

## REVIEW

# Frustrated clathrin-mediated endocytosis – causes and possible functions

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## ABSTRACT

Clathrin-mediated endocytosis is the main entry route for most cell surface receptors and their ligands. It is regulated by clathrin-coated structures that are endowed with the ability to cluster receptors and to locally bend the plasma membrane, resulting in the formation of receptor-containing vesicles that bud into the cytoplasm. This canonical role of clathrin-coated structures has been shown to play a fundamental part in many different aspects of cell physiology. However, it has recently become clear that the ability of clathrin-coated structures to deform membranes can be perturbed. In addition to chemical or genetic alterations, numerous environmental conditions can physically prevent or slow down membrane bending and/or budding at clathrin-coated structures. The resulting ‘frustrated endocytosis’ is emerging as not merely a passive consequence, but one that actually fulfils some very specific and important cellular functions. In this Review, we provide an historical and defining perspective on frustrated endocytosis in the clathrin pathway of mammalian cells, before discussing its causes and highlighting the possible functional consequences in physiology and diseases.

**KEY WORDS:** Clathrin, Endocytosis, Mechanics, Mechanobiology, Actin, Cell signaling

## Introduction

Endocytosis is the process by which eukaryotic cells internalize substances from their surroundings. Although different endocytosis pathways have been described, they all rely on the formation of a plasma membrane invagination that matures into a vesicle or tubule budding off in the cytosol (Doherty and McMahon, 2009; Mayor et al., 2014; Mettlen et al., 2010). These invaginations exhibit huge variations in size, shape and dynamics, depending on the molecular machinery involved in shaping them. This reflects the specialization of different endocytosis pathways in regulating the uptake of substances that can also dramatically vary in nature, composition and size (Maldonado-Báez et al., 2013; Traub, 2009). In any case, the ultimate fate of bona fide endocytic invaginations is to detach from the plasma membrane, carrying the internalized substances in their lumen or at their limiting membrane. However, this process is not necessary unidirectional as some invaginations, such as caveolae, can flatten up in the plasma membrane, thus aborting the undergoing internalization (Sinha et al., 2011). It appears that, in most cases, the formation and maturation of invaginations are tightly regulated and active processes that require energy in order to overcome the resistance of the plasma membrane to bending

(Haucke and Kozlov, 2018). Numerous parameters, such as high membrane tension, internal hydrostatic pressure and properties of the to-be-internalized cargo, can make membrane bending a challenging task. Thus, the successful completion of endocytosis depends on a balance of forces, some of which favor membrane bending and some others that oppose it (Kaksonen and Roux, 2018).

Among the many endocytosis pathways described so far, clathrin-mediated endocytosis (CME) is probably the most studied and the best understood. The small plasma membrane invaginations associated with CME were first identified in 1964 (Roth and Porter, 1964), although the major component of the electron-dense coat seen on their cytosolic side was only identified a decade later and named clathrin (Pearse, 1975). These structures are also often referred to as clathrin-coated pits (CCPs) because of the peculiar shape of these invaginations as seen by electron microscopy (Pearse, 1975). The clathrin coat, comprising clathrin and associated adaptors and regulators, generates forces, which leads to the formation of an invagination and maturation of the pit (Liu et al., 2010). The forces needed to bend membranes are in the order of picoNewtons (Stabley et al., 2012) and are generated by multiple factors involved in CCPs, including BAR-domain proteins such as the FCHO proteins (Henne et al., 2010) and amphipathic helix-carrying proteins, such as epsin (Boucrot et al., 2012), as well as also through clathrin coat polymerization itself, which has been shown to induce membrane curvature, at least *in vitro* (Dannhauser and Ungewickell, 2012). Finally, budding can be assisted by Arp2/3-mediated actin polymerization at the base of nascent CCPs (Kaksonen et al., 2006; Perrais and Merrifield, 2005).

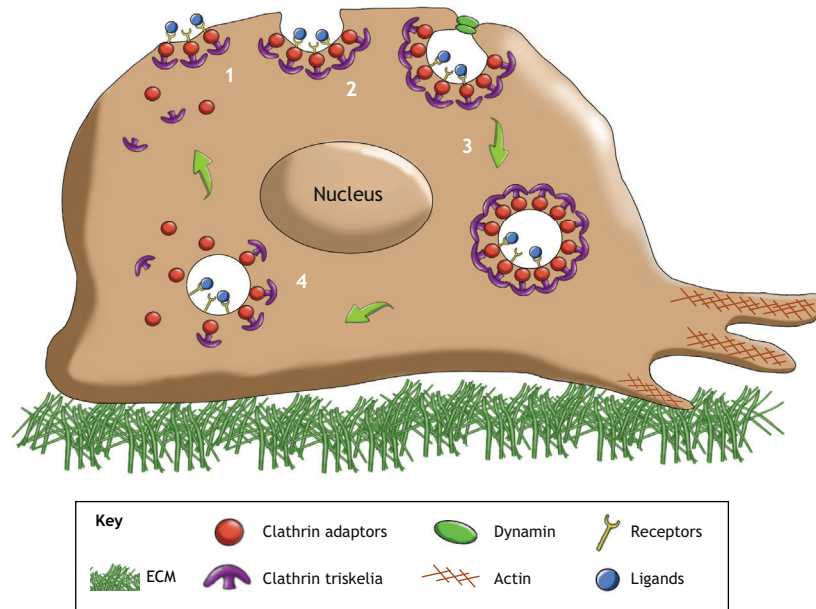
The different steps of nucleation, maturation and scission of CCPs are now quite well defined in molecular terms (Kaksonen and Roux, 2018) (Fig. 1). In addition to generating membrane invaginations, the clathrin coat is endowed with the capacity to recruit cell surface receptors and other proteins, leading to their packaging into CCPs that bud into the cytosol as endocytic clathrin-coated vesicles (CCVs) (Pearse, 1975). Cargoes internalized into CCVs are then delivered to the endosomal system, from where they can either be recycled to the cell surface or addressed to lysosomes for degradation (Cullen and Steinberg, 2018).

