cargo, Yap1801/2p appear to act as selective adaptors for Snc1p (139, 157).

In mice, individual knockouts of epsin1 and epsin2 genes result in viable animals but the double knockout is lethal at E9.5-10, at the onset of organogenesis (158). Analysis of cells from double knockout embryos indicated that clathrin-mediated endocytosis is generally intact. However, knockout mice exhibit defects in signaling by Notch, which requires ligand endocytosis for activation of signaling (159). This has led to the suggestion that epsin1/2 play a cargo-specific role in Notch ligand internalization. Mouse knockouts of AP180/CALM or Eps15 have not been reported.

5.3. Syp1p

Syp1p is a member of the muniscin protein family that includes human FCHO1 and FCHO2 and SGIP1 (160)(Figure 4a). Syp1p contains an N-terminal F-BAR (Fes/Cip4 homology; Bin, Amphiphysin, Rvs) domain that can induce membrane curvature, a central proline-rich domain, and a C-terminal mu homology domain (muHD) that is similar to the C-terminal cargo binding domain of the mammalian AP-2 mu subunit (Figure 3a). Syp1p binds to Ede1p, and like Ede1p is one of the earliest proteins to arrive at endocytic sites, departing prior to vesicle invagination (152, 160). Overexpression of Syp1p partially suppresses endocytic defects in *ede1*Δ cells, providing evidence for some overlap of function between Syp1p and Ede1p (160). However, deletion of *SYP1* alters the polarized distribution, but not the overall number, of endocytic patches, suggesting a role for Syp1p in localization rather than initiation of endocytic sites (152). Syp1p also inhibits Arp2/3-mediated actin polymerization *in vitro*, and overexpression of Syp1p reverses the decrease in Sla1p-GFP lifetimes caused by expression of a hyperactive Arp2/3 complex (161), suggesting that one function of Syp1p is to regulate the timing of endocytosis by preventing premature actin polymerization. In addition, Syp1p appears to act as a cargo specific adaptor for uptake of the plasma membrane stress sensor Mid2p, through an interaction between the Syp1p muHD and the cytoplasmic domain of Mid2p (160). Like Syp1p, the mammalian muniscins arrive early during formation of clathrin coats

and bind the Ede1p homologue Eps15 (162). Knockdown of FCHO1 and FCHO2 reduces the number of clathrin coated pits, indicating a role in initiation of coat assembly that is attributed to Ede1p in yeast (163). However, because effects of FCHQ1/2 knockdown on levels of clathrin coat formation vary (163), it is unclear whether these proteins serve as master initiation factors for encodytic clathrin coat initiation. Mouse knockouts of these proteins have not been described.

6. MONOMERIC TGN-ENDOSOME ADAPTORS

6.1. Gga1p and Gga2p

The absence of overt trafficking defects in cells deficient in AP-1 raised the possibility of additional sorting mechanisms/pathways acting in TGN-endosome transport. Discovery of the GGA (Golgi-localized gamma ear-containing, ARF-binding) proteins provided candidates for such sorting processes. GGA proteins were initially identified by several groups through database searches for homologues of AP subunits (164-166) or as interaction partners of mammalian Arf3 (167). There are three GGA proteins in mammals and two in yeast (Figures 3b, 4b). All are prominently localized to the TGN although there is evidence for some degree of endosome localization as well (4, 168-170).

Yeast Gga1p and Gga2p are about 60% identical, with Gga2p expressed at 5 to10-fold higher levels (82). Deletions of *GGA1* and *GGA2* yield defects in TGN-endosome clathrin-dependent transport processes including alpha-factor maturation, CPY transport, maturation of the vacuolar membrane protein carboxypeptidase S (CPS), and localization of the late endosomal t-SNARE Pep12p (82, 164, 165, 171-173). Furthermore, deletion of the predominant Gga protein, Gga2p, accentuates the growth and alpha-factor maturation defects in *chc1-521* cells (82), providing additional evidence for a role in clathrin-mediated traffic. In accord with the view that Gga proteins contribute to TGN-endosome traffic in AP-1-deficient cells, *gga2*Δ also exhibits synthetic genetic interactions with deletions of AP-1 subunits (82, 172), and cells lacking both Gga proteins and the beta subunit of AP-1 are severely compromised for growth (82).

In general, trafficking defects in Gga-deficient yeast are most consistent with a role for Gga proteins in transport from the TGN to late endosomes (Figure 1). For example, analysis of the t-SNARE Pep12p identified a motif, ESDSPEF, necessary for sorting from the TGN to late endosomes. Sorting to late endosomes directed by this sorting signal was dependent on Gga proteins and clathrin (171). Additionally, in a cell free transport assay that directly measures delivery to late endosomes, Gga proteins but not AP-1 were required for transport directed by the cytoplasmic domains of the CPY sorting receptor Vps10p or Kex2p (174).

