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Spoilt for choice: Diverse endocytic pathways function at the cell surface

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Abstract

Endocytosis has long been identified as a key cellular process involved in bringing in nutrients, clearing cellular debris in tissue, and maintaining cell membrane compositional homeostasis. While clathrin-mediated endocytosis (CME) has been the most extensively studied, a number of clathrin-independent endocytic (CIE) pathways are continuing to be delineated. Here we provide a current survey of the different types of endocytic pathways available at the cell surface and discuss a new classification and plausible molecular mechanisms for some of the less characterized ones. Along with an evolutionary perspective of the origins of some of these pathways, we provide an appreciation of the distinct roles these pathways play in various aspects of cellular physiology, including the control of signalling and membrane tension.

Keywords

clathrin-mediated endocytosis; clathrin-independent endocytosis; caveolae; CLIC/GEEC; dynamin; evolution; signalling

Introduction

Over a century ago, Elie Metchnikoff's observations of phagocytosis laid the foundation to the field of endocytosis (Metschnikoff 1884). It has been proposed that the capacity for engulfment as a process has led to the acquisition of bacteria, allowing mitochondrial assimilation in the eukaryotic lineage (Cavalier-Smith 1987). Now, we understand that endocytosis is necessary for nutrient uptake, extracellular milieu sensing, maintaining and homeostasis of plasma membrane (PM) composition (chemical aspect), surface area and tension (physical aspect), by providing control mechanisms for these processes.

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Several attempts to classify endocytic pathways have been made over the years (Conner & Schmid 2003, Doherty & McMahon 2009, Mayor et al. 2007, Schmid et al. 2014). Our knowledge of endocytosis has been framed mainly by early advances made in Clathrin Mediated Endocytosis (CME), where endocytosis is accomplished by the formation of a Clathrin coated pit and the endocytic vesicle is derived from the scission of the endocytic vesicle by a Dynamin-mediated process (Fig 1, 2). However, multiple endocytic mechanisms operate at the PM (Fig. 1), and endocytic pathways have been classified based on based on their requirement (or not) for Clathrin, therefore the term Clathrin Independent Endocytosis (CIE) (Kirkham & Parton 2005; Mayor et al. 2007, 2014). As depicted in Fig. 1, the presence or absence of dynamin encompasses a broader set of pathways and offers a different way to parse endocytic pathways. While dynamin-dependent mechanisms appear to accompany endocytic pathways that recruit membrane coats, dynamin-independent pathways rely on the association of actin with the membrane for their operation, and may represent a distinct class of membrane processes. Therefore, we propose an alternate way to categorize these pathways that distinguishes endocytic processes by the type of scission machinery utilized (Fig.1).

The absence of precise mechanisms, a general medley of molecules with pleiotropic functions that operate in regulating different CIEs, and the lack of codified information have helped fuel a perspective that CIE pathways are a consequence of perturbation of CME (Bitsikas et al. 2014). On the other hand, to understand these processes, there is no choice but to embrace the complexity that exists. Here, we will focus on a number of dynamin-dependent and independent pathways and provide evidence for their role in different physiological processes to highlight the importance of multiple endocytic pathways in a cell. We also provide an evolutionary argument for their existence. We list different mechanisms and provide a nomenclature that we believe will address some of the confusion. We also point out relevant features that help to distinguish the different pathways. We hope this will allow scientists in their areas of specialization to look at the role of the different endocytic pathways in multiple contexts and help in the interpretations of the phenomenon they observe.

Types of endocytic pathways

Clathrin-mediated Endocytosis

First described using Electron Microscopy (EM) (Pearse 1976, Roth & Porter 1964) (Fig.1), decades of work showed that CME governs endocytosis of receptors (Brown & Goldstein 1979) assisted by adaptor protein complex. This complex recognizes specific sequences in the cytoplasmic tails of receptors and also aids in Clathrin recruitment to the forming endocytic pit (Edeling et al. 2006, Robinson 2004). This begins with Clathrin recruitment and polymerization to form Clathrin-coated pits (CCP) (David et al. 1996, Neumann & Schmid 2013, Qualmann et al. 2011, Taylor et al. 2011), accompanied by local bending/ invagination of membrane accomplished by curvature sensing/stabilising Bin-amphiphysin-Rvs (BAR) domain containing proteins such as amphiphysin, FBP17 and sortin nexin 9 (Taylor et al. 2011). Dynamin, a large GTPase, is subsequently recruited and oligomerizes at the neck of a CCP and powers vesicle scission via GTP hydrolysis (David et al. 1996,

Shpetner & Vallee 1989). Following scission, these CCPs lose their coats and fuse with the early endosomal sorting compartment (Kirchhausen et al. 2014, Schmid 1997). The endocytosis paradigm of cargo recruitment via adaptor complexes, membrane coat formation, membrane bud formation and finally scission is derived from studies of CME (See Fig. 2; steps 1A, 2A, 3A). We refer the readers to several recent reviews for extensive details on CME (Kaksonen & Roux 2018, Mettlen et al. 2018, Robinson 2015).

Clathrin-independent (CI) endocytosis (CIE)

Inhibition of CCP formation failed to prevent Ricin intoxication and fluid phase uptake invoking the existence of CIE processes in a cell (Moya et al. 1985, Sandvig 1987). Several cargoes have been shown to be internalised in a similar fashion (Fig.1; (Mayor et al. 2014, Otto & Nichols 2011)), and via multiple pathways, making it difficult ascertain whether cargo chooses endocytic pathways or vice versa (Supplementary Table S1). Later, the fluid phase was also found to be internalized independent of dynamin function (Damke et al. 1994, 1995). Studies over the last three decades indicates a plethora of CIE pathways both dynamin-dependent or independent. CIE is an emerging field and we again refer the readers to excellent reviews on the different CIE pathways. Here we focus on only few of the CIEs whose molecular mechanisms have been worked out in detail, recently. For the others, we provide greater detail in Supplementary Figure S1.

Dynamin-dependent CIE pathways

Caveolar endocytosis: Caveolae, one of the earliest plasma membrane structures observed in EMs, are coated, flask-shaped invaginations (Fig. 1 and 3). They are implicated in a variety of cellular processes such as trans-cellular transport, membrane repair and mechanotransduction (Kirkham & Parton 2005, Sinha et al. 2011). In most cell types caveolae contain ~ 140 molecules of the integral protein caveolin 1, 30–70 molecules of caveolin-2, and a complex of membrane proteins termed cavins (Hansen & Nichols 2010, Hayer et al. 2010, Parton & Del Pozo 2013). Cavins 1, 2, and 3 associate with the caveolae of mammalian non-muscle cells. In skeletal muscle cells, caveolin-3 replaces the caveolin-1/2 complex and an additional cavin family member, cavin4/MURC, associates with the cavin complex (Bastiani et al. 2009, Ogata et al. 2008, Tagawa et al. 2008, Way & Parton 1996). Caveolae undergo a dynamin-dependent scission (Henley et al. 1998, Parton & Richards 2003). The molecular machinery involved in caveolar uptake also is emerging where the ATPase EHD2 acts as a negative regulator by interacting with pacsin, an F-BAR domain containing protein that promotes caveolar endocytosis (Moren et al. 2012).