The canonical role of CCPs in regulating cargo endocytosis is central to cell homeostasis because it controls nutrient acquisition and the composition of the plasma-membrane in time and space, as well as cell surface receptor signaling (Antonescu et al., 2014; Polo and Di Fiore, 2006; Reider and Wendland, 2011). However, over the years, it has become increasingly clear that the budding ability of CCPs can be perturbed, resulting in shallow invaginations or flat clathrin lattices (Baschieri et al., 2018; Bucher et al., 2018a; Elkhatib et al., 2017; Ferguson et al., 2017). Rather than being just a passive consequence, the resulting CME ‘frustration’ can actually be used as a mechanism to convey information to the cell, or as a means to achieve specific functions (see below). Furthermore, beyond genetic and chemical

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**Fig. 1. Sequential steps of clathrin-coated vesicle formation.** Clathrin-mediated endocytosis relies on the assembly of clathrin-coated pits (CCPs) at discrete regions of the plasma membrane. These small structures (~100 nm in diameter) are mostly composed of clathrin and clathrin-adaptors. Clathrin itself assembles into a triskelion composed of three clathrin heavy chains and three clathrin light chains. Several clathrin adaptors are found at CCPs and their role is to recruit clathrin to the plasma membrane as well as cell surface receptors in order to package them into nascent CCPs. The sequence of events leading to the release of a fully formed, receptor-containing endocytic vesicles into the cytosol can be divided into four steps: initiation (step 1), maturation (step 2), scission (step 3) and uncoating (step 4). Initiation is proposed to occur randomly at the internal leaflet of the plasma membrane through the recruitment of the clathrin adaptor AP-2, which might already be bound to some clathrin triskelions. In addition, the F-BAR domain FCHo proteins are also recruited at this step and start to bend the membrane. Many additional adaptors are recruited during step 2, including but not limited to, epsin, Hip1R, CALM, Dab2, Numb and ARH, which, in turn, recruit specific receptors. This maturation phase is accompanied by the progressive bending of the plasma membrane; this is promoted by clathrin polymerization, as well as by membrane-bending proteins, such as FCHo proteins and epsin. At the end of the maturation step, CCPs are still connected to the plasma membrane by a thin neck that needs to be resolved in order for the vesicle to bud into the cytosol. This is achieved through the recruitment and action of dynamin that polymerizes around the neck and constricts it until scission occurs. Finally, shortly after budding of the CCV into the cytosol, uncoating occurs; this removes clathrin and clathrin adaptors from the vesicle, thus allowing the vesicle to fuse with endosomes, into which receptors are delivered. If unperturbed, this sequence of events typically takes 30 to 90 s from initiation to uncoating.

alterations, several environmental factors can prevent or slow down the membrane-bending process occurring at CCPs (Baschieri et al., 2018; Baschieri et al., 2019 preprint; Elkhatib et al., 2017; Ferguson et al., 2017). We propose that frustrated CME is part of the homeostatic response and can help the cell to adapt to the changing conditions of its environment. In this Review, we first provide an overview of the work that has led to the description of frustrated endocytosis. Focusing on CME, we then describe the different potential causes and functional consequences of frustrated endocytosis has in both normal physiology and diseases.

### What is frustrated endocytosis?

The use of the adjective ‘frustrated’ associated with endocytosis processes dates back to the 1970s when investigators analyzed the behavior of phagocytic cells, such as neutrophils or macrophages, seeded onto immune complexes that were immobilized on a glass coverslip (Henson, 1971; Michl et al., 1979). While these cells are able to generate large invaginations, called phagocytic cups, in order to internalize large particles opsonized with immune complexes, they obviously cannot internalize the glass coverslip. As a consequence, the phagocytic cup spreads out on the glass in a failed attempt to internalize it. This system has been used consistently to study the dynamics of molecular factors involved in phagocytosis, as it offers great advantages for imaging purposes and control of the experimental system (Henson, 1971; Michl et al., 1979; Rabinovitch et al., 1975).

Although experimental frustrated phagocytosis is clearly artificial, it illustrates the fact that the cell environment can impede endocytosis. This notion is particularly important when attempting to provide a definition of frustrated endocytosis. In a dictionary, besides its psychological meaning, frustration is defined as ‘the fact that something prevents plans or efforts from being successful’. We thus envisage frustrated endocytosis as the impossibility for an otherwise perfectly fit endocytic structure to perform endocytosis. This definition excludes any kind of perturbations that affects *a priori* the molecular or chemical composition, and thus functions, of the considered endocytic machinery. For instance, we do not consider experimental or pathological genetic alterations (such as mutations in CME players or their overexpression or knockdown), nor abortive CCPs that quickly disassemble before producing a vesicle (Loerke et al., 2009) as proper causes of frustrated endocytosis. Rather, frustrated endocytosis is considered here as a mechanical obstruction that prevents the forces developed by endocytic structures from forming an invagination or proceeding until the scission of the invagination from the plasma membrane. The phenotypic consequence is that clathrin-coated structures experiencing frustration are longer-lived as compared to unperturbed CCPs. The extended lifetimes can be more or less pronounced, sometimes even giving rise to extremely long-lived and flat structures called clathrin-coated plaques (see below). Frustration may also be local or global, depending on

whether only some or all of the considered type of endocytic structures experience frustration in one given cell.

