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Clathrin-independent internalization and recycling

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Abstract

The functionality of receptor and channel proteins depends directly upon their expression level on the plasma membrane. Therefore, the ability to selectively adjust the surface level of a particular receptor or channel protein is pivotal to many cellular signalling events. The internalization and recycling pathway plays a major role in the regulation of protein surface level, and thus has been a focus of research for many years. Although several endocytic pathways have been identified, most of our knowledge has come from the clathrin-dependent pathway, while the other pathways remain much less well defined. Considering that clathrin-independent internalization may account for as much as 50% of the total endocytic activity in the cell, the lack of such knowledge constitutes a major gap in our efforts to understand how different internalization pathways are utilized and co-ordinated. Recent studies have provided valuable insights into this area, yet many more questions still remain. In this review, we will give a panoramic introduction to the current knowledge of various internalization and recycling pathways, with an emphasis on the latest findings that have broadened our view of the clathrin-independent pathways. We will also dedicate one section to the emerging studies of the clathrin-independent internalization pathways in neuronal cells.

Keywords: clathrin-independent internalization • recycling • receptor and channel proteins • neuronal cells

Introduction

Most plasma membrane receptor and channel proteins are only functional when they remain on the cell surface. Undoubtedly, a 'functional level' of these receptor and channel proteins needs to be not only accurately regulated to maintain physiological homeostasis, but also flexible to changes in response to various stimuli, such as hormones, stresses, nutrients, etc. Compared to the regulation at the level of transcription or translation, the regulation of protein trafficking works in a more prompt manner, and therefore is crucial in assisting the cell to cope with a dynamic environment. For example, extensive stud-

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ies have established a critical role of amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor trafficking in long-term potentiation (LTP) and long-term depression (LTD), two prominent neuronal phenomena respectively linked to memory formation and motor learning [1–4]. Abnormalities in protein trafficking often cause serious consequences; for example, Bartter syndrome (a hypertension disorder), Andersen's syndrome (a muscle disorder), or hyperinsulinism may be caused by mutations affecting the surface delivery of inwardly rectifying potassium (Kir) channels Kir1.1 [5], Kir2.1 [6] or the ATP-sensitive potassium channels composed of Kir6.2 and SUR1 [6], whereas trafficking of several G protein-coupled receptors (GPCRs) has been implicated in drug addiction [7–9]. The importance of receptor and channel protein trafficking has been further underscored by a recent report that 14 out of 20 Kir1.1 mutations associated with Bartter syndrome display defective trafficking and fail to reach the cell surface [5].

Proteins destined for the plasma membrane share a common pathway: they are synthesized in the endoplasmic reticulum (ER), processed by the quality control system, exported to the *cis*-Golgi network (CGN), modified in the Golgi cisternae, and eventually sorted at the *trans*-Golgi network (TGN) and exported to the plasma membrane. Upon reaching the cell surface, the fate of a plasma membrane protein comes under strict regulation. The protein may remain on the surface for an extended period, or it may, in response to specific signals, become enriched in specific membrane subdomains and rapidly internalized in endocytic vesicles. Once internalized, the cargo protein may then be recycled back to the cell surface, either directly or indirectly through recycling endosomes or TGN, or it may enter the internal vesicles in the multi-vesicular body (MVB), which eventually leads to cargo protein degradation in the lysosome. By adjusting the itinerary of endocytosis and downstream events, the cell can efficiently adjust the surface level of a plasma membrane protein according to its needs.

Generally, there are two types of endocytosis: phagocytosis and pinocytosis. Phagocytosis predominantly occurs in professional phagocytes such as macrophages, monocytes and neutrophils [10], and will not be discussed in this review. In contrast, pinocytosis occurs in most mammalian cell types, and can be categorized into at least four different pathways: the clathrin-dependent internalization pathway, and three clathrin-independent internalization pathways (*i.e.* macropinocytosis, caveolae-dependent internalization and clathrin-and-caveolae-independent internalization) [10]. In the following sections, we will give a brief introduction to each of the four internalization pathways, with a special emphasis on the less understood clathrin-independent pathways, which may represent up to 50% of total endocytic activity [11]. Furthermore, we will also review the current progress in the studies of clathrin-independent internalization pathways in neuronal cells. For more detailed information on each subject, the following several excellent reviews and the references therein ([12–20]) are recommended.

Clathrin-dependent internalization

Among all the internalization pathways, clathrin-dependent pathway is the best characterized, primarily due to its high selectivity conferred by specific trafficking motifs [21]. Known motifs for clathrin-dependent internalization include the tyrosine-based motif, di-leucine-based motif, NPXY and mono-/multi-ubiquitination [21]. These trafficking motifs are recognized by various adaptor proteins, most notably the adaptor protein 2 (AP-2), which binds to tyrosine-based motif, di-leucine-based motif, and NPXY motif. Other adaptor proteins include epsin and EGFR pathway substrate clone 15 (Eps15) (both bind to the ubiquitin moiety of cargo proteins), β-arrestin 1/2 (binds to phosphorylated ligand-activated GPCR family proteins), disabled-2 (Dab2) (binds to the NPXY motif of low-density lipoprotein (LDL) receptor family proteins), etc [22]. These adaptor proteins also interact with clathrin, either directly or indirectly. During internalization, the adaptor proteins link the membrane cargo proteins to clathrin, concentrating them in clathrin-coated pits. The clathrin-coated pits invaginate into the cytoplasm, and eventually pinch off from the plasma membrane to form clathrin-coated vesicles (80–120 nm in diameter) [10]. The large GTPase dynamin is required to facilitate the fission process, a role that is also essential in several other internalization pathways. Several studies have proposed that clathrin-coated pits tend to assemble repeatedly at specific sites on the plasma membrane called 'coated-pit zones' [23–25], however this hypothesis has been under debate. In a recent study,

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the initial formation of clathrin-coated pits was shown to be a random event, and the frequent coincidences of the nucleation sites can be better explained by an alternative model in which the nucleation events happen randomly in an active field that is surrounded by an inactive rim maintained by the cytoskeleton [26].

Eps15, AP180 (the neuronal isoform of the ubiquitous protein CALM [27]), and transferrin are three commonly used diagnostic proteins unique to the clathrin-dependent internalization pathway. Eps15, a component of clathrin-coated pits, associates with AP-2 and is required for its docking onto the plasma membrane [28]; AP180 simultaneously binds to phosphatidylinositol (4,5)-bisphosphate (PIP2) and clathrin, and may help to tether clathrin to the membrane [29]. The truncated mutants of Eps15 or AP180 have been reported to specifically block clathrin-dependent internalization without affecting other pathways. Transferrin is well established to be specifically internalized *via* clathrin-dependent pathway, and therefore can be employed as a marker for clathrin-dependent endocytic compartments. These diagnostic tools may be collectively utilized to determine whether a plasma membrane protein is internalized *via* the clathrin-dependent pathway.