Fast Endophilin A- Mediated Endocytosis (FEME): Recently, an Endophilin A (EndoA) dependent pathway has been described that makes tubulo-vesicular compartments (<1µm) that derive from EndoA positive assemblies (EPAs) in a ligand, actin and dynamin-dependent fashion (Boucrot et al. 2015). EndoA is proposed as a versatile molecule, capable of cargo recognition (via its SH3 domain), inducing curvature with the aid of its BAR domain and driving scission by insertion of multiple amphipathic helices contained within its structure. Interestingly, high concentrations of epidermal growth factor (EGF) results in the down-regulation of CME but is needed for the full stimulation of EPAs. Cargoes such as GPCRs (α2a- and β1-adrenergic receptors), Interleukin-2, Epidermal Growth Factor

Receptor (EGFR) and Shiga toxin (STx-B) traffic via FEME (Boucrot et al. 2015, Renard et al. 2015). FEME is downregulated by inhibitors of dynamin and phosphatidylinositol 3 Kinase (PI3K). The regulators of actin polymerisation, the small GTPases of the Rho family, RhoA, and Rac1 are also necessary for FEME functioning, while there are conflicting claims about whether the RhoGTPase, CDC42 activation, which positively or negatively regulates FEME (Boucrot et al. 2015, Chan Wah Hak et al. 2018).

Phagocytosis: Phagocytosis, one of the most important elements of innate immunity, mediates engulfment and destruction of invading microorganisms ($>0.5\mu\text{m}$) (Flannagan et al. 2012, Gordon 2016). The phagosome cup closes with the help of dynamin-2 and actin (Gold et al. 1999, Marie-Anaïs et al. 2016, Tse et al. 2003). Recently, in a mammalian genome-wide CRISPR screen, where Bassik and co-workers examined multiple phagocytic cargoes, many new regulators (such as NHLRC2 which participates in the Rac1 signalling cascade, and very long chain fatty acid synthesis essential albeit only for certain substrates) have been identified (Haney et al. 2018). Understanding the role of these regulators in phagocytic processing will undoubtedly become a focus of research in this area.

Dynamin-Independent CIE pathway

CLIC/GEEC (CG) endocytosis: Endocytic vesicles generated independent of Clathrin are called CLathrin-Independent Carriers (CLICs) and when they are formed independent of dynamin have a highly pleomorphic tubular structure. They are often formed in a polarised manner at the leading edge of migrating cells (Howes et al. 2010). The CG pathway is regulated by the small GTPases, ARF1 and CDC42 (Chadda et al. 2007, Guha et al. 2003, Gupta et al. 2009, 2014; Kumari & Mayor 2008, Sabharanjak et al. 2002) and internalises several cargoes including GPI-anchored proteins (GPI-APs), and a major fraction of the fluid-phase; several other cargo are detailed in Supplementary Table SI. The CG pathway is distinct from macropinocytosis (see next section), in that it is not sensitive to inhibitors of Na^+/H^+ exchange (NHE) inhibitors (Kalia et al. 2006). The CLICs/GEECs are high capacity endocytic carriers and can turn over the entire membrane surface of fibroblasts within 12 minutes (Howes et al. 2010). CG endocytosis is also postulated to generate a tubular vesicular endocytic network during cytokinesis (Kettle et al. 2015). GEECs are highly acidic (Kalia et al. 2006) and inhibiting vacuolar ATPase downregulates the CG pathway (Gupta et al. 2014). The CG pathway is sensitive to even slight changes in temperature, unlike CME (Thottacherry et al. 2018) and is reminiscent of the fast clathrin-independent response seen in neurons (Delvendahl et al. 2016).

The earliest events in the CG endocytic pathway begins with the recruitment of the GEF for ARF1, GBF1, to a membrane patch followed by the actin nucleator ARP2/3, CDC42 and filamentous actin (Sathe et al. 2018). The CG machinery also includes two BAR domain proteins (BDPs), PICK1 and IRSp53, which interact with ARP2/3. In the first phase, PICK1 inhibits ARP2/3 while in the second phase, CDC42/IRSp53 effector complex enables ARP2/3 activation and subsequent F-actin polymerisation. It is tempting to speculate that the initial repression of the ARP2/3 complex and therefore actin polymerization, allows membrane budding whereas its subsequent activation assist its pinching off.

Galectin-3 based CLIC biogenesis: The absence of a coat and an obvious adapter complex raises the question as to how are cargo proteins sorted and membrane bending is accomplished. In this context, Galectin-3 mediated CLIC biogenesis was put forward as one of the mechanisms in which sorting and membrane bending could be achieved (Johannes & Mayor 2010). Gal-3 is a member of N-glycan binding protein, capable of binding to carbohydrates via its C-terminus and other Gal-3 monomers via its N-terminus to form oligomers. In a recent study, Johannes and colleagues suggested that monomeric Gal-3 is recruited by glycosylated cargo to the membrane wherein it oligomerizes with neighbouring Gal-3 (Lakshminarayan et al. 2014). This co-clustering of cargo proteins with glycosphingolipids induces a local mechanical strain on the membrane, leading to buckling and subsequent generation of CLIC carriers. Further research is necessary at this point to establish if the Galectin-based mechanism is universal for initiation of CG vesicles or if it is part of a collection of mechanisms.

Macropinocytosis: Macropinocytosis is characterized by the formation of large endocytic vesicles ($>0.5 \mu\text{m}$) that originate from dorsal actin ruffles at the plasma membrane. Macropinosomes are generated by EGFR and PDGR based signaling and not found in resting cells. It is non-selective and is specifically inhibited by NHE inhibitors (West et al. 1989). Additionally, it is regulated by ARF6, Rac1 and requires PtdIns (4,5)P2 turnover (Brown et al. 2001, Koivusalo et al. 2010, West et al. 2000). CtBP1/BARS translocates to the macropinocytic cup and its surrounding membrane upon EGF stimulation and has been implicated in its scission (Bonazzi et al. 2005). Some reports implicate dynamin using dynasore perturbation (but not genetic perturbation) (Gold et al. 2010), that has many off target effects (Park et al. 2013, Preta et al. 2015). Thus, the role of dynamin in macropinocytosis should be interpreted with caution.

