Molecular mechanisms of clathrin-independent endocytosis

Carsten G. Hansen and Benjamin J. Nichols*

MRC-LMB, Hills Road, Cambridge, CB2 0QH, UK

*Author for correspondence (e-mail: ben@mrc-lmb.cam.ac.uk)

Journal of Cell Science 122, 1713-1721 Published by The Company of Biologists 2009
doi:10.1242/jcs.033951

Summary

There is good evidence that, in addition to the canonical clathrin-associated endocytic machinery, mammalian cells possess multiple sets of proteins that are capable of mediating the formation of endocytic vesicles. The identity, mechanistic properties and function of these clathrin-independent endocytic pathways are currently under investigation. This Commentary briefly recounts how the field of clathrin-independent endocytosis has developed to date. It then highlights recent progress in identifying key proteins that might define alternative types of endocytosis. These proteins include CtBP (also known as BARS), flotillins (also known as reggies) and GRAF1. We argue that a combination of information about pathway-specific proteins and the ultrastructure of endocytic invaginations provides a means of beginning to classify endocytic pathways.

Key words: CTBP, Caveolin, Clathrin, Dynamin, Endocytosis, Flotillin

Introduction

Endocytosis – the internalization of components of the plasma membrane, associated ligands and fluid – is a fundamental process in eukaryotic cells. As such, it has a key role in many different areas of cell biology, ranging from the uptake of nutrients to regulation of intercellular signaling.

Historically, the chief means of classifying endocytic pathways has been to consider them as either clathrin-dependent or clathrin-independent. The network of protein-protein interactions that is responsible for endocytosis in clathrin-coated pits is being understood in ever-increasing detail (Schmid and McMahon, 2007). Key players in this process include adaptor proteins and the large GTPase dynamin, which is thought to be directly involved in pinching off endocytic vesicles from the plasma membrane (Bashkirov et al., 2008; Pucadyil and Schmid, 2008; Roux and Antonny, 2008). The interactions between clathrin, adaptor proteins and endocytic cargo proteins are being analyzed at the level of protein structures (Edeling et al., 2006; Kelly et al., 2008; Pryor et al., 2008), and the dynamics of the recruitment of these components to forming coated pits are beginning to be understood (Ehrlich et al., 2004; Merrifield et al., 2005). Other forms of endocytosis, including macropinocytosis, uptake in CLIC/GEEC (clathrin-independent carriers/GPI-enriched early endosomal compartments) structures, and caveolar endocytosis remain much less well-characterized (Conner and Schmid, 2003; Mayor and Pagano, 2007; Sandvig et al., 2008; Stagg et al., 2007).

In this Commentary, we first provide a very brief account of the history of the field of clathrin-independent endocytosis in mammalian cells. We go on to discuss different approaches to defining or classifying endocytic pathways, before presenting recently published data that suggest that the defining protein components of these pathways are now being identified. We conclude by highlighting still largely unanswered questions about the physiological functions of clathrin-independent endocytosis.

A history of clathrin-independent endocytosis

It is useful to outline how the field of clathrin-independent endocytosis has evolved, as this sets in context current experimental approaches and problems. Up until the early 1990s, there was controversy as to whether clathrin-independent endocytosis existed at all. Pioneering studies on cellular uptake of bacterial toxins showed that pharmacological perturbations that were thought to interfere with the formation of clathrin-coated pits did not block all endocytosis (Sandvig et al., 2008; Sandvig and van Deurs, 1990); however, quantitative approaches that measured plasma-membrane uptake suggested that clathrin-coated-pit activity could account for all detectable membrane uptake (Doxsey et al., 1987; Watts and Marsh, 1992). The transition away from pharmacological perturbations to genetic tools, which were derived from knowledge about the proteins required for clathrin-coated-pit formation, resolved this issue. In 1995, it was shown that the expression of a dominant-negative form of the GTPase dynamin, which was known to be required for budding of clathrin-coated pits (Chen et al., 1991; van der Bliek and Meyerowitz, 1991; van der Bliek et al., 1993), did not block the internalization of fluid (Damke et al., 1995). Subsequently, the use of dominant-negative mutants of other clathrin-coated-pit proteins, such as epsinR (Chen et al., 1998), eps15 (Benmerah et al., 1998) and AP180 (Ford et al., 2001), revealed that the endocytosis of various ligands and plasma-membrane components does not depend on the canonical clathrin-associated machinery (Lamaze et al., 2001; Nichols et al., 2001; Puri et al., 2001; Sabharanjak et al., 2002). By the late 1990s, the idea of clathrin-independent endocytosis was well established, prompting further efforts to define the relevant mechanisms.