Internalization from cell surface is not the end of the journey for cargo proteins. Once internalized, the nascent clathrin-coated endocytic vesicles shed their coats and undergo homotypic fusion before fusing with sorting endosomes. From sorting endosomes, internalized proteins are either recycled to the cell surface, directly or indirectly (*via* recycling endosomes or TGN), or targeted to degradative compartments such as late endosomes and lysosomes [30]. In neurons, similar endosomal compartments have been observed, which are the potential sorting and recycling stations for internalized receptors and channels [31–33].

This recycling pathway requires the activity of several small GTPases of the Rab subfamily, which act as molecular switches to control various intracellular trafficking steps by cycling between the active GTP-bound and inactive GDP-bound states [34–36]. Among them, Rab4, 5, 7, and 11 regulate the traffic through the sorting and recycling endosomes [34–36]. Rab5 mediates the homotypic fusion between endocytic vesicles and the fusion between endocytic vesicles and sorting endosomes. Rab4 and Rab11 respectively modulate the direct and indirect (*via* recycling endosomes) recycling from sorting endosomes to the plasma membrane, whereas Rab7 likely controls the transport from sorting endosomes to late endosomes. Mutating a conserved Ser (S) or Thr (T) residue of a Rab protein to Asn (N) favors the inactive GDP-bound state and leads to a dominant negative effect, whereas mutating a conserved Gln (Q) residue to Leu (L) stabilizes the active GTP-bound state and results in constitutive activation of a Rab protein [37]. These Rab mutants are valuable tools for dissecting the trafficking itinerary of an internalized membrane protein. For example, overexpression of the GTPase-defective Q79L mutant of Rab5 increases clathrin-dependent internalization and decreases the exit of internalized proteins from sorting endosomes [38]. In consequence, proteins internalized *via* the clathrin-dependent pathway accumulate in sorting endosomes, which eventually leads to the enlargement of these endosomal structures.

Clathrin-independent internalization

Although the research of clathrin-independent pathways is still at an early stage, our understanding in this area has significantly advanced in recent years. The past decade has seen many insightful studies directed at clathrin-independent pathways, and several novel pathways have been characterized to be independent of both clathrin and caveolae [39–43]. For simplicity, we will put those novel pathways under the same category of clathrin-and-caveolae-independent internalization pathways. Moreover, as our knowledge of clathrin-independent pathways grows, it becomes clear that the endocytic vesicles internalized *via* distinct internalization pathways may eventually converge at the same endosomal structures and come under the regulation of the same recycling machinery. Therefore, whenever applicable, the internalization and recycling process will be separately introduced for each pathway.

Macropinocytosis

Macropinocytosis usually occurs at a special type of membrane subdomains termed membrane ruffles, a wavy form of lamellipodia [44]. There are at least two different types of ruffles: cell edge ruffles and

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circular dorsal ruffles [45, 46]. The endocytic vesicles of macropinocytosis pathway, also known as macropinosomes, are formed through the folding back and closure of membrane ruffle structures. Unlike the small vesicles (<200 nm in diameter) derived from other internalization pathways, macropinosomes are large vesicles, often >500 nm in diameter. Although in multiple cell types dynamin activity does not seem to be required for the fission of macropinosomes [17, 47], the dominant-negative dynamin-2 mutant Dyn2K44A has been reported to block an internalization pathway in endothelial cells, which shares some but not all of the characteristics of the conventional macropinocytosis [48]. Whether this pathway represents a novel clathrin-and-caveolae-independent pathway or a cell-type-specific macropinocytosis with a different dynamin requirement has yet to be addressed. Moreover, Dyn2K44A was shown to inhibit the PDGF-induced macropinocytosis in NIH3T3 cells and Rat1 cells, presumably by altering the activity and localization of Rac, a small GTPase which can be activated by PDGF and is involved in macropinocytosis [49]. Fluorescence imaging studies have shown that F-actin [50] and D3 phosphoinositide (including PI (3,4,5) P3 and PI (3,4) P2) [51] are transiently enriched on the forming macropinosomes. It has been proposed that actin polymerization is necessary for membrane extension, whereas D3 phosphoinositide is required for closure of macropinosomes. Additionally, the activation of the small GTPase ADP-ribosylation factor 6 (Arf6) facilitates the formation of membrane ruffles and the accumulation of macropinosomes, presumably by stimulating the synthesis of PIP2 [15], a well known regulator of actin cytoskeleton [52].

Our current understanding of macropinocytosis has lagged far behind that of clathrin-dependent internalization, and has been derived primarily from studies using cells displaying constitutively high levels of macropinocytosis, such as macrophages, dendritic cells, and *Dictyostelium* [53, 54]. Macropinocytosis has historically been viewed as a non-selective process for the uptake of nutrients and solute macromolecules [44, 54]. Subsequent studies, however, have revealed an unexpected but critical role of macropinocytosis in the sampling of antigens by antigen-presenting cells [55, 56]. It was shown that in dendritic cells, large quantities of solutes are internalized by constitutive macropinocytosis and delivered to MHC class II-rich vesicles, a degradative compartment, where the solutes are presumably degraded to peptides, before being recycled back to cell surface as MHC II-peptide complexes [55]. Additionally, clinically important pathogens such as *Salmonella* [57] enter the host cells *via* macropinocytosis.

In contrast to those cell types discussed above, macropinocytosis remains relatively inactive in most other cell types, but can be transiently stimulated by growth factors [58–60] or phorbol esters that activate protein kinase C (PKC) [53, 61]. Surprisingly, although the constitutive macropinocytosis in NIH 3T3 fibroblasts is sensitive to cytochalasin D, an inhibitor of membrane ruffling [62], the macropinocytosis induced by epidermal growth factor (EGF) or phorbol esters does not show similar sensitivity [58, 63]. This finding suggests there might be a difference in actin cytoskeleton involvement between constitutive and transient macropinocytosis, and that macropinocytosis might serve different functions in non-haematopoietic cells.

Macropinosome-like structures have also been observed at the nerve terminals of several types of neurons [64–69]. Interestingly, formation of macropinosomes in frog motor neurons appears dependent on the stimulus frequency associated with the onset of synaptic depression [70], implicating a possible role of macropinocytosis in synaptic plasticity [69].

A few experimental methods have been developed to study macropinocytosis. Macropinocytosis can be induced in relatively inactive cells *via* treatment with phorbol 12-myristate 13-acetate (PMA, 100 nM for 30 min), which stimulates the PKC pathway. Due to the extraordinary size of macropinosomes, they can be preferentially labeled after a brief (5–10 min) exposure to an extracellular fluid-phase marker, such as fluorophore-conjugated 70 kD Dextran [71]. Macropinocytosis can be specifically blocked by inhibitors of Na^+/H^+ exchange (*e.g.* amiloride) [45, 59] or PI3 kinase (*e.g.* wortmannin) [72], without disrupting clathrin-dependent internalization.