Other dynamin-independent pathways

ARF6 dependent endocytosis of MHC Class 1 and other receptors: Cargoes such as MHC Class I, CD1a, GPI-APs such as CD55 and CD59 are internalised via a pathway that requires ARF6 and cholesterol (Maldonado-Báez et al. 2013, Radhakrishna & Donaldson 1997). ARF6 activation and inactivation modulates recycling rather than internalisation. Other molecular machinery is detailed in Supplementary Figure S1, and in an extensive review (Grant & Donaldson 2009).

Rac1 and Src-dependent endocytosis: Nicotinic acetylcholine receptor (nAChR) internalisation is important for neuromodulation at neuromuscular junctions. When stimulated, nAChR internalisation occurs via narrow-tubular carriers formed independently of dynamin. Instead, it requires c-Src, Rac1 and actin polymerization (Kumari et al. 2008). A similar dynamin-independent mechanism involving c-Src and Rac1 activation is also utilized by the HIV-Nef protein in down-regulation of the costimulatory molecules CD80/CD86 (Chaudhry et al. 2005, 2007).

Flotillin mediated endocytosis: In HeLa cells, GPI-AP and Cholera Toxin B (a marker of cross-linked GM1) internalization occurs via a dynamin-independent manner (Glebov et al. 2006). Depletion of flotillin inhibits their uptake, suggesting that flotillins may outline yet

another dynamin-independent pathway. However, contrasting reports suggests that flotillins regulated endocytosis of some cargoes is dynamin-dependent mechanism (Aït-Slimane et al. 2009, Compeer et al. 2018). It is therefore, suggested that flotillins could act as adaptors deciding the fate of specific cargo, (Otto & Nichols 2011).

General principles of endocytosis at the cell surface

An overview of multiple endocytic pathways in eukaryotic cells highlights the variety by which cargo can be internalized (Fig. 1), and suggests ways by which this process may be broken down into the steps delineated in Fig 2. A few general issues need to be taken on board and are listed below.

Membrane bending and vesicle scission

At the cell surface where different cytosolic coats assemble (clathrin, caveolin, endoA), the coats along with accessory proteins bend the membrane and subsequently dynamin is recruited for vesicle scission. However, to generate an endocytic carrier, neither a canonical coat nor dynamin as a scission protein is necessary. Physical mechanisms such as membrane domain formation can result in line tension driven membrane bending causing constriction and eventual budding in the absence of membrane coats (Johannes et al. 2015, Sens et al. 2008). Dynamin is not involved at most sites in intracellular membrane compartments where vesicles are generated (ER, Golgi, endosomes), and here dynamin-independent mechanisms operate where COPI and COPII coated vesicles bud and detach in the absence of dynamin (Campelo & Malhotra 2012). BDPs can also drive vesicle scission by inserting their amphipathic helix into the membrane. Recent in vitro experiments suggest that EndoA coated tubules facilitate scission by creating friction between rigid scaffolds and the membrane (Simunovic et al. 2017). The breakage occurs at the border of scaffolding and bare membrane. Such a mechanism provides an attractive alternative hypothesis for dynamin independent pinching. Scaffolding led membrane fission may also occur due to mechanisms like protein crowding (Snead et al. 2017), or by assisting in building up membrane bulges adjacent to sites of membrane scission, as observed in recent work on scission catalyzed by the ATP-driven EHD1 protein (Deo et al. 2018).

In the FEME pathway, STx-B induced tubules undergo scission in an actin-dependent fashion wherein actin polymerization on the STx-B induced tubules drives membrane reorganization (inferred from changes in the membrane order) and results in scission (Römer et al. 2010). Microtubules along with dynein and dynactin have also been implicated in facilitating tubulation and scission in CIE (Day et al. 2015). This may provide the pulling force necessary for the friction based scission mechanism proposed by (Simunovic et al. 2017). Thus, it is not surprising, that at the plasma membrane, multiple mechanisms, serve to help build and scission membrane buds (Fig 2).

Role of actin

While there is no generalizable mechanism for endocytosis, all endocytosis has to negotiate the cortical actin meshwork. Different endocytic processes depend on actin for different reasons. Actin polymerization helps counteract higher apical membrane tension relative to

the basal side in CME (Boulant et al. 2011). Among the CIE pathways, CG endocytosis requires dynamic actin as both actin depolymerizing and stabilizing drugs inhibit it (Chadda et al. 2007). Other CIEs regulate the actin machinery required for endocytosis using small Rho family GTPases, RhoA (FEME), CDC42 (CG), and Rac1 (FEME and macropinocytosis) using known upstream regulators of the actin cytoskeleton (Boucrot et al. 2015, Koivusalo et al. 2010, Sabharanjak et al. 2002).

Mechanistic understanding for the role of actin in endocytosis comes primarily from studies in budding yeast where endocytosis is difficult to classify as either CME or CIE. The endocytic process begins with a membrane invagination followed by tubule elongation. This is exquisitely coordinated between actin polymerization and motor activity. Clathrin-recruitment precedes actin and is postulated to aid in the initial assembly of actin (Kaksonen et al. 2005). In the first phase, nucleation of actin and its crosslinking causes membrane invagination for which, a physical link between PM and the actin network is necessary. Crosslinking is speculated to tolerate larger stresses that occur during initial membrane deformation. During the second phase, nucleation ceases, while the actin network expands/rearranges. This rearrangement is thought to release the stress stored in the cross-linked actin mesh, thereby leading to vesicle scission (Picco et al. 2018). Hence, actin may play multiple roles in endocytosis depending upon the context. Actin may scaffold proteins, actin filaments may push against the membrane, and actin-based motors may pull the membrane.

Cargo sorting among different endocytic pathway

In CME, AP2 is traditionally known to act as a primary adaptor that recognises cargo via a specific sequence based motif on one hand while connects the cargo to clathrin assembly (Robinson 2004). Despite this, several CME cargoes can traffic independent of AP2 utilizing co-adaptors. For example, Epsin family proteins (ubiquitinated cargo) (Polo et al. 2002), β -arrestins (phosphorylated GPCR) (Laporte et al. 2000), Dab2 and ARH (Low Density Lipoprotein) (Maurer & Cooper 2006), Numb (Notch) (Santolini et al. 2000) are some of the diverse co-adaptors that are used by CME cargoes. Notably, GPCR are also internalised by FEME pathway, independent of β -arrestins that raises the question about the factors that govern cargo sorting to the different pathways (Boucrot et al. 2015). Ubiquitylation may serve as a signal for CIE via the FEME, and the capacity to bind Galectins for the CG pathway. Identifying the sorting mechanisms for each of the CIEs must serve as a major focus.