The domain organization of Gga proteins fits well with roles expected of adaptor proteins. Gga proteins contain an N-terminal VHS (Vps27, Hrs, STAM) domain followed by a GAT (GGA and Tom1) domain, flexible linker, and a C-terminal gamma-adaptin ear (GAE) domain homologous to the appendage (ear) domain of the AP-1 gamma subunit (the basis for the homology with AP subunits that led to discovery of Gga proteins). The VHS domain binds to PI4P (175, 176) and, in the case of mammalian Gga proteins, to acidic dileucine sorting motifs with the consensus DxxLL (4). However, key residues in the mammalian Gga VHS domain that contact the DxxLL sorting motif are not conserved in yeast Gga VHS domains (177, 178) and no cargo (including Pep12p, see below) has been described that specifically interacts with the yeast Gga VHS domains. VHS-mediated interactions with the scaffold protein Mon2p/Ysl2p and the PI4-kinase Pik1p suggest roles for this domain in Gga protein localization and regulation of PI4P at the TGN (168, 170).

The GAT domain binds to the GTP-activated form of Arf GTPases and, at a separate site, ubiquitin (164, 167, 179-181). Although Arf binding is essential for membrane association of mammalian Gga proteins, yeast Gga proteins are less dependent on this interaction for proper localization (182). In contrast, Arf is required for localization of AP-1 in both yeast and mammals (4, 83). Ub binding by the GAT domain plays a role in sorting certain membrane proteins from the TGN into the multivesicular body (MVB) pathway to the vacuole lumen (82). In this pathway, ubiquitinated proteins delivered to the endosomes from either the TGN or from the endocytic pathway are incorporated into vesicles that bud into the interior of the endosome and are then delivered to the lumen of the vacuole. Initially, analysis of Gga GAT domain-dependent sorting of the amino acid permease Gap1p led to the proposal that GAT recognition of ubiquitinated Gap1p was important for sorting Gap1p from the TGN to endosomes (179, 183). However, more recent studies favor a revised model in which GAT engagement of Ub on membrane proteins is required at late endosomes for efficient targeting into the MVB pathway while another Gga function serves to sort membrane proteins from the TGN to late endosomes (184, 185). A possible TGN to endosome sorting signal has been defined on the Arn1p siderophore transporter consisting of two separate tripeptides, THN_{29-31} and YGL_{38-40} (184). The relationship between this signal and the Pep12p sorting signal is unclear and the binding partner has not been identified.

C-terminal to the GAT domain is a region predicted to be flexible and relatively unfolded that, like the AP-1 beta subunit linker region, contains clathrin box motifs. In both Gga proteins and AP-1, these motifs participate in clathrin binding but proteins lacking these motifs are still mostly functional, indicating the presence of other clathrin binding sequences in the adaptors and/or indirect interactions with clathrin through adaptor-binding partners (11, 173).

The Gga C-terminal GAE domain is homologous to the gamma appendage domain in AP-1 and serves as a binding platform for interaction with other clathrin coat-associated proteins (186). The only two Gga GAE interaction partners identified in yeast are the epsin-related proteins Ent3p and Ent5p (9). Both contain GAE acidic-

phenylalanine binding motifs with the consensus $[D/E]_nFxx\Phi$ that bind to a hydrophobic pocket on the GAE domain. The structural basis for these interactions is conserved in mammalian Gga GAE domains (9, 187). In contrast to the VHS and GAT domains, yeast Gga GAE domains are not required for Gga function, even in the absence of the AP-1 gamma appendage domain (172, 173). These findings imply that other binding interactions contribute to Ent3p and Ent5p membrane recruitment, a possibility that has been established for Ent5p (81).