In the frame of this definition, different examples of frustrated endocytosis have been described among the different endocytosis pathways. While experimentally induced frustrated phagocytosis is used as a tool, it actually also reflects a pathological situation in which phagocytes cannot proceed to the full engulfment of extracellular particles that are too large. This has been suggested to be the case for the uptake of long fibers, such as asbestos (Dörger et al., 2001; Hansen and Mossman, 1987) or carbon nanotubes (Poland et al., 2008), as well as also for amyloid fibers (D'Andrea et al., 2004) and even for some large bacterial biofilms (Leid, 2009; Thurlow et al., 2011). The incomplete engulfment of the foreign body leads to the secretion of toxic compounds that damage nearby healthy tissues and triggers a detrimental inflammation reaction (Donaldson et al., 2010; Dostert et al., 2008). While frustrated phagocytosis is toxic for the organism, frustration of other uptake pathways may play a role in homeostasis. Caveolae are small plasma membrane invaginations that have long been proposed to support the endocytosis of various cargoes (Palade and Bruns, 1968; Parton, 2018). However, at steady state, most caveolae are quite stable and seemingly stay invaginated for long periods of time (Parton, 2018). Besides their endocytic functions, caveolae are also known to regulate plasma membrane tension by flattening up in the membrane in order to buffer a sudden increase in tension (Sinha et al., 2011). Conversely, a reduction in plasma membrane tension leads to caveolae assembly and may also promote the budding of caveolae from the plasma membrane (Parton et al., 2019). Thus, caveolae may be constitutively frustrated by membrane tension at steady state, as their primary role is likely to fine tune this tension (Parton et al., 2019). In other instances, frustrated endocytosis also plays fundamental, regulatory roles that help the cell to interpret and adapt to its environment, as will be discussed below for the case of frustrated CME.

When applied to CME, the above-mentioned definition of frustrated endocytosis may help to draw a clearer understanding of the different dynamic behaviors that are exerted by populations of clathrin-coated structure at the plasma membrane, not only in different cell types, but also often within the same cells. Indeed, from the initial recruitment of the clathrin coat at the plasma membrane to scission of a fully formed CCV, the lifetime of canonical CCPs typically ranges from 30 to 90 s (Ehrlich et al., 2004; Loerke et al., 2009). However, the average lifetime of CCPs varies from cell type to cell type (Doyon et al., 2011), as well as within a given cell type (Loerke et al., 2009), depending on the subcellular localization of the respective CCPs (Liu et al., 2009; Pelassa et al., 2014), the composition of the substrate on which cells are seeded (Batchelder and Yarar, 2010) and the progression of the cell cycle (Hinze and Boucrot, 2018). In addition, besides CCPs, many cell types display other large or flat clathrin lattices that are more long-lived – from several minutes to hours (Saffarian et al., 2009). These particular structures have been named clathrin-coated plaques (hereafter called plaques) and, as discussed below, most likely represent an extreme example of frustrated CME.

### Causes of endocytosis frustration in the clathrin pathway

CCPs dynamics have been extensively studied and the sequence of events leading to the formation of CCVs is quite well understood (Cocucci et al., 2012) (Fig. 1). The clathrin coat together with clathrin adaptors exert inward forces, orthogonal to the plasma membrane, in order to form an invagination. Plasma membrane bending rigidity and tension, as well as cell turgor pressure, are

critical factors that oppose membrane deformation (Kaksonen and Roux, 2018). Although in yeast and plants, the turgor pressure is an important parameter that CCPs have to cope with, this is not the case in mammalian cells and thus will not be discussed here. Bending rigidity depends on the chemical composition of the membrane, which is known to vary over the lifespan of a cell (Atilla-Gokcumen et al., 2014). However, apart from an experimental depletion of cholesterol, which is known to affect membrane rigidity and has been shown to impair CME (Subtil et al., 1999), to our knowledge, there are no available data that link a modification of the lipid composition to potential CCPs frustration in either a physiological or pathological situation. The apparent bending rigidity of the plasma membrane can also be increased by adhesion of the actin cortex to the lipid bilayer (Charras et al., 2008; Murrell et al., 2011), and a stiff actin cortex has been linked to a stall in endocytosis in *Drosophila* (Lee and Harris, 2013). However, interactions between the actin cortex and the plasma membrane also impact on the measured membrane tension (Diz-Muñoz et al., 2013), a critical parameter affecting membrane bending in living cells. Indeed, if the membrane rigidity is considered to be constant, the largest energetic barrier to invagination formation is the in-plane membrane tension (Charras et al., 2008). As a consequence, an effective way to obtain frustrated CCPs is through an increase in membrane tension. In addition, another way CME could experience frustration is when attempting to internalize cargo, which, for different reasons, cannot be accommodated into the small, spherical invaginations formed by CCPs. Below, we will describe and provide examples of how membrane tension and cargo properties can cause CME frustration.