Caveolae-dependent internalization

Lipid rafts are commonly defined as membrane subdomains containing concentrated cholesterol, saturated lipids, and more importantly, various signalling proteins, such as glycosylphosphatidylinositol (GPI)-anchored proteins, growth factor receptors, integrins,

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G proteins, Src family kinases, etc [19]. Those signalling proteins usually have a high affinity to specific lipids within the lipid raft subdomain, and are thus enriched in that region. The existence of lipid rafts has been confirmed by many studies, although there is still much controversy concerning their physiological size, distribution, and contents [19]. A recent review has made excellent efforts to propose a new model of lipid rafts to accommodate various observations made with differing techniques [73]. Due to their unique composition, lipid rafts remain insoluble after cold Triton X-100 extraction [14], and the resulting detergent-resistant membrane (DRM) fraction is studied as a crude counterpart of physiological lipid rafts. Lipid rafts are believed to function as a signalling platform, and endocytosis occurring within lipid rafts may serve as a negative regulation mechanism of the signalling events. As discussed below, some clathrin-independent pathways appear to be lipid raft-dependent.

Caveolae are a special type of lipid rafts, which differ from other types of lipid rafts in two aspects: (1). they display a unique morphology of flask-shaped invaginations (50–80 nm in diameter) on the cell surface; (2). they contain a high level of caveolin proteins, which are essential for caveolae formation. There are three caveolin isoforms: caveolin-1, caveolin-2, and caveolin-3. Caveolin-1 and caveolin-2 are mainly found in non-muscle cells, while caveolin-3 is expressed in skeletal and smooth muscle cells [14]. Caveolins insert into the cytoplasmic leaflet of the membrane lipid bilayer and form a hairpin structure, leaving both the N- and C- termini facing the cytosol [74]. The hairpin structure is speculated to increase the surface area of the cytosolic leaflet, and therefore stabilize a positive curvature of the membrane [74]. Caveolin-1 is also palmitoylated at its C-terminus, the significance of which is not fully understood. According to recent studies, caveolin-1 proteins start to form SDS-resistant oligomers right after they are synthesized in ER [74], and are also phosphorylated at Serine 80 by an unknown protein kinase, which prevents high-order polymerization [74]. When they reach the Golgi apparatus, the caveolin-1 oligomers are dephosphorylated at Serine 80, which enables the further polymerization of caveolin-1 and the clustering of cholesterol [74]. This caveolin-enriched structure is implicated as an export carrier for a subgroup of cargo proteins, and might be controlled by a different mechanism than other export carriers [75]. Caveolin-1 and caveolin-3 are required for the formation of caveolae in their corresponding cell types [76, 77], whereas depletion of caveolin-2 has no apparent effect on caveolae formation [78]. Previous studies showed that caveolin-1 itself can form oligomers, whereas caveolin-2 remains in monomer/dimer form in the absence of caveolin-1 [79]; furthermore, overexpressing caveolin-2 alone leads to its accumulation in the Golgi apparatus, suggesting the coexpression with caveolin-1 is critical for caveolin-2 to be transported to the plasma membrane [80, 81]. Based on the above findings, after depletion of caveolin-2, caveolin-1 alone may still be able to form oligomers and reach the cell surface as usual, whereas without caveolin-1, caveolin-2 may have problems in both forming oligomers and reaching plasma membrane normally. This may partly explain the different phenotypes caused by the depletion of caveolin-1 and caveolin-2. Caveolins directly interact with cholesterol [82, 83], and cholesterol depletion abolishes the formation of caveolae [84]. Importantly, the introduction of caveolin-1 into cells that do not normally express caveolins causes the *de novo* formation of caveolae [85], which suggests caveolin-1 is both necessary and sufficient to mediate caveolae formation.

The abundance of caveolae varies among different cell types: for instance, they are not found in lymphocytes and most neurons, while they are highly abundant in muscle cells, endothelial cells and adipocytes, occupying up to 35% of the cell surface [86]. Similar to the clathrin-dependent pathway, dynamin is also required for the internalization of caveolae [87]. Although caveolae-dependent internalization has been reported to be a constitutive pathway in endothelial cells, the budding of caveolae are commonly regarded as a regulated process in most other cell types. In these cells, after forming invaginations, the caveolae remain attached to the plasma membrane for a long time before budding off. This process may be stimulated by additional signals, such as Simian Virus (SV40), phosphatase inhibitors, exogenous sterols, and glyocosphingolipids [14]. Even after activation, caveolae are slowly internalized (half-time > 20min), and carry little fluid-phase volume [10]. Several cargoes have been reported to enter the caveolae-dependent pathway, such as SV40, cholera toxin, GPI-anchored proteins, transforming growth factor β (TGFβ) receptor, etc. However, most of the cargoes appear to go through other internalization pathways as well.

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Furthermore, the caveolin-1-knock out mice have no detectable caveolae and fail to display apparent defects in development [76].

Given their essential role in caveolae formation, caveolins were initially speculated as positive regulators of the caveolae-dependent internalization pathway. However, many subsequent studies appeared to contradict this proposal. Overexpression of caveolin-1 has been reported to inhibit lipid raft-based endocytosis in some cases [20]; in contrast, knockdown of caveolin-1 in transformed NIH-3T3 cells leads to increased lipid raft-mediated uptake of autocrine motility factor, a process which is normally mediated by caveolae [88]. Based on these findings, caveolin-1 was proposed to be a negative regulator of caveolae-dependent internalization by stabilizing the caveolae structure on the plasma membrane [89]. It is likely that caveolin-1 functions to restrain the internalization which may otherwise be a constitutive process, until dynamin is recruited to the caveolae by cellular signals and releases the caveolae vesicles from the cell surface. The coordinated utilization of caveolin-1 and dynamin may therefore constitute an additional regulation mechanism on the internalization process [13]. According to this model, one intriguing possibility is that in those cells without detectable caveolae, there might be an internalization pathway which has a similar formation mechanism and machinery as the caveolae-dependent pathway, except that it could be a constitutive process, and independent of caveolin and dynamin. This hypothesis is consistent with a study of SV40 internalization through the caveolae-dependent pathway and a clathrin-and-caveolae-independent pathway [90]. The two pathways display different kinetics and dependence on dynamin: the caveolae-dependent SV40 uptake is slower and dynamin-dependent, whereas the SV40 uptake *via* the clathrin-and-caveolae-independent pathway appears to be faster and dynamin-independent [90]. However, the two SV40 internalization pathways also share several common characteristics, such as vesicle morphology, post-internalization trafficking route, and sensitivity to protein tyrosine kinase inhibition or cholesterol depletion. Further studies on the machinery proteins and cargo proteins involved in these two pathways will help define the relationship between them.

