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Spotlight

Endocytosis in *Giardia*: Evidence of Absence

Nahuel Zamponi,[1] Constanza Feliziani,[1] and María C. Touz[1,*]

Zumthor *et al.* recently reported a novel function for clathrin coatomer in *Giardia lamblia* endocytosis. On the basis of old and new data, we propose an updated model of the participation of clathrin function in this parasite.

Unlike the distinct but connected network involving endosomes, multivesicular bodies, and lysosomes, the primary functions of which are discrete in other eukaryote cells, *Giardia* trophozoites possess polarized peripheral vacuoles (PVs) that assemble the functions of endocytosis, recycling, and degradation into a single entity [1]. It is therefore not unexpected that the molecules that participate in PV homeostasis and function retain some features present in other eukaryotes, but display particularities. A new study by Zumthor *et al.* [2] recently highlighted the presence of plasma membrane (PM) invaginations previously reported by Lanfredy-Rangel and collaborators [1], and questioned whether they might be clathrin-coated pits. Although vesicles of the size of clathrin-coated vesicles (CCVs) (~80 nm) have been observed in *Giardia* [1], the lack of an appropriate anti-clathrin antibody to perform transmission electron microscopy (tEM) has made it thus far impossible to show an association between these vesicles and clathrin in this parasite. In agreement with previous observations [1], Zumthor *et al.* observed PM invaginations in close proximity to some PVs by applying tEM, scanning electron microscopy (SEM), and focused ion beam-SEM (FIB-SEM) microscopy. Using a fusion protein between the giardial clathrin heavy chain (GlCHC) and a 28 kDa soybean ascorbate peroxidase mutant APEX2 (an enzymatic reporter for tEM) with a 2XHA-tag (GlCHC::APEX2-2HA), they went on to show that these invaginations are clathrin-containing structures that completely bridge the ~70 nm gap between the PM and the PV membranes, and contact both. Supporting their claim, in an elegant series of experiments using fluorescence recovery after photobleaching (FRAP) and inverse FRAP microscopy (iFRAP), the researchers demonstrated no measurable turnover of the GlCHC::GFP reporter, suggesting that GlCHC associated structures are highly stable. Similar results were observed for a putative clathrin light chain (GlCLC::GFP). The role of these static structures is unclear, but Zumthor and colleagues suggested that they function as unregulated points of entry of extracellular material into the cell. They extend upon these findings by identifying a small number of conserved and novel GlCHC-associated proteins. Using an anti-GlCHC polyclonal antibody (pAb) raised in mouse by the same group, it was shown that native GlCHC localized to defined points of the endoplasmic reticulum (ER) [3,4], in addition to the PVs and the bare zone [5,6]. Using the same pAb [5], and another anti-GlCHC pAb raised by Feliziani *et al.* [6], it was shown that native GlCHC interacts with the AP-1 adaptor complex and the epsin-like monomeric adaptor (GlENTHp), respectively, demonstrating a clear function of clathrin in lysosomal protein trafficking from the ER to the PVs [6,7]. Strongest evidence for the participation of GlCHC in association with the PVs, and the mechanism of receptor-mediated endocytosis (RME) involves its interaction with AP-2 and GlENTHp [6,8]. However, there are some drawbacks to tagging