### Plasma membrane tension

It has long been recognized that CME is particularly sensitive to membrane tension. Experimental increase of membrane tension through increasing the swelling of cells by placing them in low osmolarity buffers completely stalls CCP dynamics (Ferguson et al., 2017; Wu et al., 2017). The dramatic increase of CCP lifetime observed under these conditions likely reflects the difficulties of the clathrin coat to transition from a flat to a curved topology when tension is high (Bucher et al., 2018a). Although it is highly debated whether CCPs initially grow in a flat conformation before they generate curvature (Avinoam et al., 2015; Bucher et al., 2018a; Larkin et al., 1986), or whether they immediately bend the membrane as soon as they nucleate (Kirchhausen et al., 2014; Willy et al., 2019 preprint), it is clear that increasing membrane curvature is more difficult when tension is high (Hassinger et al., 2017; Saleem et al., 2015). Membrane tension can be perturbed by many physiological or pathological factors. One of the most striking examples is during mitosis when an increased osmotic pressure causes the cell to round up (Stewart et al., 2011). This leads to an increased membrane tension that has been shown to negatively impact the capacity of CCPs to perform endocytosis in mitotic cells (Kaur et al., 2014; Raucher and Sheetz, 1999). Accordingly, ultrastructural analyses have shown that clathrin-coated structures are often flat in mitotic cells (Pypaert et al., 1987, 1991). Membrane tension is also likely systemically increased in cells subjected to compressive forces (He et al., 2018). This is, for example, the case in cancers when the growing tumor is compressed by the surrounding tissue (Seano et al., 2019), or in asthmatic patients, in which the airway epithelium constriction squeezes epithelial cells (Park et al., 2015). Applying uniaxial pressure on adherent cell lines of different origin has been shown to result in stalling CCP dynamics *in vitro* (Baschieri et al., 2019 preprint; Ferguson et al., 2017), most likely as

a consequence of the elevated membrane tension. In these two examples of high membrane tension (owing to osmotic pressure and compression), CCPs can experience a strong frustration with lifetimes exceeding several minutes on average (Baschieri et al., 2019 preprint; Ferguson et al., 2017), as measured during mitosis (Wood et al., 2017).

Apart from the systemic effects of an increase in membrane tension owing to osmotic pressure or compression, local variations in membrane tension could also modulate CCPs dynamics. Indeed, using cell lines of epithelial and mesenchymal origin, as well as in primary neuronal and endothelial cells, it was recently shown that membrane tension is not necessarily homogenous over the entire plasma membrane of a cell (Shi et al., 2018). Furthermore, in migrating mesenchymal cells, membrane tension has been proposed to be higher at the leading edge than at the rear of the cell (Fogelson and Mogilner, 2014; Hetmanski et al., 2019; Lieber et al., 2015). This most likely results from pushing forces that are applied on the plasma membrane by the polymerizing actin cytoskeleton at cellular protrusions. Accordingly, CCPs have been shown to be longer lived in the vicinity of cell protrusions than at the cell rear (Willy et al., 2017). The differential dynamics between cell front and rear may also explain why CCPs tend to accumulate over time at the front of migrating cells (Montagnac et al., 2013; Rappoport and Simon, 2003). Polarized epithelial cells provide another example of local CME frustration, which is also driven by differential membrane tension. Indeed, membrane tension is higher at the apical side compared to the basolateral side of epithelial cells (Dai and Sheetz, 1999). Strikingly, the lifetime of CCPs at the apical side is longer as compared to those at the basolateral side, and this was shown to depend on the difference in membrane tension (Boulant et al., 2011). In this case, CCP frustration is rather mild, with average CCP lifetime being ~40 s on the basolateral side and ~55 s on the apical side, where tension is higher. This obviously depends on the strength of the stress applied on the membrane, which is most likely weaker within a polarized cell than in the case of cell compression or raised osmotic pressure in mitotic cells. However, this could also reflect specific adaptive mechanisms developed by the cell to counteract an elevation in membrane tension as discussed below.

### Cargo properties

Besides membrane tension, the second main cause of CME frustration in mammalian cells lies in the properties of the cargoes that the CCPs attempt to internalize. The term cargo generally refers to receptors or other proteins found at the cell surface that have the capacity to bind to the coat comprising clathrin and its adaptors in order to be recruited and internalized at CCPs. We will here extend this definition to any object that may be linked to the clathrin machinery through a classical cargo, such as for example extracellular matrix (ECM) proteins that bind to CCP-localized integrins. Indeed, while the nature of classical cargoes themselves could be a cause for a delay in CCP maturation, most often, the mechanical properties, shape or size of the object they bind to represent a dramatic limitation for successful endocytosis.

It is now well accepted that cargoes play an active role in CCP maturation and lifetime. For instance, overexpression of the transferrin receptor reduces the occurrence of abortive CCPs (CCPs that disassemble early after nucleation, without forming a vesicle) and helps them to progress through the sequential steps of maturation (Loerke et al., 2009). This is most likely the consequence of a stabilization of clathrin adaptors at the plasma membrane through cargo engagement (Ehrlich et al., 2004). In addition, different cargoes recruit different sets of adaptors, and this impacts

on CCP lifetime (Mettlen et al., 2010; Puthenveedu and von Zastrow, 2006). However, any such effects are not included in our definition of endocytosis frustration, as such cargoes modulate the composition and/or activity of the clathrin machinery, and thus, this issue will not be further discussed here.