Our understanding of the mechanism of the caveolae-dependent internalization is still limited. A model for caveolae assembly as seen in clathrin-coated pits is lacking. Although the importance of caveolins in the biogenesis of caveolae has been well established, their precise roles in the formation of caveolae are still unclear. Caveolins may be directly responsible for membrane deformation like clathrin, or alternatively, these proteins may be involved only in the stabilization of the caveolae after they are formed. The actin cytoskeleton also seems to be involved, as the caveolae-dependent internalization can be blocked by disrupting actin assembly [91]. Okadaic acid, a phosphatase inhibitor, stimulates caveolae-dependent internalization, which suggests protein phosphorylation may play a role that has not been fully characterized. To further underscore the importance of protein phosphorylation, PKC and Src kinases have also been shown to play critical roles in this pathway [14].

The green fluorescent protein (GFP) fusion of caveolin-1 has been widely used to monitor caveolae-dependent internalization [92]. Depletion of caveolin-1 abolishes the formation of caveolae, and therefore can be used to specifically inhibit the caveolae-dependent pathway. Previously, cholesterol depletion was also employed to distinguish the caveolae-dependent internalization from clathrin-dependent internalization, but the result needs to be interpreted with caution, since cholesterol depletion has a broad impact, and severe cholesterol depletion can also disrupt clathrin-dependent internalization [13].

After pinching off from the plasma membrane, a fraction of the caveolae vesicles fuse with the sorting endosome in a Rab5-dependent process, whereas the majority of caveolae fuse with a novel endosomal structure, the caveosome [14]. Originally identified as an endocytic compartment containing SV40-labelled caveolae vesicles but not transferrin-labelled clathrin-dependent vesicles, the caveosome is rich in cholesterol and sphingolipids, and has a neutral luminal pH, in contrast to the acidic pH of sorting endosomes [92]. Subsequent studies have shown that the caveosome functions as a trafficking hub for caveolae-dependent internalization pathway, from where SV40 is delivered to the ER [92], whereas sphingolipids and GPI-anchored proteins are transported to the Golgi apparatus [93, 94]. These findings imply that the caveosome functions as a cargo sorting organelle. Interestingly, caveolae can also bypass the sorting organelles, and directly fuse back with the plasma membrane in a 'kiss-and-run' manner, a process regulated by protein kinases KIAA0999 and MAP3K2 [95]. Most caveolae in the 'kiss-and-run' mode only travel for a very limited distance between

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docking events (mostly confined in an area of ~3–8 μm^2), and the physiological significance is unclear.

Conventionally, the sorting endosome was regarded as the primary sorting station where the fate of cargo proteins is decided. The existence of the caveosome as an alternative cargo sorting organelle brings up some obvious questions: now that several more internalization pathways have been identified, what is the corresponding recycling pathway for each of them? Or can one internalization pathway connect with several recycling pathways? Is there any communication between the caveosome and the sorting endosome? And more importantly, will the same cargo protein be delivered to different destinations when it enters different sorting organelles? Indeed, making use of a subgroup of cargo proteins which can be taken up by several pathways, several studies have argued that the same protein, when internalized *via* different pathways, can be sorted to different destinations and therefore carry out different physiological functions. For example, cholera toxin is taken up by both clathrin-dependent pathway and caveolae-dependent pathway. There is little change in toxicity when the clathrin-dependent internalization of cholera toxin is blocked. However, the toxicity is largely reduced when the caveolae-dependent internalization of cholera toxin is blocked [96]. Furthermore, the TGFβ receptor can be internalized *via* both clathrin-dependent pathway and caveolae-dependent pathway, and subsequently merge into sorting endosomes and caveosomes, respectively [97]. The internalization through clathrin-dependent pathway facilitates TGFβ signalling, while the receptors entering caveosomes are subject to rapid turnover [97]. These findings also lead to an intriguing possibility that there may be a switch mechanism that can change the distribution of cargo proteins in different pathways in response to specific cellular signals.

Clathrin-and-caveolae-independent internalization

GPI-enriched endosomal compartments (GEEC) pathway

GPI-anchored proteins were originally thought to be internalized through the caveolae-dependent pathway. This model was challenged when several reports showed that GPI-anchored proteins are not actively enriched in caveolae [98–100]. Further studies demonstrated that GPI-anchored proteins were only preferentially directed to caveolae when they are cross-linked, which is usually an artefact in immunofluorescence experiments [100]. Moreover, the endocytic pathway for GPI-anchored proteins can also be changed when they are associated with other molecules, such as ligands or interacting proteins [12]. In the absence of cross-linking or additional association, GPI-anchored proteins were found to form nanoscale clusters on the plasma membrane, and become internalized *via* the GEEC pathway, a novel clathrin-and-caveolae-independent endocytic pathway [39].

The GEEC pathway was first established in Chinese Hamster Ovary (CHO) cells [39]. In CHO cells, GPI-anchored proteins were found to be predominantly internalized in tubular elements (~28–40 nm in diameter) which do not colocalize with transferrin or caveolin-1. These primary carrier vesicles, termed clathrin-independent carriers (CLICs), have been comprehensively characterized by Kirkham and colleagues using a novel electron microscopy approach [101], and represents one of the earliest attempts to characterize the initial carriers in a clathrin-and-caveolae-independent internalization pathway. Besides GPI-anchored proteins, cargo proteins like cholera toxin B subunit (CTB), and SV40 are also reported to be internalized *via* CLICs [90, 101].

Unlike the clathrin- or caveolae-dependent pathway, the GEEC pathway does not rely on dynamin activity. This pathway appears to be also responsible for a major fraction of fluid-phase uptake, as fluid markers FITC-conjugated Dextran and Lucifer yellow primarily colocalize with the tubular elements containing GPI-anchored proteins, instead of those labeled by transferrin [39]. The existence of GEEC pathway was later confirmed in several other mammalian cell types, as well as in *Drosophila melanogaster* haemocytes, which suggests it is an evolutionarily conserved pathway [12].

The Rho family GTPase Cdc42 has been reported to play a critical role in the GEEC pathway [39]. The inhibition of Cdc42 causes the fluid-phase uptake to decrease, and GPI-anchored proteins to be redirected to the clathrin-dependent pathway. Consistent with the regulatory role of Cdc42 in actin polymerization, the GEEC pathway can also be inhibited by incubation with modifiers of actin polymerization, such as latrunculin A, cytochalasin D and jasplakinolide

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[102]. The requirement of Cdc42 is highly specific, since the inhibition of two other Rho family GTPases, RhoA and Rac, fail to incur similar effects. Another small GTPase Arf6 is also not required for the GEEC pathway [103]. Similar to other lipid raft-based internalization pathways, the GEEC pathway also shows high sensitivity to cholesterol depletion. Mild cholesterol depletion disrupts the GEEC pathway, probably by inhibiting Cdc42 activation [102]. A recent study reported that the depletion of sphingolipids disrupts the GEEC pathway by affecting the recruitment of Cdc42 to the plasma membrane, however the phenotype can be rescued by adding exogenous sphingomyelin, a subset of sphingolipids, but not glycosphingolipids [104].