Post-endocytic trafficking itineraries

Multiple endocytic pathways take in different cargoes and deliver them to different destinations within the cell. Endocytic vesicles derived from CME undergo fusion with a sorting endosome which in turn undergo homotypic fusion. Cargo is then directed towards recycling compartments or it matures to late endosomes and then fuses with lysosomes (Fig. 2). There is also a direct recycling route from the sorting endosome, and endosomes become progressively more acidic from early to late to lysosomes (Huotari & Helenius 2011). It appears that most cargo derived from dynamin-dependent endocytosis follow this itinerary, where different endocytic vesicles converge on sorting endosomes. On the other hand, dynamin-independent endocytic processes appear to form distinct early endosomal

compartments which deliver only part of their material to the endosomal system that carries cargo derived from the dynamin-dependent system. CLICs eventually fuse to form an early endosomal pathway called GEECs. The resulting GEECs subsequently fuse with the sorting endocytic vesicles via a Rab5/phosphatidylinositol-3-kinase-dependent mechanism (Kalia et al. 2006) linking up with endosomal cargo from CME and caveolar pathway (Fig 1). The GEECs which are highly acidic, even more so than the sorting endosome (Kalia et al. 2006), recycles much of its membrane contents before fusing with the classical sorting endosome (Howes et al. 2010). Recycling of membrane from these compartments is likely to involve distinct recycling systems and machinery. This is an active area of research and the reader is referred to several excellent reviews in this area (Cullen & Steinberg 2018, Grant & Donaldson 2009, Huotari & Helenius 2011, Maxfield & McGraw 2004).

Endocytosis in HeLa cells, a cautionary note

It must be admitted that the CIE field has been plagued by contradictory data from different groups. For instance, as mentioned earlier, CG is a high capacity pathway as visualized using EM based quantitative imaging (Howes et al. 2010). However, in a study done primarily in a HeLa cell line, CIE(s) were found not to contribute significantly to endocytic flux (Bitsikas et al. 2014). Furthermore, it was argued that if CIE exists, it is a compensatory mechanism when CME is perturbed. This extreme view is unlikely since CIE is observed without perturbation of CME in many systems (Guha et al. 2003, Hemalatha et al. 2016, Howes et al. 2010, Renard et al. 2015, Sabharanjak et al. 2002) and in real time (Sathe et al. 2018). Moreover, removal of all three dynamin isoforms increases fluid uptake via the CG pathway in mouse embryonic fibroblasts (Park et al. 2013, Thottacherry et al. 2018), contrary to a reduction in fluid-uptake upon dynamin inhibition in HeLa cells (Bitsikas et al. 2014). Interestingly, while AP2 knock down inhibited CME in many different cell lines and upregulates fluid phase uptake, in HeLa cells it also reduces fluid uptake (Holst et al. 2017, Thottacherry et al. 2018). These observations along with previous studies (Kalia et al. 2006) indicate that HeLa cells do not have a functional CG pathway.

An important point to note is that many endocytic studies have been carried out in cell lines long adapted to tissue culture. In the case of HeLa cells, they appear to have diverged into unique cell lines in culture over the years, with different genomic, transcription and proteomic profiles (Frattoni et al. 2015, Liu et al. 2018). An example highlighting this divergence is endocytosis of EGFR in HeLa cells. EGFR can traffic using CME and CIE and the discrepancy about the existence of a CIE route for EGFR was attributed to variation between different laboratory strains of HeLa cell line (Sigismund et al. 2013). Another example of variability in HeLa is the endocytic dependence on dynamin. On one hand, HeLa shows a predominant dynamin-dependent endocytosis (Bitsikas et al. 2014, Thottacherry et al. 2018) while on the other hand, one of the initial observations of dynamin-independent fluid uptake was made in a HeLa line (Damke et al. 1995). Hence, it is necessary to confirm inferences made in HeLa line, particularly, those pertaining to the role of CIE (Doherty et al. 2011, Francis et al. 2015, Holst et al. 2017, Krag et al. 2010, Lundmark et al. 2008), by doing experiments in other characterized cell systems.

Cross talk between Endocytic Pathways: A role for lipids?

Different endocytic pathways functioning at the cell surface appear to influence each other, indicating a cross-talk between the pathways. In the context of caveolae, the presence and expression of caveolins and cavins, regulates a number of CIEs by changing membrane fluidity and cholesterol levels at the plasma membrane, respectively (Chaudhary et al. 2014). Loss of dynamin function exhibits an increased activity of the CG pathway, indicating again cross talk between the dynamin-dependent and independent pathways (Guha et al. 2003, Thottacherry et al. 2018). However, the precise means for cross talk between different pathways is not understood.

Apart from the protein components, lipids in the plasma membrane are crucial for endocytosis. For example, cholesterol is important for the transbilayer interaction of membrane lipids (Raghupathy et al. 2015) and is necessary for making phosphatidylinositol phosphate (PIP)-rich domains (Jiang et al. 2014, Kwik et al. 2003). Cholesterol depletion affects almost all CIE pathways with varying efficacy (Boucrot et al. 2015, Chadda et al. 2007, Rodal et al. 1999, Subtil et al. 1999). Cholesterol, has been shown to regulate CDC42 activity and consequently CG endocytosis (Chadda et al. 2007); at extreme levels of depletion, it also affects CME (Subtil et al. 1999). It is unlikely that all CIE are affected by similar mechanisms upon cholesterol perturbations and the specific details need to be worked out.

Polyphosphoinositides are implicated in various aspects of cell physiology (Balla 2013). Phosphatidylinositol (PI) and its phosphorylated products, polyphosphoinositides (PIPs) are crucial signaling lipids that mark specific membrane domains and have been shown to differentially influence multiple endocytic pathways. There are seven PIP species depending on the phosphorylation status at 3-, 4-, and 5-OH positions and they are interconvertible by specific enzymes that phosphorylate or dephosphorylate them. Since many of these lipid modifying enzymes are localized to specific compartments, it is likely that their products are generated locally thereby providing a unique PIP identity to specific membrane compartments or sub-domains. During migration, for example, Class I PI3K is localized to the plasma membrane at the leading edge generating sub-domains of PI(3,4,5)P₃ in a polarized fashion, helping in polarized migration (Funamoto et al. 2002, Servant et al. 2000). Class II PI3K β on the late endosome or lysosomes in response to nutrient deprivation produces PI(3,4)P₂ which clusters lysosomes and suppress mTOR activity (Marat et al. 2017). Class III PI3K (Phosphatidylinositol 3 kinase), Vps34, generates most of PI(3)P at the early endosomes that is important for early endosomal identity via regulating Rab5 recruitment (Simonsen et al. 1998). While the role for PI(3)P in endosomal maturation via a sequential cascade of recruitment of kinases and phosphatases by the Rab proteins is well appreciated (Wandinger-Ness & Zerial 2014), the role for different PIPs in regulating different forms of endocytosis is only recently being recognized. For an overview of PIPs in the endo-lysosomal system, the reader is referred to a recent review (Wallroth & Haucke 2018).