CCPs are used for the uptake of many extracellular objects, such as viruses, bacteria and nanoparticles (Bonazzi et al., 2011; Ding and Ma, 2012; Hackett and Cherry, 2018). These objects often have a size that impedes their efficient clathrin-mediated uptake. Indeed, larger cargoes require a longer time in order to be internalized by CCPs, most likely owing to steric hindrance issues (DeGroot et al., 2018; Hackett and Cherry, 2018). Because bacteria are much larger than the average diameter of CCPs, which is 100 nm (Haucke and Kozlov, 2018), they should be unable to use CME to infect cells. Nevertheless, *Listeria monocytogenes*, *Escherichia coli* and other bacteria have been shown to require the clathrin machinery at their entry sites (Bonazzi et al., 2011; Cossart and Veiga, 2008; Veiga and Cossart, 2005; Veiga et al., 2007). Depletion of several components of CME not only delays bacterial infection, but the lifetime of clathrin-coated structures that form at bacterial entry sites is considerably longer than the 30 to 90 s that is characteristic for classical CCPs (Veiga et al., 2007). At least for some bacteria, clathrin structures that form at contact sites may not directly control their uptake, but instead provide a platform from which the actin cytoskeleton reorganizes to form a bacteria-engulfing structure known as the actin pedestal (Bonazzi et al., 2011; Veiga et al., 2007). In addition to the size of the cargo, its shape is also important. For instance, clathrin-coated structures that form around vesicular stomatitis virus, a bullet-shaped virus with a high length-to-width ratio (here a length of 180 nm but a width of 70 nm), are twice as long-lived compared to CCPs not associated with the virus (Cureton et al., 2009; Johannsdottir et al., 2009), reflecting the difficulties of the clathrin coat to adapt to such a non-spherical shape. Similar to what is seen with viruses, the efficiency of clathrin-dependent uptake of opsonized nanoparticles depends on their size and also on their shape (Foroozandeh and Aziz, 2018). Indeed, spherical nanoparticles are internalized faster than rod-shaped particles (Chithrani and Chan, 2007).

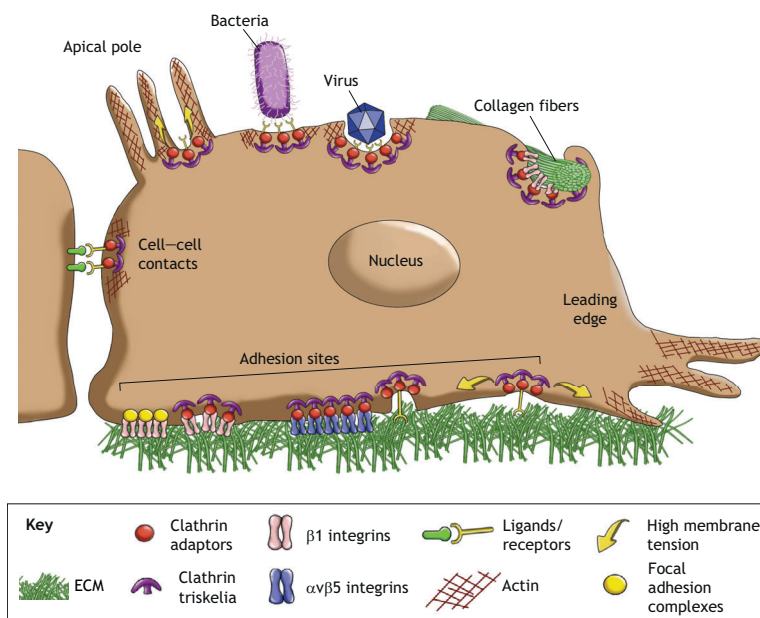
One of the most common causes of frustration occurs when the clathrin machinery is tethered to the substrate through adhesion receptors (Baschieri et al., 2018; Batchelder and Yarar, 2010; Zuidema et al., 2018). In this scenario, the substrate can be considered a cargo that cannot be internalized owing to its extremely large size. For instance, nonspecific attachment of the plasma membrane to a glass coverslip completely stalls CCPs (Batchelder and Yarar, 2010). It is likely that a strong adhesion to a non-pliable, large substrate prevents the clathrin and adaptor coat from bending the membrane and thus its maturation into proper CCPs and CCVs. More relevant to a physiological setting, adhesion receptors, such as some integrins, are known to accumulate at CCPs. Indeed, a population of  $\beta 1$ -integrin-enriched CCPs in the vicinity of focal adhesions shows an increased lifetime compared to CCPs that are located at other areas of the plasma membrane further away from focal adhesions (Batchelder and Yarar, 2010). In addition, overall CCP dynamics slows down and CME is inhibited when cells are plated on glass covered with an ECM that engages  $\beta 1$ -integrin (Batchelder and Yarar, 2010). These data suggest that integrin-mediated attachment of the clathrin coat to the substrate prevents CCPs from budding efficiently. Similarly, we have shown that clathrin-coated structures accumulate along collagen fibers in cells seeded in 3D conditions (Elkhatib et al., 2017). This also depends on  $\beta 1$ -integrin, and leads to the formation of peculiar, long-lived