Based on the above features, several methods have been routinely utilized to study the GEEC pathway. Fluorophore-conjugated Dextran is used as a fluid-phase marker [102]. The endocytosis of GPI-anchored proteins can be monitored by using a fluorescently tagged GPI-anchored protein, such as GPI-GFP [102]. To study the effect of Cdc42 on the GEEC pathway, the GTPase-deficient mutant Cdc42-L61, and the GTP-binding deficient mutant Cdc42-N17 have been constructed. Furthermore, overexpression of the Cdc42/Rac-interactive-binding (CRIB) motif from the neural Wiskott-Aldrich syndrome protein (N-WASP) can serve as a dominant-negative inhibitor of Cdc42/N-WASP signalling pathway [102].

A majority of GPI-anchored proteins are internalized *via* the GEEC pathway [12]. They are first internalized into CLICs, which then fuse with one another to form a tubular-vesicular compartment in a Rab5-independent manner. This novel endosomal compartment is known as GEEC, and does not overlap with transferrin labelling. The fate of GPI-anchored proteins after entering GEECs varies in different cell types. In most cell types, GPI-anchored proteins are delivered to recycling endosomes and subsequently recycled to the plasma membrane, while in baby hamster kidney (BHK) cells and *Drosophila melanogaster* haemocytes, they are targeted to late endosomes for degradation. The trafficking of GPI-anchored proteins has been reviewed in more detail elsewhere ([12]).

Circular dorsal ruffle (CDR)-dependent pathway

Receptor tyrosine kinases (RTKs) are required for many important signalling events, such as those involved in cell growth, mitosis, and migration [105]. For a long time, the clathrin-dependent pathway has been regarded as the major internalization pathway for RTKs, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). As our studies of clathrin-independent pathways advance, the picture gets more complicated. A report published in 2006 described a novel CDR-dependent internalization pathway, which was argued to be a major mechanism for the sequestering and internalization of EGFR following EGF stimulation in a variety of cell types [40].

The CDR, also known as dorsal waves, is a dynamic membrane structure formed after cells are incubated with RTK agonists, such as EGF, PDGF and hepatocyte growth factor (HGF) [106]. The CDR is generated at the dorsal plasma membrane, often close to the site of RTK activation, or the leading edge in the case of migrating cells [106]. It usually appears minutes after RTK activation, and lasts about 10–20 min. Similar to many other dynamic membrane structures, actin cytoskeleton remodelling is required for the CDR formation, as well as the recruitment of a handful of proteins, such as dynamin, Arp2/3, WASP family proteins, cortactin, Rho family small GTPases, etc. A more complete introduction of the proteins involved in CDR formation can be found elsewhere ([105]). Although the CDR structure has been independently confirmed in several different cell types [105], its function remained undefined for a long time. A recent study demonstrated that after EGF stimulation, 50–60% of the total surface EGFR proteins were selectively sequestered and internalized by the CDR-dependent pathway within 10–20 min in cell lines such as PANC-1 and NR6 [40]. This internalization process requires the activity of PI3 kinase and dynamin, but is independent of clathrin and caveolae, as CDR exhibits little colocalization with the coat components of clathrin and caveolae, and functional blockade of either internalization pathway doesn't affect the CDR-dependent internalization of EGFR [40]. This process is highly specific since the transferrin receptor and inactive PDGF receptor are not preferentially enriched in the EGF-induced CDR [40]. Given that CDR can also be induced by other agonists such as PDGF and HGF, a natural question to pursue next is whether those ligands can also induce the CDR-dependent internalization of their corresponding receptors.

Since macropinosomes are also reported to be induced by EGF and formed along the membrane

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ruffle structure, it is suspected that the CDR may serve as a precursory structure for macropinocytosis. However, a recent review made a comprehensive comparison between the two pathways, and reported multiple differences [17]. For example, dynamin is required for the CDR-dependent pathway, but generally not for macropinocytosis. The WASP family proteins are important players in actin polymerization, however the family members involved in the two pathways are different: N-WASP and WAVE1 appear to function in the CDR-dependent pathway, while WAVE2 functions in macropinocytosis. The two pathways also show different requirements for small GTPases: Rab5 is an important player in the CDR-dependent pathway, while Arf6, Cdc42, and RhoA are involved in macropinocytosis [17].

Whereas the above study unveils CDR-mediated internalization as an efficient mechanism for the down-regulation of activated EGFR, it should be noted that the formation of CDR is dependent on both the EGF concentration and the cell type. Thus, other internalization pathways can still play an important role in the down-regulation of the receptor. Indeed, in Hela cells which do not form CDR [40], clathrin-mediated endocytosis appears to be the predominant internalization pathway for activated EGFR [40, 107, 108]. Several studies have also suggested that caveolae function in the internalization of EGFR, although the extent of their involvement is somewhat controversial [107, 109]. One group reported that internalization of EGFR in HeLa cells is insensitive to filipin at low concentrations of EGFR (1.5 ng/ml) but the same treatment inhibits the EGFR internalization by about 40% under a higher level of EGF (20 ng/ml) [109], implying a ligand dose-dependent switch of internalization mode. Consistent with this, the authors observed that EGFR was localized primarily in clathrin-coated pits at low EGF concentrations but was equally partitioned between coated pits and caveolae at higher EGF concentrations [109]. On the other hand, another group has found that methyl-β-cyclodextrin (MβCD) and nystatin have little effect on the internalization of EGFR in HeLa cells, irrespective of the EGF concentration (1–100 ng/ml, [107]). Moreover, a high concentration of EGF (100 ng/ml) fails to increase the accumulation of EGFR in caveolae in HEp2 cells [107]. They further utilized live cell imaging to show that EGF stimulation neither changes the organization of caveolae on the plasma membrane nor induces the internalization of caveolae in HEp2 cells [107]. It is unclear whether part of the difference is caused by the different drugs or cell types used. Future studies will be needed to clarify the role of caveolae in the internalization of EGFR.

As discussed above, the TGFβ receptor can be internalized *via* both a clathrin-dependent pathway and a caveolae-dependent pathway, which eventually lead to different fates for the internalized receptor [97]. Given the observation that EGFR can be internalized *via* clathrin-, CDR-, and possibly caveolae-mediated mechanisms, it will be interesting to investigate the physiological consequences associated with each internalization pathway. For example, what are the impacts on the cell growth caused by the different internalization kinetics of EGFR in different pathways? What factors regulate the EGFR distribution among these internalization compartments? Does the CDR-mediated internalization represent a common entry port for other RTKs and cell surface signalling proteins? Given the intimate functional link between RTKs and integrins, how does the CDR internalization pathway affect cell adhesion and migration?