The first structures to be considered as mediators of clathrin-independent endocytosis were caveolae, which are invaginations of the plasma membrane with a defined and characteristic flask-shaped morphology (Stan, 2002). The first molecular component of these structures to be identified was caveolin 1, which was also called VIP26 in early papers (Rothberg et al., 1992). One significant advance linking caveolae to endocytosis was the finding that

dynamin is recruited to caveolae as well as to clathrin-coated pits (Henley et al., 1998; Oh et al., 1998). As dynamin is involved in vesicle budding, this suggested that caveolae can bud from the plasma membrane. Recent identification of further caveolar components is discussed later in this Commentary.

Several observations argued against simply equating clathrin-independent endocytosis with budding of caveolae. Not all endocytosis is blocked by the dynamin-2 dominant-negative mutant (which blocks clathrin-dependent and caveolar endocytosis) (Damke et al., 1995), and early endocytic compartments that arise without clathrin-coated pits and are devoid of caveolin can be identified (Sabharanjak et al., 2002). Photobleaching studies revealed that caveolin 1, and hence most caveolae, is strikingly immobile in the plasma membrane of common tissue-culture cells and does not bud into the cell frequently enough to account for estimated levels of clathrin-independent endocytosis (Thomsen et al., 2002). These observations have led to the use of the designation 'clathrin- and caveolin-independent endocytosis' for further endocytic mechanisms. This is clearly an unsatisfactory negative definition but, until very recently, progress in better-defining endocytic pathways has been hampered by a number of factors, as discussed below.

Criteria for defining endocytic pathways

The number and molecular identity of clathrin- and caveolin-independent endocytic mechanisms is still not completely resolved. A number of tools and approaches for defining different endocytic pathways have been used, and these are discussed critically below. Different types of endocytosis can also be defined by following the endocytosis of different viruses. This large field has been covered in recent reviews (Marsh and Helenius, 2006; Rojek and Kunz, 2008; Smith and Helenius, 2004), and is not covered in detail here.

Morphology

The ultrastructural morphology of nascent endocytic intermediates at the plasma membrane provides the simplest way of defining endocytic pathways. Four different types of structure have been reported: clathrin-coated pits, flask-shaped structures without an electron-dense coat (which can be defined morphologically as caveolae), more polymorphous or tubular membrane invaginations and vesicles, and larger macropinocytic vesicles (Fig. 1). These observations provide the basis for the classification of endocytic pathways that is used in this Commentary. Invaginations in the plasma membrane do not, however, always represent forming endocytic intermediates, and a purely ultrastructural classification does not necessarily reflect specific molecular mechanisms. Also, structures that appear as elongated or 'tubular' by light microscopy may well not be uniform tubes when visualized at the ultrastructural level – the image shown in Fig. 1 of an invagination involved in the internalization of Shiga toxin exemplifies this point, as discussed below (Romer et al., 2007). Ultrastructural data therefore need to be combined with other approaches, most obviously the identification of pathway-specific and functionally relevant proteins. Several recent papers provide evidence that, in the case of both endocytic tubes and caveolae, alternate sets of protein machinery can lead to the formation of morphologically similar structures (Frick et al., 2007; Lundmark et al., 2008; Romer et al., 2007; Zhao et al., 2002). This is discussed in detail below.

Pathway-specific cargos

Clathrin-coated pits are extremely efficient sorting machines, and can drive the concentration of membrane receptors (for example, the transferrin receptor) in the forming vesicle to at least an order of magnitude higher than that in the rest of the plasma membrane (Hansen et al., 1992; Merrifield et al., 2005). For this reason, it makes good sense to use uptake of transferrin as a read-out of the activity of clathrin-coated pits. Unfortunately, it is not yet clear whether there is similarly efficient sorting during other types of endocytosis.