structures, termed tubular clathrin-AP-2 lattices (TCALs), that wrap around and pinch collagen fibers (Elkhatib et al., 2017). By pinching collagen fibers, TCALs function as adhesive structures that help cells to migrate in complex three-dimensional environments (Elkhatib et al., 2017). Although topologically different from a glass coverslip, collagen fibers are far too long to be internalized into CCVs and thus similarly trigger frustration. However, for both TCALs and CCPs located close to focal adhesions, it is not clear whether these structures eventually proceed to a bud after a period of frustration, possibly internalizing  $\beta 1$ -integrin, or whether they disassemble without generating any CCVs. The situation appears to be clearer for another class of clathrin-coated structures, plaques, which are large, flat clathrin lattices that are highly stable at the plasma membrane (Baschieri et al., 2018; Grove et al., 2014). Although some canonical CCPs are detected at the rim of plaques and mediate CME, the core of plaques is generally considered to be unable to produce CCVs (Gaidarov et al., 1999; Lampe et al., 2016). Several recent studies showed that plaque formation depends on  $\alpha \nu \beta 5$  integrin, which clearly accumulates at these structures (Baschieri et al., 2018; Bucher et al., 2018b preprint; Vassilopoulos et al., 2014; Zuidema et al., 2018). Accordingly, we showed that acute inhibition of  $\alpha \nu \beta 5$  binding to the substrate results in a dissolution of the plaques into budding CCPs that take up  $\alpha \nu \beta 5$  (Baschieri et al., 2018).  $\alpha \nu \beta 5$  integrin appears to be more potent in triggering frustration compared to  $\beta 1$ -integrin, as plaques are much longer-lived (up to several hours) than TCALs or focal adhesion-localized CCPs (several minutes). The underlying reasons are not clear, but this could be based on the differences in affinity of these integrins for the substrate or the clathrin coat. As a matter of fact, the binding of  $\alpha \nu \beta 5$  to a large substrate is not strictly sufficient to mediate efficient frustration. Indeed, we reported that the elasticity of the substrate is a key parameter for the cell to develop plaques, and the stiffer is the substrate, the more plaques assemble (Baschieri et al., 2018). Integrins establish a peculiar interaction modality with their substrate, termed catch bond (Kong et al., 2009). Catch bonds strengthen when a pulling force is applied to them and as a consequence, integrin activation is linked to substrate rigidity, with stiffer substrates activating them to a greater extent (Puklin-Faucher

and Sheetz, 2009). Thus it is possible that  $\alpha \nu \beta 5$  integrin has a reduced affinity on a soft substrate that is not sufficient to support frustration. Overall, CME frustration can have multiple mechanical causes (summarized in Fig. 2), but although it may lead to reduced endocytosis fluxes, is not simply a passive process, as stalled clathrin-coated structures give rise to cellular responses that may help the cell to adapt to its changing environment.

### Functional consequences of frustrated CME

As outlined above, it is conceivable that a number of conditions a cell experiences results in frustrated CME and that the resulting defective endocytosis may be detrimental for the cell or for the cargo (Fig. 2). Indeed, CME is crucial for cell homeostasis, and prolonged inhibition of endocytosis is lethal (Mitsunari et al., 2005). Accordingly, frustrated endocytosis is often restricted to a subset of clathrin-coated structures and might be dampened by compensatory mechanisms. For example, CCPs located at the apical side of polarized epithelial cells rely on increased Arp2/3-mediated actin polymerization at their base to produce the extra forces required to bud under the high membrane tension at this position (Biancospino et al., 2019; Boulant et al., 2011). Actin polymerization is also increased when CCPs experience frustration while attempting to internalize large viruses (Cureton et al., 2009). Similarly, clathrin-coated plaques are often associated with an actin network that appears to help vesicular budding in its vicinity (Leyton-Puig et al., 2017). It has been proposed that frustrated endocytosis may actually be sensed at the level of individual CCPs through unknown mechanisms, thus triggering local actin polymerization to overcome the mechanical resistance (Cureton et al., 2009). Such a frustration-sensing module may not only prove useful in ensuring successful endocytosis, but also in applying forces for regulatory, endocytosis-independent purposes (discussed below). Indeed, it appears that frustration is not just a consequence, but can be used by the cell to gather information about its surroundings and to perform specific functions. For instance, CME is instrumental in regulating receptor-mediated signaling, and frustrated endocytosis modulates these signaling pathways. In addition, because adhesion receptors accumulate at clathrin-coated structures, frustrated CME can be used by the cell as a means to



**Fig. 2. Overview of the different causes of frustrated CME.**

Examples of clathrin-coated structures experiencing frustration at different cellular locations, such as close to adhesion sites, at the leading edge of migrating cells and at the apical pole of polarized epithelial cells, as well as potentially also at cell-cell contacts. In most cases, the origin of frustration could be an elevated membrane tension or an engagement of the clathrin coat with a substrate that cannot be easily accommodated into the small invaginations formed by CCPs.

adhere to its environment. Below, we will discuss the known potential functional consequences of frustrated CME and provide examples of how they affect different cellular processes.