The functional relationship between Gga proteins and AP-1 has been an important unresolved issue in understanding the basis for clathrin-mediated traffic between the TGN and endosomes. In both yeast and mammalian cells, the adaptors share interaction partners, display partial colocalization, and interact with each other (4). However, the genetic and *in vitro* analyses of Gga and AP-1 provide evidence that Gga and AP-1 adaptors provide at least partially distinct functions, among which are different cargo selectivities (10, 82, 85, 171, 174). Recently, live cell imaging of strains expressing fluorescent protein fusions to Gga2p and AP-1 beta revealed that the bulk of these adaptors assemble at the TGN in a sequential process in which Gga2p is recruited prior to AP-1 (168). Super-resolution images are consistent with semi-synchronous assembly of multiple Gga-enriched clathrin coats followed by assembly of AP-1-enriched coats at nearby sites. The process of adaptor progression is dependent on PI4P; AP-1 assembly does not occur until Gga-mediated recruitment of the Pik1p PI4 kinase provides sufficient levels of PI4P for AP-1 recruitment. These results provide evidence for a model in which Gga proteins and AP-1 primarily drive the assembly of distinct clathrin coats at the TGN, allowing clathrin coated vesicles with different cargo specificities to form at the same organelle. In this way, Gga-enriched CCV would transport proteins directly to late endosomes whereas AP-1 enriched CCV would act to retrieve proteins back to the TGN and/or transport proteins to early endosomes.

Like the yeast Gga proteins, mammalian GGA1-3 function in CCV-mediated transport from the TGN to endosomes (4) and GGA3 has been implicated in Ub-dependent sorting into the MVB pathway (169). Knockout mice deficient in GGA proteins have not been reported.

6.2. Ent3p and Ent5p

Ent3p and Ent5p were identified in a yeast 2-hybrid screen for proteins that bind to the GAE of Gga2p and the AP-1 gamma subunit (9). Both proteins have an N-terminal lipid binding domain (ENTH domain in Ent3p and ANTH domain in Ent5p), followed by a predicted unstructured region containing the acidic-phenylalanine GAE binding sequence and clathrin box motifs (Figure 3b). Both Ent3p and Ent5p bind to and colocalize with clathrin at the TGN (9, 168, 188). *ent3*Δ *ent5*Δ cells display reduced clathrin localization (9), suggesting a role in clathrin coat assembly. Ent5p but not Ent3p binds to AP-1 (9). Deletion of *ENT3* or *ENT5* does not cause trafficking defects but deletion of both affects alpha-factor maturation as well as trafficking of CPY, CPS, and Chs3p without significant effects on AP-3-dependent ALP transport (9, 81, 188, 189). These results suggest that Ent3p and Ent5p provide overlapping functions in clathrin-mediated TGN-endosome traffic. Yeast encode one additional epsin-related protein, Ent4p, which is less well characterized. Like *ent3*Δ, *ent4*Δ cells mislocalize the Arn1p siderophore transporter from the TGN to the plasma membrane (184). Additionally, *ent3*Δ *ent4*Δ cells missort CPY (189). Considering these results, Ent4p may also function like Ent3p and Ent5p in TGN-endosome traffic.

There are a number of functional distinctions between Ent3p and Ent5p. Ent3p localization depends on Gga proteins whereas Ent5p localizes in the absence of Gga proteins or AP-1 (81). Consistent with the physical relationships, genetic interaction studies provided evidence that Ent3p acts primarily with Gga proteins while Ent5p functions in both AP-1 and Gga-mediated transport but is more important for AP-1 pathways (81, 188)(Figure 1). In live cells, fluorescent protein tagged Ent3p assembles with Gga2p whereas Ent5p displays a bimodal assembly in which about 20% assembles with Gga2p and the remainder with AP-1, results that correlate well with the physical interaction studies (168).

Accumulating evidence supports a role for Ent3p in cargo selection for Gga-mediated transport. The Ent3p ENTH domain interacts with the SNARE proteins Vti1p, Syn8p, and Pep12p involved in fusion at late endosomes (189, 190). In the case of Pep12p, Ent3p ENTH domain binding depends on the ESDSPEF sorting motif required for TGN to late endosome transport (190). In addition, both Ent3p and Gga2p can be cross-linked to Pep12p in intact cells and the Gga2p interaction depends on the presence of Ent3p (188). Deletion of *ENT3* but not *ENT5* altered localization and stability of Pep12p (188, 190). Together these results support a role for Ent3p as a cargo adaptor that recognizes the Pep12p sorting signal and directs Pep12p into Gga-containing CCV for transport to late endosomes.

A similar role for Ent3p in localization of Vti1p is likely; the Ent3p ENTH domain binds to a surface acidic patch in Vti1p and this interaction is required for optimal localization of the SNARE (191). The Ent3p binding sequences in Pep12p and Vti1p are both present in the N-terminal Habc domains but located at different positions within these domains. Also, although both SNAREs bind to the same surface on Ent3p ENTH, the individual contact sites on the ENTH domain appear to be distinct (192). Thus, the Ent3p ENTH domain can recognize multiple SNARE cargo through distinct interactions.