Flotillin-1-dependent pathway

Flotillin-1, also known as reggie-2, is a member of the Stomatin/Prohibitin/Flotillin/HflK/C (SPFH) protein superfamily. Similar to its homologue, flotillin-2/reggie-1, flotillin-1 is predominantly found on the plasma membrane, although the localization patterns of the two flotillins are not always the same. Both flotillin-1 and flotillin-2 contain a C-terminal flotillin domain, which participates in the homo- and probably also hetero- oligomerization [110]. Flotillins form stable clusters within lipid rafts and are often used as lipid raft markers. The membrane localization probably results from palmitoylation and a hydrophobic domain which is inserted into the membrane. The hydrophobic domain does not span the lipid bilayer, but forms a hairpin structure, with both the N- and C-termini facing the cytosol [110]. The palmitoylation, the oligomerization, and the hairpin structure are all reminiscent of caveolin features, implying that flotillins may also have a structural function [74]. Flotillins were previously reported to colocalize with caveolae, but recent studies appear to argue against that [41, 110, 111]. Besides the cell surface, flotillins are also found at the recycling endosomes, lysosomes, MVB and even the nucleus [111]. Although flotillins were identified more than 10 years ago, their physiological functions have been under debate.

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A recent study provided some valuable insight into the function of flotillin-1, implicating flotillin-1 as a marker protein for a novel clathrin-and-cavelolae-independent internalization pathway [41]. Flotillin-1-labelled endocytic vesicles were shown to have little colocalization with caveolin- or transferrin-labelled vesicles. Both CTB and the GPI-anchored protein CD59 can be partially internalized *via* this novel pathway, although neither appears to be its specific cargo protein. The depletion of flotillin-1 inhibits the uptake of CTB and CD59 without reducing the internalization of transferrin, suggesting that rather than just being a cargo protein, flotillin-1 may be required for this new pathway to function normally. The overexpression of the dynamin dominant-negative mutant is known to block all of the dynamin-dependent pathways, such as clathrin-dependent pathway and caveolae-dependent pathway, and therefore inhibit the uptake of CTB *via* these pathways. However, flotillin-1 knockdown causes further decrease in the uptake of CTB on top of the effect of the dynamin mutant, implying that the flotillin-1-dependent pathway does not require dynamin activity [41] (however, see below).

A subsequent study further identified proteoglycans as specific cargos for the flotillin-1-dependent pathway [111]. Unlike CTB or CD59, proteoglycan-containing endocytic vesicles predominantly colocalize with flotillin-1, and their internalization is significantly inhibited by siRNA-mediated knockdown of flotillin-1. The preteoglycan-containing vesicles have little colocalization with transferrin, and neither clathrin siRNA treatment nor caveolin siRNA treatment significantly changes the uptake of proteoglycans. Once internalized, preteoglycan-containing vesicles bypass the EEA1-labeled sorting endosomes, and directly merge with Rab9-labelled late endosomes. Interestingly, in contrast with the previous finding that dynamin is not required in the flotillin-1-dependent pathway, the internalization of proteoglycans is apparently a dynamin dependent process [111]. Whether there are more than one flotillin-dependent pathway with different requirements for dynamin remains a question.

Dynamin/RhoA/cortactin-dependent pathway

The interleukin (IL) 2 receptor β subunit is the first receptor protein found to follow a clathrin-and-caveolae-independent internalization pathway [42]. It is concentrated to DRM domains and constitutively internalized *via* a lipid raft-based internalization pathway even without activation. When the ligand IL2 is added, the enrichment of the IL2 receptor at DRM domains and the internalization of ligand-bound IL2 receptor are further increased. The internalization process is not affected by Eps15Δ95/295, a dominant negative Eps15 mutant which specifically blocks the clathrin-dependent pathway, but is dependent upon dynamin, as well as a Rho family small GTPase RhoA. It should be noted that RhoA is involved in many cellular processes, including the clathrin-dependent internalization pathway. However, compared to the clathrin-dependent pathway, the endocytosis of IL2 receptors shows different dependence on RhoA activity: it is not affected by the constitutively active mutant RhoA Q63L, but is inhibited by either the dominant-negative mutant RhoA T19N or RhoGDI, a negative regulator for Rho family GTPases. In contrast, the clathrin-dependent internalization of transferrin receptors is blocked by RhoA Q63L, but not by RhoA T19N or RhoGDI [42].

Subsequent studies have shown that the γc cytokine receptor and the aggregated high-affinity immunoglobulin E (IgE) receptor (FcεRI) are also enriched in lipid rafts and internalized *via* a dynamin-dependent, clathrin-and-caveolae-independent pathway [112, 113]. However, since our knowledge of this novel pathway is still very limited, the internalization mechanism for these newly found cargo proteins is not necessarily the same as the IL2 receptor β subunit. Colocalization studies of these cargo proteins in early endocytic vesicles may help to clarify this question. Additionally, cortactin is found to be required for the endocytosis of both γc cytokine receptor and IL2 receptor β subunit. Cortactin binds to both F-actin and Arp2/3 and functions as an activator of Arp2/3, and is suggested to link cytoskeleton organization with signal transduction [114]. Cortactin has been found at the lamellipodia, invadopodia, as well as the clathrin-dependent pathway and CDR-dependent pathway. This finding may help shed light on the mechanism of F-actin organization during the endocytosis of IL2 receptor β subunit and γc cytokine receptor.

Arf6-dependent pathway

Arf6 is a member of the Arf family small GTPases. In contrast to Arf1, another well-studied Arf family pro-

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tein localized predominantly at the Golgi apparatus, Arf6 usually functions at the interface of the plasma membrane and endosomal structures, and has been implicated in several membrane trafficking events [16, 115]. Besides the afore mentioned function of inducing membrane ruffles and macropinocytosis, it has also been reported to regulate clathrin-dependent internalization [116], exocytosis [115], and as will be discussed below, the clathrin-and-caveolae-independent internalization and recycling [16].

Since its discovery 10 years ago by Donaldson and colleagues, more and more proteins have been added to the cargo protein list of the Arf6-dependent internalization pathway, such as the IL2 receptor α subunit (Tac) [43], E-cadherin [117], β1 integrins [118], M2 muscarinic acetylcholine receptor (M2 mAChR) [119], peripheral myelin-membrane protein (PMP22) [16], metabotropic glutamate receptor 7 (mGluR7) [120] and possibly one TWIK family potassium channel (TWIK1) [121]. At least in HeLa and COS-7 cells, the internalization appears to be independent of clathrin or caveolae, as endocytic vesicles containing these proteins do not colocalize with vesicles decorated with transferrin or caveolin. The uptake of cargo proteins such as GPI-anchored protein CD59 and MHCI seems to occur at DRM domains and require free membrane cholesterol, suggesting it is a lipid raft-based process [122]. A GPI chimera protein, Tac-GPI, was also demonstrated to take the same endocytic pathway as CD59, which implicates the Arf6-dependent pathway as a common pathway for GPI-anchored proteins [122].

