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The long life of an endocytic patch that misses AP-2

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Abstract

Endocytosis is the process by which cells regulate extracellular fluid uptake and internalize molecules bound to their plasma membrane. This process requires the generation of protein-coated vesicles. In clathrin-mediated endocytosis (CME) the assembly polypeptide 2 (AP-2) adaptor facilitates rapid endocytosis of some plasma membrane receptors by mediating clathrin recruitment to the endocytic site and by connecting cargoes to the clathrin coat. While this adaptor is essential for early embryonic development in mammals, initial results suggested that it is dispensable for endocytosis in unicellular eukaryotes. The drastic effect of depleting AP-2 in metazoa and the mild effect of deleting AP-2 subunits in *Saccharomyces cerevisiae* have prevented a detailed analysis of the dynamics of endocytic patches in the absence of this adaptor. Using live-cell imaging of *Schizosaccharomyces pombe* endocytic sites we have shown that eliminating AP-2 perturbs the dynamics of endocytic patches beyond the moment of coat assembly. These perturbations affect the cell growth pattern and cell wall synthesis. Our results highlight the importance of using different model organisms to address the study of conserved aspects of CME.

Keywords: AP-2, Clathrin adaptor, Endocytosis, Live-cell imaging, *Schizosaccharomyces pombe*, Yeast

Clathrin and clathrin adaptors.

Clathrin, which plays a major role in vesicle trafficking, was initially described in 1976 (Pearse 1976) and is generally comprised of three units of clathrin heavy chains and three units of clathrin light chains able to autoassemble to form triskelions. Triskelions form a cage surrounding a small region of membrane producing a clathrin-coated pit where cargoes are sorted and the vesicles originate (Vigers et al. 1986a). Clathrin cannot bind directly to either cargoes or membranes and, therefore, requires the presence of different adaptors that associate to membrane lipids, recognize specific sequences in the cargoes, and bind clathrin for the generation of coated vesicles. The best-characterized adaptors are the Assembly Polypeptides (AP) complexes (Pearse and Bretscher 1981; Vigers et al. 1986b). Five AP adaptors (AP-1 to AP-5) have been described; they are heterotetramers composed of two large adaptins, a small subunit, and a medium size subunit. AP-2 is involved in endocytosis and is formed by the α and β adaptins, the μ 2 medium subunit and the σ 2 small subunit (figure 1). The N-terminal regions of adaptins bind the μ and σ subunits forming the “head” of the adaptor that participates in cargo recognition, while the C-terminal end of the adaptins form an appendage involved in the binding of accessory proteins. Accessory proteins promote the progression of the clathrin-coated pit into a vesicle and its scission from the donor membrane. The head and appendages are separated by flexible hinges, with the clathrin-binding motif being located at the β subunit hinge (for reviews about clathrin adaptors see Edeling et al. 2006; Hirst and Robinson 1998; Merrifield and Kaksonen 2015; Reider and Wendland 2011; Robinson and Bonifacino 2001).

1 Significant advances in any science are frequently associated with the development of
2 new tools and techniques. While the initial and basic results in the field of CME relied
3 on the use of electron microscopy and biochemical techniques, the arrival of molecular
4 biology and the incorporation of crystallography and structural modelling in the 80's led
5 to a better characterization of the molecules involved in the process, and to the
6 confirmation of predictions made in earlier works (Kirchhausen 1999; Roth 2006). The
7 studies of CME using metazoan models were soon complemented with research
8 performed in *Saccharomyces cerevisiae*, a model organism with powerful genetics,
9 leading to the identification of numerous proteins involved in endocytosis. In fact, some
10 aspects of the process were first identified in yeast, and later shown to be conserved in
11 higher eukaryotes (Goode et al. 2015). In spite of this, initial results indicated that AP-2
12 did not play a role in endocytosis in yeast because the uptake of the α -factor mating
13 pheromone was not affected by AP-2 mutations, and because no physical or genetic
14 interaction between AP-2 components and clathrin was found (Boehm and Bonifacino
15 2001; Yeung et al. 1999). These results impeded the study of yeast AP-2 for a time.
16 Later, the availability of collections of viable yeast null mutants permitted genome-wide
17 studies to flourish. In 2009, a massive screening for mutants with altered sensitivity to
18 the K28 killer toxin led to the identification of strains resistant to this toxin; some of
19 these strains were deleted for AP-2 subunits. Thus, resistance to K28 was the first
20 phenotype associated with yeast AP-2 mutants (Carroll et al. 2009). A few years later it
21 was established that yeast AP-2 interacts with the stress sensor Mid2 and the protein
22 kinase Pkc1, and that deletion of the μ subunit causes defects in cell growth patterns
23 (Chapa-y-Lazo et al. 2014; Chapa-y-Lazo and Ayscough 2014). These results argued
24 against the notion of AP-2 being dispensable for CME in yeast and prompted us to

1 continue our research using a lower eukaryote model to further investigate the function
2 of this adaptor.