The ENTH domain of Ent3p and the ANTH domain of Ent5p are predicted to bind phosphoinositides based on their homology to cognate domains in mammalian epsins and AP180s. The Ent3p ENTH domain displays preference *in vitro* for PI3P and $PI(3,5)P_2$, and the Ent5p ANTH domain for $PI(3,5)P_2$ (189, 193, 194). Considering that these phosphoinositides are associated with trafficking at endosomes (195), the reported phosphoinositide specificities of Ent3p and Ent5p are not entirely consistent

with data indicating primary functions for Ent3/5p at the TGN, which is enriched for PI4P. Indeed, the binding preferences for Ent3p and Ent5p have been questioned (196). Biochemical fraction experiments indicated that a minor amount of Ent3p and Ent5p were displaced from membranes in cells lacking the sole PI3 kinase Vps34p or PI3P 5-kinase Fab1p in yeast, and an overexpressed fluorescent protein fusion to Ent3p was mislocalized to the cytoplasm in *fab1*Δ cells (194). In contrast, in live cells lacking the PI3 kinase [and therefore deficient in PI3P and $PI(3,5)P_2$] no significant effects on recruitment of endogenously expressed Ent3/5p, AP-1 or Gga1/2p were observed, whereas a PI3P-binding reporter was completely cytosolic (168). Inactivation of the Golgi PI4 kinase Pik1p did not have strong effects on Gga2p and Ent3p assembly but uncoupled the sequential assembly of AP-1 and released most Ent5p into the cytosol. Based on these experiments it seems that *in vivo*, Ent3p does not require a specific phosphoinositide for membrane recruitment while Ent5p depends primarily on PI4P.

Effects of Ent3p and Ent5p mutations on sorting into the MVB pathway have been described (193, 194). Roles for Ent3/5p in MVB sorting would be compatible with binding to PI3P and $PI3,5P_2$, lipids that function in this process (195). However, MVB sorting defects were observed in cells carrying a temperature-sensitive allele of *ENT3* but deletion of *ENT3* did not affect the MVB pathway (194), raising the possibility that the Ent3p mutant exerts effects beyond the normal functions of the wild-type protein. MVB sorting defects were also observed in *ent3*Δ *ent5*Δ cells (193) but given the strong defects in TGN-endosome transport in these mutants, indirect effects on MVB sorting cannot be discounted.

The apparently discrepant results with Ent3p and Ent5p may be reconciled if these proteins, like Gga proteins, function in both TGN-endosome transport and MVB sorting. Considering the weak affinities of Ent3p and Ent5p for phosphoinositides, it seems possible that lipid binding is not a primary determinant for membrane recruitment but instead, specific protein-protein interactions govern the site of assembly. For example, Ent3p interacts with Gga2p, which binds specifically to PI4P and assembles at the TGN (168, 175). Both Ent3p and Ent5p also appear to bind to Vps27p (193), a PI3P-binding component of the MVB machinery, which would allow assembly at the MVB. Tests of this scenario will require mutations in Ent3p or Ent5p that affect MVB function without altering TGN-endosome transport.

A single TGN-endosome epsin-related protein, EpsinR, has been identified in mammals that preferentially binds PI4P and localizes primarily to the TGN [Figure 4b; (197)]. EpsinR seems to incorporate elements of both Ent3p and Ent5p. It contains an N-terminal ENTH domain that binds to the SNARE vti1b, a homologue of yeast Vti1p (189, 198) and epsinR plays a role in sorting vti1b into CCV (199). However epsinR binds AP-1 and strongly interacts with clathrin similar to Ent5p. Like Ent3p and Ent5p, epsinR acts in traffic between the TGN and endosomes (199-201). An epsinR knockout mouse has not been described.

7. CLATHRIN, ADAPTORS AND DISEASE

Perturbations of clathrin-dependent trafficking pathways have been associated with a variety of human diseases, as might be expected of processes fundamental to eukaryotic cell biology. Additionally, a wide variety of pathogens take advantage of clathrin-mediated endocytosis to enter cells and also co-opt clathrin machinery to promote other aspects of infection. Here we highlight examples of disease association and microbial pathogenesis involving clathrin and adaptors as well as the use of yeast as a model system to address disease mechanisms (Table 2).

A role for clathrin in human disease was first discovered through the ground-breaking work of Brown and Goldstein on the molecular basis of familial hypercholesterolemia (202). Their studies revealed that defects in the low-density lipoprotein receptor (LDL-R) are genetically associated with autonomic dominant forms of hypercholesterolemia. In one unusual case, although the LDL-R efficiently bound its substrate, it did not localize to clathrin-coated pits, resulting in a defect in internalization (203). This observation provided seminal evidence that receptor internalization is dependent on incorporation into clathrin-coated vesicles and offered a molecular explanation for the high levels of blood cholesterol in the patient.