Some of the best evidence for clathrin- and caveolin-independent endocytosis comes from studies on glycosylphosphatidylinositol (GPI)-anchored proteins (Mayor and Riezman, 2004; Nichols et al.,

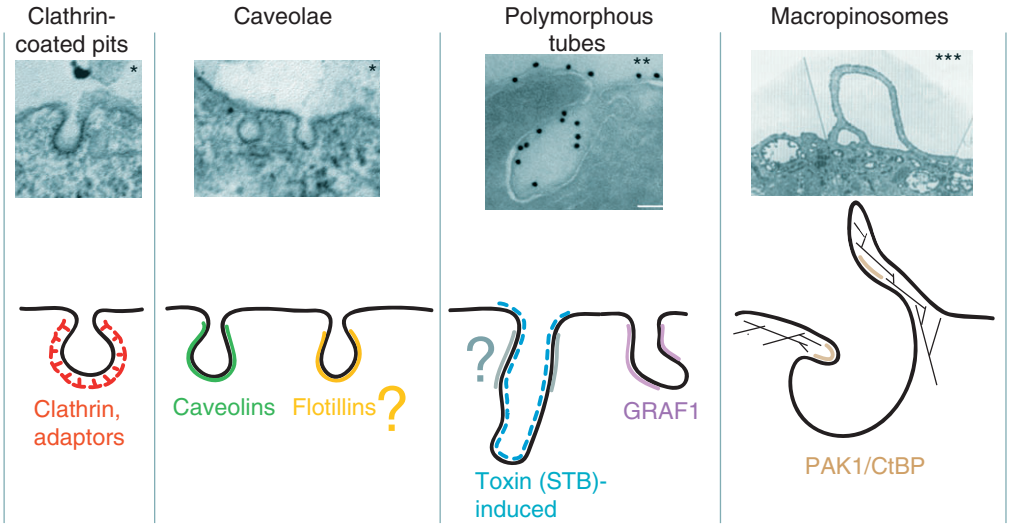

Fig. 1. Molecular mechanisms for endocytosis. Endocytic pathways can be classified by the ultrastructure of the relevant membrane-transport intermediates as they form at the plasma membrane, coupled with currently emerging information about the molecules that are directly involved in the biogenesis of these intermediates. The electron micrographs shown are our unpublished images (*), or are reproduced from Romer et al. (Romer et al., 2007) (**) and Swanson and Watts (Swanson and Watts, 1995) (***), with permission. Note that the micrograph showing a putative macropinosome is not at the same scale as the others – caveolae are around 60 nm across, the coated pit shown is about 80 nm across, and macropinosomes can be more than 5 μm in diameter.

2001; Sabharanjak et al., 2002), and the literature on endocytic sorting of these proteins exemplifies the difficulties in defining clathrin-independent endocytic pathways in terms of specific cargos. In general, GPI-linked proteins are taken up slowly, with a half-time in the order of minutes to hours (Mayor and Riezman, 2004). Their uptake is not blocked by perturbations that disrupt clathrin-coated-pit formation, and they can be detected in a population of early endosomal organelles referred to as GPI-enriched early endosomal compartments, or GEECs (Sabharanjak et al., 2002). Importantly, when the extracellular domain of a specific GPI-linked protein was transferred to a heterologous transmembrane domain, it was excluded from the GEECs – hence the designation GEEC (Sabharanjak et al., 2002). It is not clear, however, that GPI-linked proteins are found at a much higher density per unit area of membrane in GEECs than in the plasma membrane, and careful analysis of the nanoscale organization of GPI-linked proteins did not reveal clustering or concentration in structures more than a few nm across (Sharma et al., 2004b). Indeed, there is some consensus that GPI-linked proteins have a more-or-less uniform distribution in the plasma membrane at the level of resolution of the light microscope (Glebov and Nichols, 2004; Kenworthy et al., 2004; Munro, 2003) and, despite intensive investigation, they have yet to be directly visualized being concentrated in nascent endocytic structures. It is thus not known what factors predominate during sorting of GPI-linked proteins, and exclusion from coated pits might plausibly have as significant a role as concentration in other types of endocytic carrier (Nichols, 2003a; Nichols et al., 2001). In light of all this, it is likely that GPI-linked proteins can enter the cell via multiple pathways.