3 4 **The life of an endocytic clathrin-coated patch**

5 The lifetime of a vesicle, from the moment when the clathrin coat assembles to the time
6 when the cargo is delivered, ranges from seconds to a few minutes (McMahon and
7 Boucrot 2011). Although some of the steps involved in the process were inferred from
8 biochemical and structural analyses, the finest details have been observed by live-cell
9 imaging. The onset of the use of fluorescent proteins represented a quantum leap in cell
10 biology because they allowed dynamic events to be monitored by time-lapse
11 microscopy in a single live cell. The use of fluorescent proteins, together with the
12 development of new microscopy techniques and image-processing software has greatly
13 facilitated the analysis of temporal and spatial dynamics of a protein of interest, the
14 physical interaction between two proteins, and the behavior of proteins at the cell
15 surface, leading to a better understanding of the function of specific proteins. In
16 particular, these techniques have aided in the advancement of knowledge of the
17 progression of a coated pit into a vesicle and its scission from the membrane. Clathrin
18 assemblies were first observed in living cells using transient expression of GFP-tagged
19 clathrin light chains (Gaidarov et al. 1999). During the following years several studies
20 analyzed the assembly of clathrin and AP-2 at the endocytic sites, the incorporation of
21 regulatory proteins, the recruitment of cargoes, and the scission of the endocytic
22 vesicles. These studies, undertaken in metazoan and budding yeast, have generated a
23 wealth of information that has allowed the temporal stages and the complex
24 choreography of proteins during endocytosis to be dissected.

1 The proteins involved in endocytosis have been grouped in different functional modules
2 according to their time of arrival at the endocytic sites, their lifetime, and their spatial
3 dynamics. The sites of the plasma membrane where the proteins involved in endocytosis
4 aggregate are known as endocytic patches. The initiation of endocytosis in a
5 fosfatidilinositol 4,5-bifosfato-enriched site of the plasma membrane is defined by the
6 arrival of “early proteins” (Eps15/Ede1p and FCHO1/Syp1p). The binding of two AP-2
7 units bridged by one clathrin triskelion to the site triggers the stable incorporation of
8 other “coat module” molecules (Cocucci et al. 2012). Although other adaptors may
9 substitute AP-2 in the endocytosis of certain cargoes (Traub 2003), recent studies using
10 genome-editing techniques in mammalian cells have shown that successful progression
11 of an endocytic patch into a vesicle requires the presence of AP-2 (Hong et al. 2015).
12 Clathrin regulates a transition point, possibly defined by cargo recruitment (Carroll et
13 al. 2012), after which the lifetime of proteins at the patch are fairly regular. The
14 endocytic coat remains basically immobile during 60-120 seconds until actin
15 polymerization is triggered. Actin polymerization requires the incorporation of the
16 “WASP/Myo module” proteins, followed by the recruitment of the “actin module”
17 proteins, which include the Arp2/3 complex and actin. Actin polymerization, interaction
18 of End4/Sla2 with actin and clathrin (Boettner et al. 2011), and the Myo1p motor
19 activity contribute to the elongation of the endocytic tubular invagination during the 10-
20 second time frame that defines the patch slow-mobile phase, which when observed by
21 live-cell microscopy appears as an inward movement of fluorescent patch markers to the
22 cytoplasm. Finally, the vesicle pinches off from the membrane and exhibits a fast
23 movement for around 5 seconds; this fast-mobile phase lasts until the coat disassembles.
24 The general features of this process are evolutionarily conserved between yeast and
25 metazoa (Boettner et al. 2012; Brach et al. 2014; Galletta and Cooper 2009; Galletta et

al. 2010; Merrifield and Kaksonen 2015; Toret and Drubin 2006; Weinberg and Drubin 2012).