7.1. Sorting signal mutations

Mutations in the Kir6.2 ATP-sensitive potassium channel that alter a YxxΦ endocytic sorting signal and impair channel endocytosis have been reported in patients with neonatal diabetes mellitus (204). Kir6.2 endocytosis was blocked in cultured cells by a dominant-negative form of the AP-2 mu subunit, providing evidence that channel uptake is normally mediated by AP-2 and clathrin. Defective endocytosis leads to increased cell surface levels of the channel in pancreatic beta cells, which is thought to alter regulation of insulin secretion.

A sorting signal mutation in another potassium channel, Kir2.1, is associated with Andersen-Tawil syndrome (ATS1), a disease characterized by periodic paralysis, arrhythmia, and developmental defects (205). The mutation blocks export of Kir2.1 from the TGN and prevents interaction with AP-1. In addition, RNAi-mediated knockdown of AP-1 gamma caused accumulation of Kir2.1 in the TGN. Based on these findings it has been proposed that defects in clathrin/AP-1 mediated transport of Kir2.1 out of the TGN reduce cell surface expression of the channel, leading to ATS1.

7.2. AP Mutations

While loss of AP-2 mediated endocytosis has been implicated in disease, mutations in AP-2 itself have not been observed, suggesting an essential function for AP2 in humans. In contrast, mutations in all of the remaining AP complexes have been linked to disease.

Table 2. Diseases associated with clathrin and clathrin adaptors

Mammalian Protein	Yeast Ortholog	Disease	References
Clathrin	Clathrin	Viral uptake	256-267
		Uptake of *Lysteria monocytogenes*	277, 280
		Enteropathogenic *E. coli* infection	283
		Uptake of bacteria toxins	284-286
		Various types of cancer	236-239
		Alzheimer's disease	242
AP-1	AP-1	Andersen-Tawil syndrome	205
		X-linked mental redardation	207-209
		Syndrome with mental retardation, enteropathy, deafness, neuropathy, icthyosis and keratodermia	206
		MHC downregulation during HIV-1 infection	275
AP-2	AP-2	Neonatal diabetes mellitus	204
		CD4 downregulation during HIV-1 infection	273, 274
		Alzheimer's disease	246
AP-3	AP-3	Hermansky-Pudlak Syndrome	117
		HIV-1 pathogenesis	269, 270
AP-4		APP sorting out of TGN	249
GGA1-2	Gga1p/Gga2p	BACE sorting out of endosomes	251-253
		HIV-1 pathogenesis	271
GGA3	Gga1p/Gga2p	BACE sorting to lysosomes	255
Epsin	Ent1p/Ent2p	Influenza virus entry	259
		Enteropathogenic E. coli infection	282
Eps15	Ede1p	Enteropathogenic E. coli infection	282
		Acute myelogenous leukemia	232
AP180	Yap1801p/Yap1802p	Alzheimer's disease	224-226
CALM	Yap1801p/Yap1802p	Alzheimer's disease	216-218, 220-223, 289
		Acute myelogenous leukemia, acute lymphoblastic leukemia	233
CD2AP	Sla1p?	Enteropathogenic E. coli infection	281
		Alzheimer's disease	219
SGIP-1	Syp1p	Obesity	231
EpsinR	Ent3p/Ent5p	Genetic susceptibility to schizophrenia	227-230

A splice site mutation in the gene encoding the AP-1 sigma 1A isoform has been identified in families with a syndrome displaying mental retardation, enteropathy, deafness, peripheral neuropathy, ichthyosis and keratodermia (206). Mutations in the second AP-1 sigma isoform, sigma 1B, are associated with X-linked mental retardation (207-209). The molecular bases for the disease phenotypes in these cases have not been defined.

Perhaps the most well-characterized adaptor role in disease derives from studies of AP-3. Humans lacking AP-3 function due to a mutation in AP-3 beta suffer from Hermansky-Pudlak syndrome type 2 (HPS2), a disease characterized by malfunction of lysosome-related organelles (LRO)(117). Platelets, melanocytes, neutrophils, cytotoxic T cells, and natural killer cells all depend on the presence of LROs; consequently, loss of LRO function due to AP-3-deficiency perturbs pigmentation, function of immune cells and coagulation (210). In at least some of these cases, specific defects in AP-3-dependent sorting have been characterized that can account for LRO-related disease symptoms. For example, albinism can be explained by a failure to properly sort the first enzyme in the melanin pigmentation pathway, tyrosinase (211). Improper sorting of neutrophil elastase in AP-3 deficient neutrophils (212) likely contributes to immunodeficiency, as does missorting of the lipid antigen-presenting protein CD1b (213). Less well-defined AP-3 roles include microtubule-based secretory granule motility in cytotoxic T cells (214), innate immune receptor TLR7 and TLR9 signaling in antigen-presenting cells (215), and formation of platelet dense granules in platelets (112).