Turnover of PIPs is crucial for the initiation and completion of a Clathrin pit during CME. The generation of PI(4,5)P₂ recruits Clathrin adaptors, FCHO, epsins and AP-2 complex.

AP-2 can, in turn, recruit more PIP5K, creating a feed forward loop (Bairstow et al. 2006, Haucke 2005, Krauss et al. 2006). However, later stages require hydrolysis of the accumulated PI(4,5)P₂ and production of PI(3,4)P₂ from PI(4)P (Rusk et al. 2003). In the caveolar pathway our understanding of the role of PIs is limited. However, a distinct pool of PI(4,5)P₂ is observed at the rim of caveolae (Fujita et al. 2009). The dynamin-related ATPase EHD2 is recruited to caveolae by binding to PI(4,5)P₂ to negatively regulate internalization of caveolae (Daumke et al. 2007, Stoeber et al. 2012). The FEME pathway is initiated by PI(3,4)P₂ produced by SHIP1/2 phosphatase from PI(3,4,5)P₃. This helps in recruiting lamellipodin and thus EndoA2, which allows local deformation of the membrane to mediate FEME pathway (Renard et al. 2015). The CG pathway is also regulated by PI(3,4,5)P₃ which recruits GBF1, a key regulator (Mazaki et al. 2012). Inhibition of Class 1 PI3K or its depletion abrogated the localization of *Drosophila* homolog of GBF1, Garz, to the plasma membrane, inhibiting the CG pathway (Hemalatha et al. 2016). These experiments, however, do not rule out a role for products of PI(3,4,5)P₃ such as PI(3,4)P₂. In addition, ARF1 is a potential activator of PI(4)P5K1 (Honda et al. 1999) and the resultant local PI(4,5)P₂ (Di Paolo & De Camilli 2006) could indirectly activate CDC42 (Rohatgi et al. 2000), another regulator of CG pathway (Sabharanjak et al. 2002). Thus by-products of PI(3,4,5)P₃ could potentially bring together two regulatory arms of CG pathway: GBF1 via PI(3,4,5)P₃ and CDC42 via PI(4,5)P₂ indicating a prominent role for PIPs in initiating the CG pathway, or regulating FEME.

PIPs along with other proteins help with the coincidence detection and provide compartment identities (Behnia & Munro 2005, Di Paolo & De Camilli 2006). This could work at the plasma membrane to give nano-scale identities for operating multiple endocytic pathways in adjacent regions of the membrane. Indeed, PI(4,5)P₂ and PI(3,4,5)P₃ are in separate nanoscale domains at the plasma membrane (Wang & Richards 2012). These domains by themselves could help to activate different endocytic pathways at the plasma membrane where signalling could spatially modulate endocytic levels by regulating the levels of the PIP species generated.

Endocytic pathways also compensate each other, resulting in substantial cross-talk among them. Could the nanodomain localization of PI-Kinases and phosphatases and feedback loops therein help coordinate this cross talk? Addressing this question will require looking at multiple pathways simultaneously, to get a system wide regulation of these pathways in the context of membrane lipid species generation and localization.

Evolutionary Antecedents of Endocytic pathways

Understanding the evolutionary history of each of the endocytic processes, will shed light on the function of each of these pathways, as well as answer questions about how ancient cells performed these tasks. Was there a primordial eukaryotic cell that lacked the capacity for specific forms of endocytosis? When did specific endocytic processes emerge? Was it operational in prokaryotes or Archaea or in FECA (first eukaryotic common ancestor) that arose out of merger?

Phylogenetic analyses are key to understanding evolutionary antecedents, since they allow us to compare molecular and other cellular processes within eukaryotes. If a trait is found in a representative species of all the super-groups, then the trait is presumed to be ancient and any absences are due to loss of these traits. On the other hand, if a trait is present in only a subset of super groups then it is considered a recent acquisition. Such genomic comparison indicates that several of endocytic proteins were present in last eukaryotic common ancestor (LECA) (Dacks et al. 2008, Field et al. 2007). A recent phylogenetic analysis of the dynamin superfamily members, revealed that there are three classes of dynamins. Class A and B were present in LECA while Class C appears to have arisen in amoebozoans and archaeplastids (Purkanti & Thattai 2015). Extant eukaryotes have two distinct Class A dynamin specialized for two distinct functions: mitochondrial division and vesicle scission. Several eukaryotic lineages, for instance *Trypanosoma brucei* have only a single Class A dynamin indicating that in these organisms both mitochondrial division and vesicle scission is performed by the same dynamin (Chanez et al. 2006). From the same excavata supergroup, *Giardia lamblia*, an amitochondriate, has a single dynamin that colocalizes with CCP (Gaechter et al. 2008). Thus, it appears that LECA possessed a single bifunctional dynamin that duplicated and specialized into mitochondrial and vesicle variants three independent times (Purkanti & Thattai 2015). Looking at other key endocytic molecules in LECA, indicates that the endocytic system of LECA was quite sophisticated (Field et al. 2007). Rabs and Syntaxins are highly conserved and some of the key early, recycling and late Rabs, (5, 7 and 11) are present in almost all the taxa. In addition to the clathrin coat and the adaptin complex (Hirst et al. 2011), SNAREs and many other fusion and endocytic machineries are present in LECA and almost all taxa (Field et al. 2007). Thus, the endocytic system is not only ancient but LECA possessed a highly sophisticated endocytic system. A general scheme of endocytosis involves formation of the vesicles which become differentiated into early and subsequently late endosomes/lysosomes, and this network is closely linked with the exocytic traffic. Most organisms have all of these features, while some have lost one or more aspects.

So far evidence suggests that LECA possessed CME but what about CIEs? A phylogenetic analysis of caveolae and caveolin, shows that it appears very late in evolution (Field et al. 2007, Kirkham et al. 2008). Regardless, CIE is likely to occur in all eukarya, given that it is prevalent in single cell eukaryotes such as yeast, and metazoa such as both animals and plants (Baral et al. 2015). Many CIEs are regulated by small GTPases (RhoA, Rac1 and CDC42; Supplementary Table S1), which participate in several other crucial cellular processes and are likely to be present in the LECA. Recently, evidence has accumulated that IRSp53, an I-BAR domain protein specifically affects the CG endocytic pathway but leaves CME unperturbed (Sathe et al. 2018). I-BAR domain of IRSp53 that is speculated to stabilize the necks of forming CG endocytic tubules, apart from being present in metazoans (Scita et al. 2008), is also present in budding yeast with orthologs in filasterea and choanoflagellates (Itoh et al. 2016). Similarly, EndoA-dependent FEME pathway could exist in other organisms since BDPs are found not only in metazoans but also in unicellular organisms like yeast. Detailed phylogenetic analysis for IRSp53, EndoA and other recently characterized CIE molecules in a fashion similar to the analysis performed for dynamin should reveal insights about the prevalence of many of these pathways in eukaryotes.