The life of *Schizosaccharomyces pombe* endocytic patches in the absence of AP-2

Although the role of AP-2 in endocytosis is well established, the dynamics of the endocytic patches in the absence of the adaptor has not been addressed in detail in metazoa, probably because of the strong phenotype associated with AP-2 depletion (Boucrot et al. 2010). In the case of *S. cerevisiae*, it was described that neither Sla1-GFP patch number nor the dynamics of this marker were altered when the Apl1 β subunit was deleted (Carroll et al. 2009). In order to obtain more information about the effect of AP-2 depletion in the dynamics of the endocytic patch, we decided to use a different model organism. The fission yeast *Schizosaccharomyces pombe* diverged from budding yeasts 500 million years ago, has less genetic redundancy, and is a well-established model for cell-cycle, polarity, and cytokinesis (Rhind et al. 2011). *S. pombe* is very helpful when studying protein trafficking because some aspects of vesicle transport are similar to those of metazoans; thus, contrary to *S. cerevisiae* that mostly has a single-cisterna Golgi apparatus (Preuss et al. 1992), this fission yeast has a stacked Golgi (Ayscough et al. 1993). Additionally, whereas in *S. cerevisiae* deletion of AP-1 subunits leads to no apparent phenotype unless combined with other mutations affecting vesicle transport (Boehm and Bonifacino 2001; Valdivia et al. 2002; Yeung et al. 1999), in *S. pombe* this clathrin adaptor is required for efficient secretion of some cargoes, and AP-1-defective mutants exhibit multiple phenotypes (Kita et al. 2011; Kita et al. 2004; Ma et al. 2009). Furthermore, clathrin is essential for survival in animals and in *S. pombe* (Bazinet et al. 1993; de Leon et al. 2013) while in *S. cerevisiae* *chc1 Δ* mutants are viable unless combined with a mutation in other *loci* and are able to secrete proteins

(Lemmon and Jones 1987; Munn et al. 1991). The convenience of using this organism to gain information about AP-2 function is supported by the facts that quantitative confocal microscopy and mathematical modelling have been used to characterize endocytosis in this organism, which has been found to generally follow the steps described for other organisms (Berro et al. 2010; Sirotkin et al. 2010), and that some new aspects of patch progression have been uncovered using this yeast (Basu and Chang 2011).

Our laboratory is interested in the use of live-cell imaging to analyze the behavior of proteins required for the regulation of cell morphogenesis. Hence, we analyzed the behavior of *S. pombe* endocytic patches in mutants lacking AP-2 using a spinning-disc confocal microscope. *apl3Δ* deleted strains were used as AP-2 mutants because in the absence of the α adaptin, which is located at/near the cell surface (figure 1), the localization of the β and μ subunits at the cell periphery was lost (de Leon et al. 2016). On the contrary, eliminating the β subunit did not completely disrupt the localization of Apl3-GFP at the plasma membrane. In order to analyze the behavior of patches in wild-type and *apl3Δ* strains, several endocytic markers fused to fluorescent proteins were used. The selected markers were Sla1, which is a “coat module” protein that interacts with the cargoes and with activators of actin, and Wsp1, Arc5, and coronin (Crn1). The latter three proteins mark stages of endocytosis that take place after AP-2 has recruited clathrin to the endocytic site. Wsp1 is an Arp2/3 activator that belongs to the “WASP/Myo” module; Arc5 is a component of the Arp2/3 complex that belongs to the “actin module”. Crn1 is recruited very late to the patch, participates in actin nucleation, and disperses after the vesicle pinches off from the membrane. Our results showed that the lifetimes of all of these markers were longer and more heterogeneous in the absence

of AP-2 than in the control strain (de Leon et al. 2016 and figure 1). As a consequence, the time required for the endocytic patch to produce a vesicle was significantly longer in the mutant. Additionally, a detailed analysis of patch ingressión showed that the very early marker Sla1 exhibited an extended immobile phase while for the Wsp1, Arc5 and Crn1 markers the mobile phase was longer in the *apl3Δ* mutant than in the control strain. Regarding the patch spatial dynamics, in the mutant strain only half of the patches exhibited a normal progression; 35% of the patches were abortive, bouncing back to the cell surface after the initial ingressión, and 13% of the patches were immobile. Thus, the effect of AP-2 depletion alters the steps of patch progression that take place well after the clathrin coat is assembled. In budding yeast, clathrin arrival and cargo recruitment define a transition point that regulates the recruitment of other components of the endocytic machinery and the progression of the pit (Carroll et al. 2012). Therefore, the existence of this transition point in *S. pombe* would explain why the lifetimes of late markers are longer in the *apl3Δ* strain, because the arrival of clathrin would be altered in the absence of the adaptor. Additionally, the longer recruitment times detected for proteins that promote actin polymerization would produce defective patch ingressión and scission, which is what we actually observed. Finally, it is also possible that a particular accessory protein that participates in vesicle ingressión/scission cannot be recruited to the patch in the absence of AP-2, leading to a reduced efficiency of these processes.