7.3. Monomeric adaptor mutations

Genetic association studies have implicated monomeric adaptors in several different types of disease. AP180, CALM and CD2AP (related to Sla1p) have been identified as risk factors for Alzheimer's disease (216-226). Additionally, epsinR is associated with schizophrenia (227-230), and SGIP1 (which has a domain structure similar to Syp1p) is linked to obesity (231). How the adaptor proteins contribute to disease remains to be established in all cases.

A number of genetic alterations of monomeric adaptors have also been detected in different cancers. Most prevalent are gene fusions that join adaptors to transcription factors. For example, fusions of Eps15 and CALM have been identified in acute lymphocytic and lymphoblastic leukemias (232, 233). However, further analysis of a CALM-AF10 fusion demonstrated that, although the fusion inhibited endocytosis when introduced into cultured cells, receptor-mediated endocytosis was not consistently altered in CALM-AF10+ leukemia cells (234). These data suggest that perturbation of endocytosis is not a major oncogenic effect of the fusion protein. An alternative hypothesis is that the adaptor fusion protein fosters oligomerization of the attached transcription factor, thereby promoting transcriptional activity, a model proposed previously for the Eps15-AF10 fusion (235). Similar uncertainties apply to fusions of the clathrin heavy chain gene found in different cancers (236-239). It should be noted however that large-scale sequencing studies have identified somatic mutations in genes encoding Eps15 and clathrin in solid tumors, though these were not the most frequent genetic changes in tumor samples (240, 241).

7.4. Functions of adaptors in trafficking of Alzheimer's disease-related proteins

In addition to the association studies described above, clathrin-mediated pathways have been implicated in the etiology of Alzheimer's disease. Clathrin has been observed in the neurofibrillary plaques in Alzheimer's disease (242). Neurodegeneration in Alzheimer's disease is thought to be caused by aggregation of beta-amyloid (A-beta), which is formed by aberrant cleavage of the Alzheimer's precursor protein (APP). A-beta production requires APP cleavage by beta-amyloid converting enzyme, or BACE (243). Both APP and BACE are transported to the cell surface and rapidly internalized via clathrin-mediated endocytosis (244, 245). In the case of BACE, endocytosis requires the AP-2 adaptor. Notably, the optimal pH for BACE function is 4.5, suggesting that the majority of cleavage by BACE occurs in endosomes (246). Consistent with this hypothesis, blocking or enhancing endocytosis reduces or increases A-beta production, respectively (247, 248). Moreover, a mutation in APP that impairs internalization reduces secretion of A-beta (245). APP also binds to AP-4, and APP mutants that impair AP-4 binding cause mislocalization of APP from endosomes to the TGN (249). Surprisingly, loss of AP-4 mediated sorting increased production of A-beta, suggesting that BACE-mediated cleavage of APP can also take place in the TGN, and that transport of APP out of the TGN may be important to prevent A-beta pathogenesis.

In addition to AP-2, BACE can also bind to GGA proteins via an interaction between an acidic dileucine sorting signal and the GGA VHS domain (250). Depletion of GGAs or inhibition of GGA function results in accumulation of BACE in endosomes and increases A-beta production (251, 252). In contrast, GGA overexpression reduces A-beta production (253). Expression of GGA1 is lower in Alzheimer's brains relative to controls, suggesting that a loss of GGA function may contribute to disease (254). GGA3, unlike GGA1 and GGA2, binds ubiquitinated BACE (169), suggesting a role in directing BACE into the MVB pathway. Consistent with this model, GGA3 depletion stabilizes BACE by impairing transport to lysosomes (255). Expression of GGA3 is also lower in Alzheimer's brains, with an inverse correlation between the levels of GGA3 and BACE(254, 255). Together these studies raise the possibility that changes in the levels or functions of clathrin adaptors can contribute to the pathology of Alzheimer's disease through effects on A-beta production.