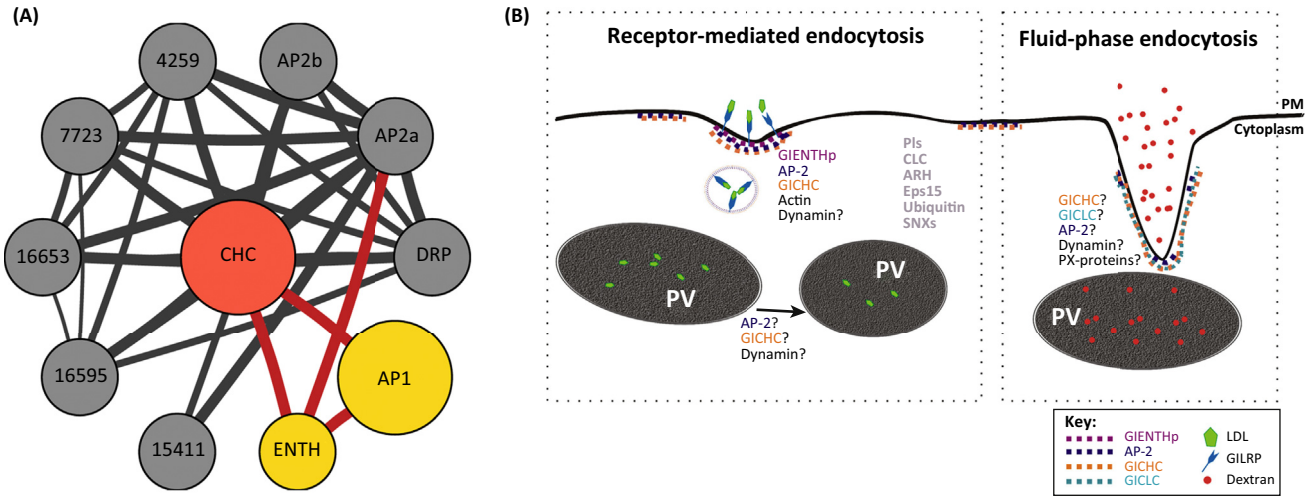

Figure 1. Molecules involved in *Giardia* endocytosis. (A) The clathrin interaction map. Nodes and links in grey correspond to core proteins and interactions described in [2]. Two experimentally validated GlCHC interactors described previously [6,8] were added in yellow. (B) Convergent model for receptor-mediated and fluid-phase endocytosis. The model shows proteins known to be involved in LDL and dextran endocytosis. (Left) Membrane invaginations for LDL endocytosis might occur from the static clathrin/AP-2 scaffolds and progress through the participation of GlENTHp, AP-2, and actin. While inside the PVs, LDL moves laterally by an unknown mechanism. Molecules that might be involved in receptor-mediated endocytosis in other eukaryotic cells but not present in the *Giardia* genome are depicted in grey. (Right) Dextran is endocytosed without the participation of GlENTHp, AP-2, GlCHC, or actin. After endocytosis, dextran remains inside the PVs before moving to the ER or nuclear envelope. Functional analysis will disclose the participation of GlCHC, GlCLC, dynamin, and PX-proteins in dextran uptake. Proteins with question marks denote presence in the PV or the membrane invaginations but whose participation in endocytosis has not been shown. Abbreviations: AP-2, adaptor protein 2; ARH, autosomal recessive hypercholesterolemia endocytic adaptor; CHC, clathrin heavy chain; CLC, clathrin light chain; Eps15, epidermal growth factor receptor substrate 15; ER, endoplasmic reticulum; GlCHC, *Giardia lamblia* clathrin heavy chain; GlCLC, *G. lamblia* clathrin light chain; GlENTHp, *G. lamblia* ENTH protein; GlLRP: *G. lamblia* low-density lipoprotein receptor-related protein; LDL, low-density lipoprotein; PIs, phosphoinositide; PM, plasma membrane; PV, peripheral vacuole; PX, phosphoinositide-binding structural domain; SNX, sorting nexin.

proteins that form structural complexes – because Zumthor *et al.* were able to see GlCHC only in association with the PM and PVs, this might account for the presence of clathrin scaffolds from which CCV are formed, as has been reported for other cells [9]. However, because the clathrin static structures were observed with a GFP reporter, and an unusual number of invaginations were observed in GlCHC::APEX2-2HA transfected cells, it is possible that the ectopic expression of these fusion proteins might alter GlCHC normal function. This might explain why known GlCHC-interacting proteins were not detected in immunoprecipitation assays using HA-tagged GlCHC, and could also explain the finding of non-canonical GlCHC-interacting proteins. Given experimental limitations, the results of Zumthor *et al.* might not constitute the complete picture of clathrin function in *Giardia*, although this study indeed provides new insights into the GlCHC interactome (Figure 1A) and on the role of clathrin in this parasite. However, several key questions remain unanswered. It was very interesting that the authors presented a model in which fluid-phase endocytosis and exocytosis are controlled by rounds of 'kiss and flush', in which the PVs periodically open to the environment, releasing their contents to the environment, and replacing this volume with extracellular medium before closing again. Evidence for this model might be assessed by functional analysis of GlCHC and GlCLC using endocytic markers. Thus, the question remains open as to whether this is an exclusive mechanism for fluid-phase endocytosis – molecules internalized by RME, such as low-density lipoprotein (LDL) and chylomicrons, were previously shown to depend on actin, AP-2, and GlENTHp, and are transported laterally between the PVs [6,8,10]. In addition, it remains to be ascertained how PVs maintain their acidic nature in a 'kiss and flush' model in which the PVs open their membranes to the external media. If clathrin forms a static structural 'bridge' between the PM and the PVs, either to internalize or to expel unspecific material, how does membrane-to-membrane fusion occur in the presence of fixed clathrin structures? How can dynamin and AP-2 make dynamic distributions in the absence of coated vesicles? What are the structures that GlCHC and the putative GlCLC form? More work will be necessary to solve these points and, perhaps, other methods such as total internal reflection fluorescence microscopy or correlative light electron microscopy, and knockdown strategies together with functional assays, will be necessary to uncover the common and unusual roles of clathrin in this parasite. In Figure 1B we depict an updated model of endocytosis in *Giardia* in which all the established and missing components conserved in other eukaryotes are included. Because, ultimately