Endocytic processes are also observed in some bacteria belonging to phylum *Planctomycetes*; *Gemmata obscuriglobus* carries out protein uptake via membrane encased vesicles (Fuerst & Sagulenko 2012). *Lokiarchaeum* postulated as the closest living ancestor of eukaryotes, encodes more eukaryotic signature proteins than any other Archaeon. *Lokiarchaeum* encodes small GTPases, BDPs, gelsolin and other proteins, and might display membrane trafficking (Dey et al. 2016). Unfortunately, members of this species are yet to be isolated and studied to confirm these exciting possibilities.

In addition to the phylogenetic analysis, it is instructive to study different cell systems that have diverged across different evolutionary time scales to provide inputs into how cells have evolved to construct endocytic processes. For example, understanding endocytosis in the budding yeast, *Saccharomyces cerevisiae*, has provided insights into what appears to be a strictly ARP2/3 (and Las17/N-WASP) dependent endocytic pathway but relies to a much lesser extent on clathrin and dynamin (Kaksonen & Roux 2018). By contrast, in another yeast, the pathogenic *Candida albicans*, membrane lipids and bulk fluid internalization were not perturbed in the absence of ARP2/3 whereas membrane receptor endocytosis via the CME machinery was compromised. This revealed the existence of two types of endocytic mechanisms that might resemble the CME and CIE processes, found in metazoan. Future comparative analysis of the endocytic mechanism among different eukaryotic organisms will provide further insights into the multiple design principles of endocytosis.

Functional roles of multiple endocytic pathways

High-throughput studies (Collinet et al. 2010, Gupta et al. 2014, Pelkmans et al. 2005) of multiple endocytic processes analyzed simultaneously, have not only advanced our understanding of the intricate molecular network involved in various endocytic pathways but have also elucidated the roles of endocytosis in maintaining cellular homeostasis. In this section, we focus on the roles of multiple endocytic pathways in nutrient uptake, setting plasma membrane tension, cell migration and cell signaling.

Membrane tension and homeostasis

Membrane tension has long been proposed to regulate endo-exocytic membrane trafficking (Sheetz & Dai 1996). The membrane responds passively and actively to changes in membrane tension. Passively by creating membrane tubules called 'reservoirs' (Kosmalska et al. 2015), blebs (Norman et al. 2010), and flattening of caveolae (Sinha et al. 2011) and actively by gating mechanosensitive channels, and altering cytoskeleton dynamics and membrane trafficking (Apodaca 2002, Diz-Muñoz et al. 2013, Gauthier et al. 2012). In terms of endocytosis, different endocytic processes respond differently to the application of force on a cell. CME resists an increase in membrane tension by recruiting actin where actin and BDPs helps offset tension to allow internalization of the CCPs (Boulant et al. 2011, Ferguson et al. 2017, Hassinger et al. 2017, Schöneberg et al. 2018) while membrane tension is involved in transition of clathrin coats from flat to curved (Bucher et al. 2018). Caveolae help to passively buffer an increase in tension by rapidly flattening the cup shaped caveolae to provide excess membrane (Sinha et al. 2011), but is restored actively using cytoskeletal rearrangements. Upon deadhering, caveolae coated region of the membrane are endocytosed

over a period of minutes to hours, resulting in an alteration of the membrane composition of the cell surface. Phosphorylation of caveolin is necessary to trigger internalization of membrane micro-domains enriched in cholesterol (del Pozo et al. 2005). This endocytosis is important for triggering anoikis and happens once the cell is in suspension. The CG pathway on the other hand rapidly and transiently responds to a decrease in tension by internalizing excess membrane (Thottacherry et al. 2018). This transient endocytic upregulation is also observed during deadhering (Thottacherry et al. 2018) potentially caused by a reduction in tension (Norman et al. 2010). It is important here to distinguish the difference between the caveolin-dependent endocytosis and CG pathway during deadhering. Contrary to the caveolar pathway, the CG pathway is downregulated in cells in suspension (Thottacherry et al. 2018) possibly due to the reduction in cholesterol at cell surface (del Pozo et al. 2004). Thus, different endocytic pathways respond differentially to changes in tension and this could be useful in different circumstances.

Integrin mediated cell-substrate detachment is accompanied by caveolar uptake of cholesterol enriched microdomains contributing to an alteration of the membrane composition as well as targeting of Rac to the plasma membrane (del Pozo et al. 2004). In parallel, the tension-regulated response of the CG pathway depends on the activation of vinculin, a key mechanotransducer downstream of cell-substrate and cell-cell adhesion (Thottacherry et al. 2018). The CG pathway acutely senses changes in tension and actively responds to the change. In addition, the CG pathway is also proposed to provide membrane during cell spreading from recycling of endocytic compartments in response to increase in tension (Gauthier et al. 2009). The high capacity and membrane tension sensitive features of CG endocytosis positions it to be a key component in maintaining membrane homeostasis.

Migration

During cell migration in 2D, multiple endocytic pathways are localized to spatially distinct sites. In migrating fibroblasts, CG endosomes are localized to the leading edge and transient ablation of these endosomes specifically inhibit efficient migration, while caveolae are at the rear end and CCPs are unpolarized (Howes et al. 2010). FEME, when induced, is observed at the leading edge of adherent BSC1 cells with CCPs remaining unpolarized (Boucrot et al. 2015).

Membrane tension is involved in coordinating multiple cellular processes during migration (Diz-Muñoz et al. 2013). For instance, the increase in the membrane tension at the leading edge maintains the polarity and helps in migration (Houk et al. 2012). The CG pathway helps in regulating membrane tension (Thottacherry et al. 2018) and key regulators of the CG pathway are localized to leading edge in neutrophils (Mazaki et al. 2012). Thus, the polarized CG pathway in migrating cells may be deployed to modulate membrane tension. In contrast, CCPs help in 3D migration of MDA-MB-231 by helping to pinch the collagen fibers (Elkhatib et al. 2017). In confined environments, migrating immune cells overcome hydraulic resistance by activating macropinocytosis at the leading edge to transport fluid across the cell (Moreau et al. 2018). Nevertheless, the deployment of a particular endocytic pathway will depend on the type of migration and/or the cell type and how regulation is

achieved remains to be resolved in the context of both physical (membrane tension) and chemical (extracellular matrix or growth factor) cues.

Nutrient Uptake and Sensing

Historically the role of endocytosis has been associated with uptake of nutrients for the cell. In fact the uptake of iron (bound to Transferrin) and lipids (associated with lipoproteins) are perhaps the most celebrated aspects of the CME function (McMahon & Boucrot 2011). In addition, the CG pathway internalizes a variety of GPI-APs allowing for the uptake of its small molecule ligands. A prime example is the folate receptor that binds to the folate ligand in the neutral pH environment of the cell surface, and is endocytosed via CIE to robustly deliver folates to cells (Ritter et al. 1995). Folate dissociates from its receptors in the extremely low pH environment of the endosomes and is transported out to the cytosol. This pathway could be of vital importance for a number of cargoes where a low concentration bulk ligand is captured at the cell surface and is concentrated and delivered into the cell via GEECs.