As discussed above, the GEEC pathway is also reported to be a major internalization pathway for GPI-anchored proteins, which appears to be Arf6-independent [103]. However, the studies of the GEEC pathway are usually done in CHO cells, instead of HeLa cells in which the Arf6-dependent pathway was previously studied. When comparing the two cell types, it was found they have different trafficking kinetics and extents in the internalization of fluid phase and GPI-anchored proteins. Moreover, when the GEEC pathway is inhibited by aluminum fluoride in CHO cells, GPI-anchored proteins are redirected to the Arf6-dependent internalization pathway, implicating a parallel Arf6-dependent internalization pathway for GPI-anchored proteins in CHO cells [103]. Therefore, it is very likely that certain cargo proteins enter different internalization pathways in different cell types.

Our knowledge about the formation mechanism and machinery proteins of the Arf6-dependent endocytic vesicles is still very limited. A recent study reported that dynamin is not required for the uptake of CD59 and MHCI in HeLa [103], while an earlier study reported the inhibition of E-cadherin internalization in MCF-7 cells by the dynamin dominant negative mutant. Whether the inconsistency results from utilizing different cell types remains to be established.

After internalization, these proteins are first transported to an intracellular Arf6-labeled compartment that is distinct from the classical Rab5-labelled sorting endosome. From there, internalized cargo proteins can be either recycled directly to the cell surface [118] or redirected to the Rab5-positive sorting endosomes [119, 123]. Expression of the GTPase-defective Arf6Q67L mutant blocks the exit of cargo proteins from the Arf6 compartment and results in their intracellular accumulation in Arf6-positive vacuoles [118]. Direct surface recycling occurs *via* an array of tubules extending from the juxtanuclear region to the plasma membrane. Both Arf6 and EHD1, an EH (Eps15 homology) domain-containing protein, are found to be associated with these recycling tubules [124]. Indeed, overexpression of EHD1 induces tubular formation and enhances Arf6-mediated recycling [124]. The Arf6 compartment is coated with F-actin [16], and acute disruption of the actin cytoskeleton inhibits the recycling of cargo proteins and shifts their distribution from plasma membrane to intracellular Arf6-associated tubules [43].

Recently, our group identified the Kir3.4 channel protein to be a cargo protein for the Arf6-dependent pathway [125]. Moreover, we discovered a class of K channel acidic cluster (KAC) trafficking motifs localized at the C-terminus of Kir3.4, which proved to be both necessary and sufficient to direct a plasma membrane protein into the Arf6-dependent pathway. Additionally, the KAC motifs may enhance the association of a membrane protein with the EFA6 family of guanine nucleotide exchange factors of Arf6 [125]. Further studies are needed in order to fully characterize the precise functioning mechanism of the KAC motifs.

Pericentrion, a novel PKC-dependent recycling endosomal structure

As suggested by the CDR-dependent pathway or PKC-activated macropinocytosis, some trafficking mechanisms may only be initiated during special cellular signalling events. The recent studies of a

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novel recycling endosomal structure, the PKC-dependent pericentrion, may represent another such example [18].

The PKC family plays an important role in various cellular processes, such as cell growth, secretion, receptor desensitization and internalization [18, 126]. Based on their dependence on calcium and phorbol esters, PKC isozymes can be categorized into three groups, the classical PKCs (cPKCs), the novel PKCs (nPKCs), and the atypical PKCs (aPKCs). The cPKCs have a C1 domain which binds to phorbol ester, and a C2 domain which binds to calcium; binding of ligands at both C1 and C2 domains are required for the activation of cPKCs.

PKC is an important player in protein trafficking, and one of its key functions is related to receptor down-regulation. In the classical model, PKC activated by the ligand binding of GPCRs not only phosphorylates the downstream effectors to mediate the signalling event, but also phosphorylates the cytoplasmic regions of GPCRs to trigger their subsequent desensitization and/or internalization [127, 128]. In addition to GPCRs, PKC has been shown to be an endocytosis modulator for growth factor receptors, transporters, channels and even viruses [18]. However, in all the above findings, PKC appears to regulate the cargo proteins on an individual basis, which mostly involves the phosphorylation of the cargo protein itself.

Recent studies have implicated a novel mechanism for PKC to regulate protein recycling on a broad scale. Normally inactive PKCs appear diffuse in the cytosol and get recruited to the plasma membrane under activation. Intriguingly, after the activation by a long-term treatment with PMA, PKCα and PKCβII, two members of the cPKC subfamily, have been found to translocate to the plasma membrane as well as a novel perinuclear region, which the authors named as ‘pericentrion’ [129]. The pericentrion represents an endosomal compartment since its formation can be blocked by inhibiting internalization [130]. Moreover, results from utilizing a specific cPKC inhibitor suggest that a sustained cPKC activity is required for the formation of pericentrion [130].

Besides PKCα and PKCβII, several recycling plasma membrane proteins, including those internalized from the clathrin-dependent pathway such as transferrin receptor, as well as from the clathrin-independent pathways such as CD59 and caveolin, can be also found in the pericentrion [130]. However, those plasma membrane proteins and lipids destined for lysosomes (*e.g.* LDL and DiIC16) seem to be excluded from the pericentrion [130]. This observation suggests that cPKC acts at a site downstream of internalization and might have a function in altering the surface expression of a group of proteins normally recycled between the plasma membrane and endosomes. Consistent with this notion, the pericentrion is labelled by Rab11, a marker protein for recycling endosomes [129].

Several interesting questions remain to be addressed. The first regards the role of PKC in the recycling of pericentrion cargo proteins. In principle, the prolonged activation of PKCα and PKCβII may facilitate the pericentrion formation by hijacking cargo proteins from their regular fast-recycling routes and re-direct them to another endosomal compartment with a slower recycling kinetics. Alternatively, the sustained cPKC activity may block the normal recycling from endosomes leading to the pericentrion formation. The second issue is the physiological significance of the pericentrion compartment. In this aspect, sustained activation of cPKC is expected to result in the trapping of a group of receptors, channels, and transporters in the pericentrion and such sequestering mechanism may have important implications in the cellular homeostatis in both physiological and pathological conditions.

Clathrin-independent pathways in neuronal cells

Protein internalization and recycling is particularly important in neuronal cells. At nerve terminals, the exocytosed synaptic vesicles need to be efficiently internalized and recycled to prepare for the next round of neuronal signal transmission in a timely manner; the surface level of receptor and channel proteins also needs to be strictly adjusted in order to accommodate exogenous agonists or antagonists of various nature and intensities, or even to induce long-term changes in synaptic efficacy (*i.e.* synaptic plasticity). One excellent example is the role of AMPA receptor internalization and recycling in LTP and LTD. LTP and LTD are two types of synaptic plasticity which have been commonly accepted as the cellular basis of learning and memory, and thus have been extensively characterized in the past few

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decades [1–4]. In LTP and LTD, a short period of stimulation (with high and low frequency, respectively) to the neuronal cells induces the change (up-regulation and down-regulation, respectively) of synaptic function that lasts for hours or even longer. Numerous studies have demonstrated that the regulated insertion or internalization of AMPA receptors on the cell surface is the underlying molecular mechanism for LTP and LTD [2]. AMPA receptors are internalized *via* the clathrin-dependent pathway, and disruption of their internalization abolishes the LTD [131, 132]. Similarly, inhibition of AMPA receptor trafficking to the plasma membrane drastically diminishes the LTP [133].