7.5. Microbial pathogenesis

Microbial pathogens often take advantage of clathrin-mediated endocytic machinery for cell entry and can engage clathrin-based intracellular trafficking pathways to advance infection. A number of viruses have exploited clathrin-mediated trafficking pathways (256). Influenza, hepatitis C, Ebola, poliovirus and SARS are all able to enter cells at least in part via clathrin mediated endocytosis (257-262). There is also evidence that HIV-1 can enter cells via clathrin-mediated endocytosis (263, 264). Influenza virus entry has been shown to require epsin, suggesting that the receptor for influenza virus entry is recruited into clathrin-coated pits using epsin as an adaptor (259). A dominant negative Eps15 blocks entry of a number of viruses, including hepatitis C, Ebola and HIV-1 but the effects are probably due to sequestration of general clathrin coat components rather than cargo selective effects expected if Eps15 served as a cargo-specific adaptor (258, 261, 263). A similar caveat applies to dominant negative AP180 blockade of foot and mouth disease virus entry (265).

The actin dependent endocytic process in yeast may be a particularly appropriate model for entry of large viruses like vesicular stomatitis virus (VSV). This bullet shaped virus induces formation of clathrin coats but enters through vesicles that are only partially coated (266), reminiscent of membrane invaginations in yeast that carry the clathrin coat only at the tip of the invagination (46). Furthermore, VSV entry is dependent on actin, a requirement imposed by the size of the virus based on the finding that actin polymerization is not necessary for uptake of a smaller mutant VSV particle (267).

Not only do viruses use clathrin-mediated endocytosis for cell invasion, but they also can manipulate clathrin-mediated trafficking pathways during their life cycle. The envelope G protein of VSV binds AP-3 delta, an interaction that is required for transport out of the Golgi (268). The HIV-1 Gag polyprotein also binds to AP-3 delta, and disrupting this interaction blocks trafficking of Gag and viral particle formation (269, 270). GGA proteins have also been implicated in trafficking of HIV Gag, as GGA overexpression impairs Gag trafficking to the PM and particle production (271).

In another example of viral interaction with clathrin-mediated trafficking, HIV-1 manipulates clathrin-mediated pathways to downregulate surface expression of both CD4 and MHC-I (272). The HIV-1 Nef protein links the HIV-1 co-receptor CD4 to AP-2, leading to CD4 downregulation, thus preventing superinfection and increasing viral production (273, 274). Nef also is able to downregulate MHC class I expression by binding to AP-1 and causing MHC-I retention in the Golgi (275).

Bacteria and fungi also take advantage of clathrin coats for entry and/or propagation (276). Internalization of pathogens such as *Listeria monocytogenes*, Rickettsia, *Candida albicans*, and enteropathogenic *E. coli* (EPEC) is clathrin dependent (276-280). However, these organisms are too large to be incorporated into conventional clathrin coated vesicles. Studies of Listeria and EPEC suggest that the bacteria induce formation of stable clathrin coated pits that serve as platforms for actin filament assembly to drive the membrane dynamics necessary for internalization (276). As in yeast, the bacterially induced clathrin coats are linked to actin through interactions between clathrin CLC and the Sla2 homologue Hip1R. Additionally, in the case of EPEC, the formation of the clathrin-dependent actin-rich pedestals involved in internalization requires Eps15, epsin, and CD2AP adaptors, but not AP-2 (281-283).

Finally, a number of bacterial toxins enter cells at least in part through clathrin-mediated endocytosis,

including Shiga, Anthrax and cholera toxins (284-286). Together, these studies highlight the essential role of clathrin and adaptor proteins during the microbial life cycle for a wide range of pathogens.

7.6. Yeast models for disease

The ease of genetic manipulation make yeast an attractive organism to model disease (287, 288). Such models for neurodegenerative diseases associated with protein aggregation have uncovered possible roles for clathrin machinery in the disease process (288).

A-beta expression and targeting to the secretory pathway of yeast inhibits cell growth, providing a model for A-beta toxicity in Alzheimer's disease (289). A genome-wide overexpression screen for suppressors of toxicity identified the AP180/CALM homologue Yap1802p and CD2AP-related Sla1p, both of which are risk factors for Alzheimer's disease. Overexpression of CALM suppressed the toxicity of A-beta in rat cortical neurons, suggesting that the findings in yeast can be extended to mammals. A-beta expression in yeast perturbed clathrin-mediated endocytosis, a defect that was partially rescued by suppressor overexpression. Together these findings provide evidence for functional roles of the endocytic machinery in A-beta toxicity.