An apparent contradiction revealed by our results was that even though the lifetimes of the endocytic markers was extended and the efficiency of patch progression reduced, the number of patches in the *apl3Δ* cells was similar to that of the control strain. A plausible explanation for this could be that the increase in the number of endocytic

1 patches, resulting as a consequence of the slow dynamics of the process, is compensated
2 by a low efficiency in the initiation of new endocytic sites, since the simultaneous
3 arrival of two AP-2 complexes and one clathrin triskelion to the membrane represent a
4 defining event for the nucleation of the clathrin-coated pit (Cocucci et al. 2012).

6 An important question to address is whether the alteration in patch dynamics is relevant
7 for the biology of *apl3Δ* cells. Through our analysis we found that FM4-64 was
8 internalized efficiently in the mutant strain; however, when AP-2 was deleted in a
9 mutant with a reduced amount of clathrin (de Leon et al. 2013) FM4-64 uptake was
10 delayed. Furthermore, genetic interaction was shown between *apl3⁺* and *clc1⁺* and
11 between *apl3⁺* and *end4⁺*. Phenotypic analysis of the *apl3Δ* strain showed that this
12 mutant was defective in polarity establishment; defects in the growth pattern have been
13 detected in other organisms where AP-2 subunits have been deleted (Chapa-y-Lazo et
14 al. 2014; Chapa-y-Lazo and Ayscough 2014). Finally, we found that the *apl3Δ* strain
15 exhibits defects in the synthesis of the cell wall, the fungal equivalent to the mammalian
16 extracellular matrix. Interestingly, in previous work we found that deletion of *clc1⁺* (the
17 gene coding for the clathrin light chain) is lethal in *S. pombe* and that this lethality was
18 rescued by sorbitol. Furthermore, a reduction in the amount of clathrin that did not
19 affect general trafficking did affect the synthesis of this structure (de Leon et al. 2013).
20 Thus, our results have shown that the synthesis of the extracellular matrix is one of the
21 biological processes most sensitive to defects in clathrin-related functions.

23 In summary, we have confirmed that AP-2 is required for normal progression of
24 endocytosis in a unicellular eukaryote, an issue that had been questioned since neither
25 genetic nor physical interaction between AP-2 and clathrin was detected in budding

1 yeast, and since the general dynamics of endocytosis was not altered in either *S.*
2 *cerevisiae* or *Dictyostelium* AP-2 defective cells (Boehm and Bonifacino 2002; Carroll
3 et al. 2009; Macro et al. 2012; Yeung et al. 1999).

5 **Perspectives**

6 Super-resolution light microscopy makes the observation of cell structures at nanometer
7 scale in living cells possible; the use of this technique will allow a better
8 characterization of protein localization and even the definition of subdomains in the
9 endocytic patch. Additionally, the generation of new and more stable fluorescent
10 proteins might help to analyze the behavior of low-abundant patch components. The
11 combination of these techniques will help to understand the relationship between AP-2
12 and other proteins involved in CME. The analysis of CME in mammalian systems has
13 relied largely on the use of techniques that either overexpress the proteins of interest in
14 plasmids or reduce gene expression using RNAi, which can be slow and incomplete.
15 The use of genome-editing technologies will permit the expression of fluorescent
16 proteins at native levels and the generation of point mutations, leading to a more precise
17 characterization of endocytosis in higher eukaryotes. Some efforts are already being
18 made regarding this issue (Grassart et al. 2014; Hong et al. 2015). However, it is
19 important to remember that the short yeast generation time in combination with the
20 rapidly developing- and powerful yeast genetics and the application of new technologies
21 has kept yeasts at the leading edge of cell biology research. Our results, together with
22 those of other laboratories, have shown that *S. pombe* is a valuable model to study
23 vesicle trafficking, and that the study of endocytosis in this organism will allow new
24 discoveries in the field.

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Figure legend

Figure 1. The dynamics of endocytic patch components are altered in the absence of AP-2.

A. Schematic representation of AP-2. **B.** Localization of Apl3-GFP in a *S. pombe* wild-type strain. The image is a medial section captured with a DeltaVision deconvolution system. **C.** Kymographs of patches containing Sla1-GFP and Crn1-Tomato were generated from two-color movies, acquired with a spinning-disc confocal microscope at a rate of one frame per 0.66 seconds, by drawing a line perpendicular to cell tips. **D.** Schematic representation of the average lifetimes of Sla1, Crn1, Wsp1, and Arc5 markers at endocytic patches in the indicated strains. The cells bore either Sla1-GFP and Crn1-Tomato or YFP-Wsc1 and Arc5-Cherry. The color bars represent the temporal map of the lifetime for each marker and the relative time of its recruitment to the endocytic patch.