Endocytic uptake of nutrients takes even more prominence in the case of tumors that are nutrient deprived. For instance, human pancreatic cancer tumors are nutrient poor because they lack the access to freely circulating amino acids due to poor vascularization (Kamphorst et al. 2015) and therefore need to actively scavenge extracellular proteins. In separate study, Ras-transformed cells upregulate macropinocytosis to provide an amino acid source by proteolytic degradation of endocytosed extracellular proteins, dependent on PTEN and AMPK (Commisso et al. 2013, Kim et al. 2018). Moreover, inhibition of macropinocytosis suppressed tumor growth (Commisso et al. 2013, Kim et al. 2018). The question then arises as to how the nutrient sensing machinery talks to the endocytic mechanisms. GAPDH comes up as an interesting candidate since it can act as a GAP for ARF1 in addition to its role in glycolysis, thereby connecting membrane trafficking to the energy state of the cell (Yang et al. 2018). Interestingly, upon GAPDH depletion, fluid-phase endocytosis increased while CME and other CIE remained unaffected. A second candidate that potentially connects nutrient sensing and endocytosis is AMPK. AMPK also phosphorylates GBF1 (Mao et al. 2013), the GEF for ARF1 and a key regulator of the CG pathway (Gupta et al. 2009). AMPK may even indirectly control ARF1, by regulating GAPDH localization to membrane compartments through its phosphorylation in response to nutrient status (Yang et al. 2018). Thus, GAPDH and AMPK open up ways to coordinate endocytic pathways with nutrient level within a cell. Nevertheless, the cell would need to take in key ligands and continue to exert basal signaling control. This can be achieved by allowing the CME to continue robustly whilst the fluid-phase uptake and secretion is blocked on GAPDH perturbation.

Endocytosis and signalling

Endocytosis of ligand-receptor pairs has been typically associated with downregulation of signalling. However, since the discovery of endosomal localization of EGFR and its signalling effectors (Di Guglielmo et al. 1994) upon EGF stimulation, numerous functions (positive, negative, and modulatory) of endocytosis have been identified in various signalling systems, and some excellent recent reviews have highlighted this (Di Fiore & von Zastrow

2014, Villaseñor et al. 2016). In this section, we focus on the roles of multiple endocytic pathways in one signalling module - Receptor Tyrosine Kinase (RTK) signalling.

Growth factors and their respective RTKs play pivotal roles in cellular functions during development as well as in pathogenesis (Lemmon & Schlessinger 2010). Typically, growth factor binding results in receptor dimerization, activation of the tyrosine kinase, rapid internalization and sorting of ligand-RTK complexes via CME into lysosomes for degradation. However, many RTKs deviate from this standard model (Goh & Sorkin 2013). Several factors, such as ligand concentration, types of ligands and receptors and cells, control the mode of ligand-RTK trafficking and subsequent downstream RTK signaling. The first evidence that EGFR is trafficked by CIEs came when it was noticed that clathrin heavy chain depletion did not affect EGF uptake in HeLa cells (Hinrichsen et al. 2003). Studies in HeLa cells showed that low concentrations of EGF (1.5 ng/ml) results in CME of EGFR while high concentrations of EGF (20 ng/ml) partitions EGFR via a cholesterol sensitive CIE pathway (Sigismund et al. 2005, 2008). The fate of EGFR internalized via the different pathways is also distinct: EGFR endocytosed via CME is recycled, thus aids in prolonged activation, while those internalized via CIE results in lysosomal degradation effecting signal attenuation. CIE trafficking is associated with Cbl mediated receptor ubiquitylation at the plasma membrane (Sigismund et al. 2013). However, the finding of CIE pathways for EGFR uptake is contested (Grandal et al. 2012, Kazazic et al. 2006, Rappoport & Simon 2009). The discrepancy could be attributed to cell specific or clone specific differences in HeLa cell lines used in these studies (Sigismund et al. 2008, 2013). The nature of the ligand in activating EGFR also directs EGFR endocytosis via different pathways: while EGF stimulated a CME uptake, more potent ligands such as Heparin binding EGF like growth factor (HB-EGF) and betacellulin (BTC) activate both CME and CIE routes (Henriksen et al. 2013). The specific CIE that EGFR utilizes requires dynamin (Henriksen et al. 2013, Sousa et al. 2012) and is identified as FEME (Boucrot et al. 2015). In cells lacking all isoforms of Endophilin, surface levels of EGFR were enhanced consistent with FEME aiding in receptor degradation.

In the *Drosophila* hematopoietic system, high concentrations of Spitz (Spi), one of the EGFR ligands in *Drosophila*, caused increased EGFR uptake via the CG pathway. Perturbation of molecules crucial for the CG pathway such as Arf1, Garz and GRAF-1 resulted in reduced EGFR uptake at high ligand concentrations and uptake was unaffected upon depletion of clathrin or by pharmacological inhibition of dynamin. EGFR ubiquitylation was also only observed only under high Spi and ubiquitylation defective EGFR failed to undergo CG endocytosis at high Spi, confirming the necessity of Cbl-mediated receptor ubiquitylation in directing EGFR to the CG pathway. This increased EGFR signalling resulting from depletion of GRAF-1 also caused over proliferation of plasmatocytes, one specific lineage of blood cells (Kim et al. 2017).

Other growth factors such as IGF, FGF and PDGF undergo CIE in different scenarios. IGF-I binding IGF-IR triggers CME or caveolar pathways based on the ligand concentration (Sehat et al., 2008; Salani et al., 2010). FGF when bound to FGFR1 undergoes uptake via CME whereas when bound to FGFR3 undergoes clathrin and dynamin independent uptake (Haugsten et al. 2011), suggesting a CG route. Trafficking of PDGFR- β also switches

between CME and CIE depending on low or high concentrations of the ligand, PDGF-BB, respectively (De Donatis et al., 2008; Sadowski et al., 2013). A recent study shows that PDGFR- β internalization via CIE requires RhoA-ROCK, CDC42, CD44 and Galectin-3 (Jastrzbski et al. 2017). The involvement of the latter three molecules suggests that PDGFR- β may be internalized via the CG pathway. The different endocytic entry routes bias PDGFR- β signaling readouts towards cell proliferation or motility. Thus, various mechanisms of entry expand the scope for the regulation of downstream RTK signalling outputs.