(paraphrasing Martin Rees): 'absence of evidence is not evidence of absence'.

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Forum

Protist Collections: Essential for Future Research

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The vouchered deposit of protist type specimens in institution-maintained collections is a prerequisite for species description, and greatly enhances the chances of sample availability and preservation for future generations. However, specimens are currently most often deposited in personal collections maintained by the individual effort of researchers. We discuss the disadvantages of such a scenario and propose a change to this arrangement.

The sad truth is that, for historical reasons, protist collections are often relegated to dusty boxes of slides stored on shelves in an obscure corner of archives. However, for protozoologists, such collections have an invaluable interest because they often comprise reference type specimens deposited since the end of the 19th century. Interest in such collections has considerably decreased in the past few years as molecular data began to pave the road for new species (re)-description [1]. There is a general challenge to preserving scientific collections across the world. A deposit only in an individual laboratory collection greatly increases the likelihood that these specimens will be unavailable for research and reduces the chances of sample preservation for future generations. Some of these collections sometimes fail to survive, either by accidents or neglect. In 2010, a fire consumed more than 500 000 specimens of snakes, scorpions, and spiders, including several type specimens at the Butantan Institute in São Paulo, Brazil [2].

With the entry in the molecular era, natural history collections have evolved to meet the challenges of current and future interdisciplinary scientific studies. Many museums and research institutions developed new collections and information databases (DNA, tissue, culture, cryobanks, photographs, ethanol-fixed specimens, publication collections, and geographical and ecological information databases), which are of a first-rate importance, offering the opportunity to conduct integrative studies, including temporal and spatial surveys. The evolution of collections from static repositories to functional information systems is in response to increasing societal and scientific demands.

The well-known order Trypanosomatida contains the majority of catalogued species of the class Kinetoplastea, including public health-relevant species such as *Trypanosoma cruzi, Trypanosoma brucei*, and *Leishmania* spp. [3,4]. A few selected examples provided below illustrate the essential role of collections for current parasitology research.

Studies on ancient human remains changed the widely accepted theory of the origin of Chagas disease in humans, approximately 8000–6000 years ago. The high prevalence of *T. cruzi* in pre-Colombian samples of desiccated mummies, some as old as 9000 years, indicated that Chagas disease is probably as old as human presence in the Americas [5].

The rapid extinction (in less than 10 years) of rats endemic to Christmas Island at the beginning of the 20th century was found to be caused by a pathogenic trypanosome, *Trypanosoma lewisi*, carried by fleas present in the recently introduced black rats, where the parasite is not lethal. Molecular analyses of museum-archived endemic rats collected before the black rat introduction revealed that they were trypanosome negative, while those collected after the introduction were positive. The long-isolated endemic rat species were immunologically naive and highly susceptible to *T. lewisi* [6]. With a similar strategy, collection-archived bat specimens would be very useful to investigate the origin and the real extent of *T. cruzi* diversity. All basal species of this clade are parasites of bats [7].

Given that collections can preserve live trypanosomatids, either frozen or through *in vitro* or *in vivo* passages with