Another class of widely used marker for clathrin-independent endocytosis is glycosphingolipids, uptake of which is tracked either by the addition of exogenous fluorescent glycosphingolipid analogs or glycosphingolipid-binding toxins, the most widely used being cholera toxin. Here again, there is good evidence for uptake through multiple mechanisms, and a substantial fraction of internalized glycosphingolipid can, at least in some cell types, enter the cell via clathrin-coated pits (Cheng et al., 2006; Puri et al., 2001). In some cells, cholera toxin is concentrated in caveolae (Parton, 1994; Pelkmans and Zerial, 2005), but in others it appears not to be (Nichols, 2003b). In short, attempts to classify different types of clathrin-independent endocytosis using specific cargos have yet to bear fruit. As more is revealed about the mechanisms of alternate endocytic pathways, this might change.

Pathway-specific inhibitors

Differential susceptibility to inhibitors provides another way of discriminating between the activities of different sets of endocytic machinery. Earlier experiments using treatments that block endosomal acidification or alter the pH of the cytoplasm to perturb coated-pit function provided the first evidence for clathrin-independent endocytosis (Moya et al., 1985; Sandvig and van Deurs, 1990). Several reports highlight that clathrin-independent endocytosis is susceptible to depletion of cholesterol from the plasma membrane (Nichols, 2003a). However, these experiments need to be carefully controlled because, if enough cholesterol is removed, effects on permeability of the membrane and its association with the cytoskeleton lead to disruption of multiple cell functions, including the formation of clathrin-coated pits themselves (Hao et al., 2001; Kwik et al., 2003; Rodal et al., 1999).

Clathrin-independent endocytic pathways can be classified as dynamin-dependent or dynamin-independent. It has been known for over 10 years that endocytosis persists in cells overexpressing a GTPase-inactive form of dynamin 2, and data from fly cells, in which genetic tools are available to acutely perturb dynamin function, are consistent with the idea that not all endocytosis requires dynamin (Guha et al., 2003). In mammals, there are three dynamin-encoding genes, which have different patterns of expression (McNiven et al., 2000), and each can be alternatively spliced, raising the possibility that different splice variants are involved in different types of endocytosis (McNiven et al., 2000). Evidence in support of this suggestion comes from experiments in which different splice variants of dynamin 2 are added back to cells that have been treated with dynamin-2-depleting siRNAs (Cao et al., 2007). The recent development of a small-molecule inhibitor of dynamin GTPases will allow further experiments that should better define dynamin-independent endocytosis (Macia et al., 2006).

Dominant-negative mutants of small Ras-superfamily GTPases (which are locked in GDP-bound forms) have differential effects on clathrin-independent endocytosis, and so these experiments provide evidence for multiple clathrin- and caveolin-independent pathways (Mayor and Pagano, 2007). Endocytosis is perturbed by the overexpression of dominant-negative Arf1, Cdc42, Arf6 and Rac1, and these mutant proteins provide a useful means of differentiating between different endocytic mechanisms under specific conditions of cell type and GTPase expression level (Kumari and Mayor, 2008; Lamaze et al., 2001; Naslavsky et al., 2004; Sabharanjak et al., 2002). However, extrapolation to more general conclusions about the specific involvement of any of these GTPases in just one type of endocytosis is hampered by reports of different effects in different experiments – for example, overexpression of mutants of Arf6 blocks both uptake via clathrin-coated pits and a clathrin-independent endocytic pathway (D'Souza-Schorey et al., 1995; Naslavsky et al., 2004; Palacios et al., 2002), and overexpression of mutant Cdc42 blocks clathrin-independent uptake of GPI-linked proteins under some conditions (Sabharanjak et al., 2002) but can also perturb clathrin-mediated uptake of E-cadherin (Izumi et al., 2004) and macropinocytosis (Amstutz et al., 2008; Dharmawardhane et al., 1997; Garrett et al., 2000). The identification of effectors downstream of small GTPases that regulate different types of endocytosis, and an understanding of how such effectors mediate vesicle formation, would clearly be a major step forward.

Molecular mechanisms for different endocytic pathways

Although ultrastructural evidence, analysis of uptake of different cargos and use of inhibitors all make useful contributions, ultimately a full understanding of the mechanism of different types of endocytosis requires information on the key molecules that interact with the plasma membrane at the 'business end' of the process of vesicle formation. Several papers published in the last 2 years suggest that such information is starting to be available (Amstutz et al., 2008; Frick et al., 2007; Karjalainen et al., 2008; Liberali et al., 2008; Lundmark et al., 2008; Romer et al., 2007). From these studies (coupled with ultrastructural information), a classification of endocytic pathways on the basis of functionally important proteins that localize to forming transport intermediates at the plasma membrane can be derived. This is presented in Fig. 1.