### Consequences for signaling

The classical view of CME-regulated signaling is that once receptors are activated, endocytosis is used to terminate the signal from the plasma membrane (Sorkin and Von Zastrow, 2009). However, many receptors keep signaling from endosomes, and the quality, strength and duration of the signal may be modulated by the different environments receptors encounter in these compartments (Sigismund et al., 2012; Sorkin and Von Zastrow, 2009). However, endocytosis does eventually result in signal termination when receptors are degraded in late endosomal compartments. In addition to the effects of intracellular trafficking, it has recently become clear that the clathrin and adaptor coat itself can modulate the signal at the plasma membrane, likely by providing a platform for the recruitment of adaptors that are relevant for signaling, such as Gab1 (Eichel et al., 2016; Garay et al., 2015; Kim et al., 2013). In addition, an increased lifetime of CCPs at the cell surface correlates with increased signaling (Eichel et al., 2016). Many signaling receptors, such as receptor tyrosine kinases (RTKs), chemokine receptors and G-protein coupled receptors (GPCRs), are recruited at CCPs and signal from there (Eichel et al., 2016; Garay et al., 2015; Kim et al., 2013; Signoret et al., 2005). In accordance with this, we have recently shown that systemic frustration of CCPs in compressed cells increases signaling through the mitogen-activated protein kinase (MAPK) pathway (Baschieri et al., 2019 preprint). Thus, besides their role in regulating signaling through endocytosis, clathrin-coated structures themselves are now emerging as signaling platforms. It is thus not surprising that the largest and most stable clathrin-coated structures, plaques, greatly affect receptor-mediated signaling. Indeed, the same receptors that accumulate in short-lived CCPs also accumulate in plaques (Baschieri et al., 2018; Grove et al., 2014; Leyton-Puig et al., 2017), and plaques were shown to boost downstream signaling pathways (Baschieri et al., 2018; Leyton-Puig et al., 2017). As mentioned above, plaques are considered mechanosensitive structures as they assemble in response to substrate rigidity (Baschieri et al., 2018). As plaques also regulate signaling events, we have proposed that they are in fact mechanoresponsive, in that they inform the cell about the substrate elasticity by transforming mechanical information into a biochemical response that supports cell proliferation on stiff substrates (Baschieri et al., 2018). Because the causes of frustrated endocytosis are most often of mechanical nature, and the resulting long-lived, frustrated clathrin structures modulate signaling pathways, a major function of frustrated CME may thus be mechanotransduction. Along these lines, compression-induced CCP frustration may participate in informing the cell about the presence of an abnormal plasma membrane tension through the increased CCP lifetime and subsequent increased signaling (Baschieri et al., 2019 preprint). Thus, frustrated CME may have an important role in physiological contexts where cell mechanics are challenged, as well as in pathological situations in which an increased tissue rigidity and/or confinement could favor the development of plaques, such as during fibrosis and cancers (Henderson et al., 2013; Levental et al., 2009; Nia et al., 2017; Stylianopoulos et al., 2012; Tse et al., 2012).

### Consequences for cell adhesion

As mentioned above, strong adhesion to the substrate is a common cause of frustrated CME. The canonical role of CME is to

internalize receptors and this requires receptors to be recruited to CCPs, which usually occurs through direct interaction between endocytic adaptor proteins and the receptors themselves (Traub and Bonifacio, 2013). Indeed, integrins can be internalized via CME (Moreno-Layseca et al., 2019), and integrin clusters at clathrin-coated structures have been observed in several cases (Baschieri et al., 2018; Batchelder and Yarar, 2010; Elkhatib et al., 2017). The clustering of integrins or other adhesion receptors at adhesion structures is crucial for mediating efficient attachment (Changede and Sheetz, 2017) and, accordingly, clathrin-coated structures can function as adhesive structures in a similar manner to focal adhesions (Elkhatib et al., 2017). This is the case for the previously mentioned TCALs, tubular clathrin-coated structures enriched in  $\beta 1$ -integrin, that are used as adhesion structures that grab and pinch collagen fibers (Elkhatib et al., 2017), thereby helping the cell to migrate in 3D collagen networks (Elkhatib et al., 2017). Similarly, plaques represent frustrated CME because  $\alpha v \beta 5$  integrin anchors the clathrin coat to the stiff substrate, impeding endocytosis. In fact, plaques have long been proposed to serve as adhesion structures (Maupin and Pollard, 1983), and have been suggested to slow down cell migration owing to the firm attachment to the substrate they provide (Saffarian et al., 2009). Yet, only recently have plaques, or highly similar structures called reticular adhesions (Lock et al., 2019), been formally demonstrated as mediating cell adhesion to the substrate (Lock et al., 2018). Reticular adhesions are  $\alpha v \beta 5$ -rich structures that do not overlap with focal adhesions and ensure a firm attachment of mitotic cells to the substrate when their focal adhesions disassemble to allow cell rounding (Lock et al., 2018). Interestingly, these structures are enriched in components of the clathrin coat and may actually correspond to clathrin-coated plaques (reviewed in Lock et al., 2019). At least in several transformed epithelial cells and in keratinocytes, the clathrin coat appears to be required for the proper formation of the  $\alpha v \beta 5$  reticular pattern (Baschieri et al., 2018; Zuidema et al., 2018), but further investigations are required to ascertain whether this is strictly the case in all cells. Nevertheless, it seems reasonable to propose that integrin clustering at clathrin-coated structures, on the one hand, causes their frustration, but, on the other hand, also provides the cell with a new means to adhere to the substratum.

### Plaques as a scaffold for the cytoskeleton

In addition to their roles as signaling platform and adhesion structures, clathrin-coated plaques also provide a scaffold for the organization of the cytoskeleton, at least in some specialized cells. It has been shown that plaques are abundant in skeletal muscles and are required for appropriate sarcomeric actin organization (Vassilopoulos et al., 2014), as well as for the arrangement of desmin intermediate filaments (Franck et al., 2019). In the absence of plaques, muscles can no longer exert forces because sarcomeres detach from the plasma membrane (Vassilopoulos et al., 2014).

It is important to note that plaques, or reticular adhesions, assemble in an actin-independent manner and lack most of the classical adhesion-related proteins known to establish links with actin fibers (Lock et al., 2019). However, other components of the clathrin coat, such as dynamin or Huntingtin-interacting protein 1-related protein (HIP1R), could engage with the actin network and modulate its organization (Kirchhausen et al., 2014; Sun et al., 2019). In light of their described role in muscles, it is possible that plaques also help to organize the actin network during mitosis, or at the end of cell division when cells re-spread on the substrate (Lock et al., 2019). In any case, it is likely that the stable nature of plaques is advantageous for the cell in providing a scaffold for the organization of the cytoskeleton.