In the context of Wingless signalling in *Drosophila* wing disc, Wingless is internalized via the CG pathway independent of its signalling receptor. Within merged endosomes, the CG internalized pool of wingless interacts with its signalling receptor that was endocytosed via the CME (Hemalatha et al. 2016). Therefore, both endocytic pathways are required for facilitating downstream signalling of Wingless, revealing yet another context where multiple endocytic mechanisms regulate signalling. Other classes of signalling systems, such as GPCRs (Boucrot et al. 2015, Delaney et al. 2002, Okamoto et al. 2000, Zhang et al. 1996), Integrins (Arjonen et al. 2012, De Franceschi et al. 2016, Paul et al. 2015), TGF- β (Di Guglielmo et al. 2003, Zwaagstra et al. 2001) and Wnts (Hemalatha et al. 2016, Yamamoto et al. 2006), also utilize similar design principles to modulate their signalling outcomes. Supplementary Table S1 summarizes some of these key findings in cargo trafficking.

In an extensive screen for modulators of the endosomal distribution of two endocytic cargoes, Tfr and EGFR, the signalling pathways such as Wnt, integrin/cell adhesion, transforming growth factor (TGF)- β and Notch were found to regulate the endocytic system by altering the number, size, concentration of cargo and position of endosomes (Collinet et al. 2010). This indicates that not only does endocytosis influence signalling, but that signalling pathways also profoundly alter the endocytic machinery.

Conclusions

Different pathways operate at the cell surface helping cells integrate information from its surrounding and respond to it. These help the cell to sense and respond to forces, nutrients, coordinate migration and allow a cell to engage with its neighbors. A single endocytic pathway probably could not have made these complex and multiple decisions. From a functional perspective, multiple endocytic pathways appear to be responsible for distinct role(s) in specific contexts in a non-redundant fashion. Indeed, our understanding of these distinct pathways of endocytosis is crucial for understanding cell physiology as well as its role in a metazoan.

While we know the CME molecular machinery in greater temporal and spatial detail, details of CIE are emerging fast and furious. Key insights into mechanisms involved in cargo capture and scission in dynamin-independent pathways are also being acquired. It is clear that there is yet a dire need for a comprehensive parts list for the multiple CIE pathways, both dynamin-dependent and independent. This will occur using sophisticated genetic screens where multiple pathways are examined at the same time, and are capable of uncovering correlated perturbations in these pathways (Collinet et al. 2010, Dey et al. 2014,

Gupta et al. 2014, Haney et al. 2018). This will also address relationships between the different CIE pathways. The cell invests a considerable amount of molecular wiring to calibrate cross talk between these pathways, and a systematic monitoring of the correlated expression of genes in different contexts may provide a handle to parse how endocytic networks are governed.

Endocytosis most likely drove eukaryotic evolution by ensnaring the major energy factory of the cell, the respiring bacterial ancestor of the mitochondrion. Endocytosis is definitely part of the core cellular plan and having multiple pathways help bring a sophistication to this plan by engaging in multiple processes like signalling, migration, nutrient sensing, cell membrane homeostasis (Scita et al. 2010, Sigismund et al. 2012). To understand how a cell works in its entirety, it would be important to have a systems view of different membrane trafficking pathways and study them in a physiological relevant context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Different endocytic pathways and their itinerary

Multiple clathrin independent endocytic pathways function at the cell surface. These can be divided based on their requirement for clathrin (brown, orange) or dynamin (green, purple) dependence. Some pathways are not constitutively active and are marked as induced. Cargoes for these pathways are listed in Supplementary Table S1. Endosomes formed from each of the dynamin-dependent pathways fuse with the sorting endosome from which material is sorted to recycling endosome. The sorting endosome matures to late endosomes which subsequently fuse with lysosome. Dynamin-independent pathways give rise to CLICs

which fuse to form GEECs. GEECS also deliver some of their contents to the sorting endosome, but independently recycle content bypassing the sorting and recycling endosome. There are clathrin and dynamin independent pathways based on cargo (see Supplementary Table S1) which remain unclassified and are denoted as other (these are noted in the section - Types of endocytosis).

Figure 2. Steps in endocytosis

The process is initiated with cargo selection and their recruitment to the forming endocytic site/pit. The cargo can come from the extracellular milieu or belong to the plasma membrane. Once selected, generation of a budding vesicle connected with the plasma membrane occurs by assembling proteins, which help with the bending and stabilization of the bud (Step 1). These proteins can be coat proteins such as clathrin/caveolin or curvature sensing/stabilization proteins. Regardless of the exact machinery used, it leads to the formation of an endocytic pit/invagination, which is, over time, sculpted to make a neck. The neck is constricted and eventually cut to release the endocytic vesicle inside the cell by a process of vesicle scission (Step 2). Here, most endocytic pathway deploys either dynamin alone (Step 2a) or dynamin in conjugation with actin or motor proteins (Step 2b). There are a class of processes that neither utilizes coat proteins nor requires dynamin for scission (Step 2c). These budding vesicles are then pinched to release an endocytic vesicle into the cell (Step 3).

Figure 3. Timeline of key findings showing contrast between the study of CME and CIE.

The time line shows key findings in different endocytic pathways. EM based observations picked up clathrin dependent pathways due to the presence of electron dense coat (1964). It took another decade before clathrin was isolated. Existence of a clathrin independent pathway was hinted from observations where Thy1 (GPI-AP) was found excluded from coated pits (1980) and later the fluid-phase and ricin toxin were endocytosed even when clathrin mediated endocytosis was inhibited (1986/87). These molecules are now internalized via multiple clathrin and dynamin-independent mechanisms (1995/1997/2002).

Clathrin independent pathways are still being discovered with the RhoA-dependent FEME being the most recent addition (2001/2015). Important milestones in the discovery of multiple endocytic pathways: **a** - (Palade & GE 1953, Yamada 1955), **b**- (Roth & Porter 1964), **c**- (Pearse 1975, 1976), **d**- (Anderson et al. 1977a,b), **e**- (Bretscher et al. 1980), **f** - (Kosaka & Ikeda 1983), **g**- (Moya et al. 1985, Sandvig 1987), **h**- (Bar-Sagi & Feramisco 1986), **i** - (Jackson et al. 1987; Kirchhausen et al. 1987a,b), **j**- (Robinson 1989, Thuriereau et al. 1988), **k**-(Rothberg et al. 1992, Scherer et al. 1996, Tang et al. 1996), **l**- (Damke et al. 1994), **m**- (Damke et al. 1994, 1995), **n**- (Radhakrishna & Donaldson 1997), **o**- (Lamaze et al. 2001), **p**- (Sabharanjak et al. 2002), **q**- (Aboulaich et al. 2004, Hill et al. 2008), **r**-(Frick et al. 2007, Glebov et al. 2006) **s**- (Gupta et al. 2009, Kumari & Mayor 2008), **t**- (Boucrot et al. 2015, Renard et al. 2015), **u**- (Sathe et al. 2018).