Although the characterization of clathrin-independent internalization and recycling pathways in neuronal cells is still in its infancy, their prominent roles have already been implicated in multiple studies. There are two mechanisms for recycling synaptic vesicles in retinal bipolar cells, the fast endocytosis mechanism and the slow endocytosis mechanism. The clathrin-independent pathway proved to be responsible for the fast mechanism, although its precise pathway has not been elucidated [134]. Glutamate, the major excitatory neurotransmitter in the mammalian central nervous system, is the ligand for two classes of glutamate receptors: ionotropic receptors and metabotropic receptors (mGluRs). So far two members of mGluRs, mGluR5 and mGluR7, have been shown to enter the clathrin-independent internalization pathway [120, 135]. Moreover, it was demonstrated that when overexpressed in HeLa cells, mGluR7 is internalized *via* the Arf6-dependent pathway. Interestingly, the M2 muscarinic acetylcholine receptor was also reported to go through the Arf6-dependent pathway, although similar to mGluR7, it was also only tested in HeLa cells [119]. A recent study by our group confirmed the existence of Arf6-positive endosomal compartments in hippocampal neurons, and also showed that exogenously expressed Kir3.4 channel protein is accumulated in such compartments as observed in HeLa and COS-7 cells, which suggests the Arf6-positive compartments might function in a similar way in neurons as in cultured cell lines [125]. There are still plenty of neuronal receptor and channel proteins whose trafficking routes have not been explored yet, and it is likely that clathrin-independent pathways participate in the modulation of their surface expression. Defects in clathrin-independent pathways have been linked to neuronal disease. The mutant Huntington's disease protein (mhtt) was found to specifically inhibit the caveolae-dependent pathway in primary striatal neurons and cause accumulation of intracellular cholesterol, which is likely to be relevant to the pathophysiology of Huntington's disease [136].

Summary

Although the studies of clathrin-independent pathways have been burgeoning for the past few years, our knowledge about several subjects is still far from comprehensive, which significantly impedes the course of systematically characterizing every pathway and understanding the coordinated utilization of these pathways together with the clathrin-dependent pathway.

First, the trafficking motifs for most of the clathrin-independent pathways have not been identified yet, and the mechanism of cargo protein recruitment still remains elusive. As discussed above, in the clathrin-dependent pathway, there are several extensively-studied trafficking motifs that mediate the interaction of cargo proteins with adaptor proteins, and therefore are both necessary and sufficient for the enrichment of cargo proteins in clathrin-coated pits. If we identified the trafficking motifs for a clathrin-independent pathway, we could not only predict whether a cargo protein will take that internalization pathway by examining its sequence for the existence of the potential trafficking motifs, but also fuse those motifs to marker proteins to study the itinerary of that pathway. Additionally, by searching the interacting partners of the trafficking motifs, we may be able to identify the machinery proteins involved in the recognition and recruitment of the cargo proteins. The GPI moiety of GPI-anchored proteins may function as a trafficking motif for the GEEC pathway, although the fact that some GPI-anchored proteins are also internalized in other pathways adds to the complications [12]. Using Kir3.4 channel protein as a model, our group recently proposed a class of KAC motifs to be candidate trafficking motifs for the Arf6-dependent internalization and recycling pathway [125]. More studies on the trafficking motifs will help bring about valuable information on the various clathrin-independent pathways.

Second, the formation mechanism and participating machinery proteins of the initial endocytic vesicles have not been well established in most cases.

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The lack of such knowledge represents a major obstacle in studying the kinetics and regulatory mechanism of the internalization process. The study on CLICs made a successful start by identifying the very first carriers in the GEEC pathway [101], which opens the door for further characterization of the molecular machinery responsible for the formation of these carriers at the plasma membrane. Another intriguing future direction would be to elucidate the nature of initial carriers involved in other clathrin-and-caveolae-independent internalization pathways. Furthermore, knowing the precise functions and specificity of machinery proteins would allow us to devise experimental methods that specifically block one internalization pathway without affecting the others, which would be an important tool for addressing questions such as the significance of a specific pathway, and whether a certain cargo protein is exclusively internalized *via* that pathway. Among all the clathrin-and-caveolae-independent pathways, only the flotillin-1-mediated pathway may be specifically blocked, as the depletion of flotillin-1 abolishes flotillin-1-dependent internalization without affecting clathrin- or caveolae-dependent internalization. However, as discussed above, two independent studies have reported different requirements of dynamin in flotillin-1-dependent internalization, and it is possible that there are more than one flotillin-1-dependent pathways. Recently, several more machinery proteins involved in clathrin-independent pathway have been identified. As discussed above, cortactin has been found in several internalization pathways; CtBP-BARS protein has been implicated in a dynamin-independent pathway, presumably functioning as a substitute for dynamin [137]. Although once thought to specifically function in clathrin-dependent pathway, Eps15 and epsin have been reported to also function in both caveolae-dependent and clathrin-and-caveolae-independent pathway [109, 138]. Considering that the machinery proteins participating in the newly identified internalization pathways are still poorly understood, and that many pathways may still remain undefined, it is not surprising if more proteins turn out to be shared by more than one pathway. Accordingly, the experimental methods we utilize to inhibit one trafficking pathway may have broader specificity than we expect, and thus need to be analysed with caution.

Finally, most studies on protein trafficking have been done in cultured cell lines, such as HeLa, CHO and COS-7 cells. These cell lines have served as excellent model systems, and many insights have been generated from them. However, a protein may be controlled by different trafficking mechanisms when examined in different types of cells. For example, as discussed above, the trafficking routes for GPI-anchored proteins might differ between HeLa cells and CHO cells [103]. The internalization of mGluR5 is dependent upon dynamin 2 when examined in hippocampal neurons, but fails to show the same dependence when examined in COS-7 cells [135]. Furthermore, some important physiological phenomena may not be detectable in cultured cells. For example, LTP has been reproduced in hippocampal slices, but there was little success in generating LTP in dissociated neurons [2]. Since our ultimate goal is always to understand what is happening in living organisms, it is highly desirable to apply the knowledge learned from model cell lines to the native systems, such as endogenous cell types, native tissue slice, or even the whole living organism. The recent advancement of various experimental methods, such as brain slice [139], two-photon imaging [140] and knockout animal preparation [141], is expected to facilitate the future progress in this area.

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