Huntington's disease (HD) is an autosomal dominant disorder caused by expansion of a polyglutamine (polyQ) track in the Huntington protein (Htt). PolyQ expansion increases the propensity of Htt to aggregate and neuronal Htt aggregates are a hallmark of HD. Expression of Htt containing disease-associated polyQ expansions in yeast results in Htt aggregates (290). In one system, polyQ Htt expansion was detrimental to cell growth. In this system, loss of endocytic components including clathrin, Sla1p, AP-2, Ede1p and End3p enhanced Htt toxicity (291, 292). Furthermore, like A-beta, polyQ-expanded Htt inhibited endocytosis in yeast. Based on the association of yeast endocytic proteins such as Sla1p with Htt aggregates, it was proposed that one mechanism of Htt toxicity is depletion of endocytic factors (291, 293).

Mutations in alpha-synuclein cause hereditary Parkinson's disease. In addition, alpha-synuclein is a major component of Lewy bodies, aggregates that are characteristic of neurons in both familial and sporadic PD. Expression of alpha-synuclein in yeast inhibits endocytosis (294), however screens for suppressors of alpha-synuclein toxicity in yeast suggest that ER to Golgi transport is a more significant pathway in growth inhibition (295). Yeast expressing alpha-synuclein are hypersensitive to oxidative stress imposed by exogenous hydrogen peroxide, and a screen for overexpression suppressors of hydrogen peroxide hypersensitivity identified Ent3p (296). Furthermore, deletion of *ENT3* enhanced oxidative hypersensitivity. These results raise the possibility that manipulation of intracellular clathrin-mediated trafficking events subsequent to ER to Golgi transport may represent an additional approach to modulate alpha-synuclein toxicity.

8. PERSPECTIVES

The observation that clathrin has been conserved from mammals to yeast nearly three decades ago allowed pioneering molecular genetic studies in yeast of clathrin-mediated protein trafficking *in vivo*. Subsequent studies have revealed extensive conservation of clathrin coat components and provided insights into mechanisms responsible for clathrin coated vesicle formation. More recently, live cell imaging of fluorescent proteins expressed at endogenous levels has offered a means to address regulation of the complex dynamics of coat assembly. With this foundation there are significant challenges for the future. One key question is how clathrin-mediated trafficking is regulated in response to different cellular and environmental states. In this regard, the extensive genome-wide genetic and protein interaction data available in yeast offer a promising starting point for systems-level analyses of clathrin-dependent trafficking. This approach holds the potential to define the global interrelationships between different cellular pathways in the context of cellular physiology (287). To complement the global approach it will be critical to define the specific molecular changes such as posttranslational modifications that alter the activity of clathrin coat components in response to different physiological cues. For example, TGN-endosome clathrin adaptors are transiently released from membranes upon acute glucose deprivation (297). Understanding the mechanism responsible for this release will address how the energy state of the cell can impact clathrin-mediated transport. In addition to approaches such as these that are focused on understanding clathrin function in normal cells it will also be important to continue development of yeast models of disease. The conserved biology, facile genetics, ease and low cost of propagation, and short generation time make yeast well-suited for high-throughput screens. Appropriately-designed models thus offer a promising strategy to identify novel therapeutic agents to combat human disease.

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Abbreviations: A-beta: beta-amyloid; ALP: Alkaline phosphatase; ANTH: AP180 N-terminal homology; AP: Assembly polypeptide; APP: Alzheimer's precursor protein; ATS: Andersen-Tawil syndrome; BAR: Bin, Amphiphysin, Rvs; CCV: Clathrin coated vesicle; CD2AP: CD2 associated protein; CHC: Clathrin heavy chain; CLC: Clathrin light chain; CPS: Carboxypeptidase S; CPY: Carboxypeptidase Y; DPAP-A: Dipeptidyl aminopeptidase A; EH: Eps15 homology; ENTH: Epsin N-terminal homology; EPEC: Enteropathogenic *E. coli*; ER: Endoplasmic reticulum; F-BAR: Fes/Cip4 homology/Bin, Amphiphysin, Rvs; GAE: γ adaptin ear; GAT: GGA and Tom1; GGA: Golgi-localized, γ ear-containing, ARF-binding; HD: Huntington's disease; HIV-1: Human immunodeficiency virus 1; Htt: Huntington protein; HPS2: Hermansky-Pudlak syndrome type 2; LRO: Lysosome related organelle; NPF: Asparagine, proline, phenylalanine; polyQ: Polyglutamine; SAM: Sterile alpha motif; SH3: Src homology 3; SHD: Sla1 homology domain; TGN: *trans* Golgi network; UBA: ubiquitin associated; UIM: ubiquitin interacting motif; VHS: Vps27, Hrs, STAM; VSV: Vesicular stomatitis virus;

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