### Frustrated CME at cell–cell contacts

We have considered above the case of substrate-immobilized cargoes, but CME cargoes can also be presented at the surface of other cells, such as at cell–cell contacts. Whether this actually leads to frustrated CME has not been formally demonstrated so far. Nevertheless, there are hints suggesting that frustrated CME is induced at cell–cell contacts and might fulfill important physiological functions. For example, both Notch proteins and their ligands are transmembrane proteins that can engage the clathrin coat (Windler and Bilder, 2010). A tug-of-war mechanism has been proposed, in which both the ligand-presenting cell and the Notch-presenting cell pull on the receptor–ligand pair. The strength of these pulling forces depends on the engagement of the clathrin and epsin coat by the ligand and the resulting force induces the cleavage of Notch and its subsequent activation in the Notch-presenting cell (Langridge and Struhl, 2017). In the absence of any force, the cleavage site of Notch, which is located in its extracellular domain, would be masked and require a force of between 3 and 5 pN to become exposed (Gordon et al., 2015); this is in the order of force magnitude that have been shown to be produced by invaginating CCPs (Stabley et al., 2012), suggesting that the membrane bending occurring during CME could be the force driving protein unfolding. However, the formation of such ligand–receptor bonds poses an energetic barrier to CCP invagination and may thus result in frustrated CME. Accordingly, endocytosis-mediated Notch activation also requires actin (Meloty-Kapella et al., 2012), which, as explained above, could provide the additional force necessary to accomplish CME in cases of mechanical frustration. Analogous to the mechanism of Notch activation, ephrin B receptors (EphB) are a family of receptor tyrosine kinases (RTKs) that interact *in trans* with their transmembrane ligand ephrin B (Kania and Klein, 2016). The most characterized function of ephrin–Eph is to modulate cellular repulsion during cell migration. To do so, cells need to internalize the EphB–EphrinB complex as a whole (Zimmer et al., 2003). The situation can be visualized as a tug of war between two cells, with one cell eventually trans-endocytosing both proteins (Zimmer et al., 2003). Some EphB interacts with the clathrin adaptor Numb (Nishimura et al., 2006), and it has recently been proposed that trans-endocytosis of EphB–ephrinB is mediated by clathrin (Evergren et al., 2018). Internalizing two membranes instead of only one is likely to be more challenging for CCPs and, accordingly, this trans-endocytosis process also depends on the actin cytoskeleton (Marston et al., 2003).

Along this line, the B cell receptor (BCR) and T cell receptor (TCR) could also rely on frustrated endocytosis. To fulfill their roles in the immune response, BCRs and TCRs need to extract an antigen from the surface of antigen-presenting cell in a process that requires forces (Ma et al., 2019; Spillane and Tolar, 2017). These forces have been proposed to come at least in part from CME (Ma and Finkel, 2010), which is the preferential way of antigen internalization by immune cells. Another potential example is the E-cadherin-dependent recruitment of clathrin-coated structures to the vicinity of adherens junctions (Levayer et al., 2011), which share similarities to the accumulation of frustrated CCPs close to focal adhesions (Batchelder and Yarar, 2010).

In all these examples, however, a formal demonstration that there is frustrated CME is needed. However, given the susceptibility of CME to experience frustration in the different examples listed across this Review, it is likely that frustrated CCPs could indeed exist at cell–cell contacts, at least in some cases, with potential implications on intercellular communication.

### Perspectives

Frustrated CME is emerging as an important regulator of many different cellular processes, from signaling to adhesion, and from cell migration to mitosis, but the underlying mechanisms and its regulation are still elusive. For instance, enhancing the stiffness of the clathrin coat could better support the development of forces on receptor–ligand complexes (Lherbette et al., 2019). Therefore, frustrated CME could be both a consequence of local mechanical resistance and a means to achieve the production of local forces that is required for specific cellular processes. This might be the case for clathrin-coated plaques where the pulling forces exerted by the clathrin coat could participate in locally activating  $\alpha\beta 5$  or stabilizing integrin–ECM bonds, thus providing a mechanosensing mechanism alternative to the ones linked to actin cytoskeleton dynamics (Lock et al., 2019). Along this line, given the role of frustrated CME in controlling signaling downstream of RTKs or GPCR (Baschieri et al., 2018; Baschieri et al., 2019 preprint; Rakesh et al., 2010; Zimmer et al., 2003) and considering that frustration is often due to mechanical causes, frustrated CME could represent a new mechanotransducing pathway. Several pathological situations are characterized by increased substrate stiffness or increased solid stress (Kalli and Stylianopoulos, 2018; Levental et al., 2009; Nia et al., 2017). Notably, hyperactive RTK signaling and high substrate stiffness have recently been shown to be sufficient to induce transformation of healthy cells into tumor-initiating cells (Panciera et al., 2020), thus raising the possibility of frustrated CME being an important factor in tumorigenesis. Still, evidence for frustrated CME *in vivo* is scarce. Thus, efforts should be dedicated in the future to explore this aspect.

One of the most intriguing observations is that, while actin is in most cases dispensable for CME itself, actin polymerization is often increased at frustrated clathrin-coated structures. Identifying a frustration-sensing module in the clathrin coat, which has been suggested to exist (Cureton et al., 2009) and that may regulate local actin polymerization at frustrated CCPs, would provide a great opportunity to investigate the cellular functions of frustrated CME. On a similar note, whether frustrated structures share a common molecular composition that would be different from canonical CCPs is an open question worth investigating to deepen our understanding of CME frustration.

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