Macropinocytosis

Macropinocytosis refers to the generation of large endocytic vesicles (up to 5 μm in diameter), which is associated with the formation

of actin-dependent membrane ruffles (Swanson and Watts, 1995). It is not clear whether this morphological definition represents just one molecular mechanism for the key step of scission of the vesicle from the plasma membrane. The absence of a defined molecular mechanism for macropinocytosis has, moreover, meant that it is not known whether smaller endocytic vesicles (less than around 500 nm in diameter) are generated in essentially the same way as macropinosomes. Recent studies, some of which are discussed in the following paragraph, are beginning to provide tools to address these issues.

C-terminal binding protein 1 [CtBP1; also called brefeldin A-dependent ADP-ribosylation substrate (BARS) (Corda et al., 2006; Weigert et al., 1999)] is a transcriptional co-repressor (Chinnadurai, 2002), and is also thought to function during membrane fission (Bonazzi et al., 2005; Colanzi et al., 2007; Hidalgo Carcedo et al., 2004; Weigert et al., 1999; Yang et al., 2005). It was originally proposed that CtBP1 mediates fission through the acylation of lysophosphatidic acid and the associated alterations in membrane curvature (Weigert et al., 1999), but further experiments revealed that CtBP1 is, in fact, unlikely to have acyltransferase activity (Gallop et al., 2005). Recently, a role for CtBP1 in macropinocytosis has been demonstrated (Amstutz et al., 2008; Liberali et al., 2008). CtBP1 is recruited to macropinosomes that are induced by high concentrations of epidermal growth factor (EGF), and loss of CtBP1 function reduces the frequency of formation of these structures (Liberali et al., 2008). In addition, CtBP1 is required for the endocytosis of the human adenovirus in macropinocytic structures (Amstutz et al., 2008). The precise role of CtBP1 in these events is unclear, but it is a substrate for the p21-activated kinase PAK1 (Barnes et al., 2003; Bokoch, 2003; Liberali et al., 2008). PAK1 is involved in the regulation of cytoskeletal dynamics, was previously shown to be present on macropinosomes and is activated by small GTPases, including Cdc42 and Rac1 (Dharmawardhane et al., 1997; Zhang et al., 1995). Overexpression of mutants of CtBP1 that lack PAK1 phosphorylation sites blocks macropinocytosis (Liberali et al., 2008), and PAK1 itself is required for the uptake of the picornavirus echovirus 1 (Karjalainen et al., 2008). A separate study on the mechanism of cell entry for another virus, vaccinia, confirms that activation of PAK1 is associated with the formation of macropinosome-like structures (Mercer and Helenius, 2008). Putting these data together, it now makes sense to talk of a PAK1- and CtBP1-dependent mechanism for macropinocytosis (Fig. 1). Further experiments will be required to elucidate precisely what CtBP1 does during the formation of macropinosomes.

Caveolin-1-positive caveolae

Caveolae have been recognized as abundant and morphologically characteristic structures at the surface of many mammalian cell types since the early days of the application of electron microscopy to cell biology. The protein caveolin 1 is an abundant component of caveolae (Rothberg et al., 1992). Progress on the mechanism by which caveolae bud from the plasma membrane has been made using simian virus 40 (SV40) as a marker because, at least in cells in which caveolin 1 is present, the virus is internalized in caveolin-1-positive vesicles (Pelkmans et al., 2001; Pelkmans et al., 2002). These and other studies using fluorescent lipid analogs (Cheng et al., 2006; Sharma et al., 2004a) show that caveolar budding is a regulated process that requires Src-family kinases, dynamin and local actin polymerization (Sverdlov et al., 2007).

The issue of whether caveolin proteins are directly required for endocytosis of any specific cargo remains, to some extent, open. SV40 clearly colocalizes with caveolin 1 when it is internalized into common tissue-culture cell lines (Pelkmans et al., 2001) yet, when uptake of the virus was followed in cells from caveolin-1-knockout mice, a surprising increase in the rate of SV40 internalization was observed (Damm et al., 2005). These data, coupled with experiments showing that expression levels of caveolin 1 actually show a negative correlation with rates of internalization of putative caveolar ligands such as autocrine motility factor (Le et al., 2002), have led to the suggestion that caveolins actually serve to stabilize particular ligands and membrane components at the plasma membrane (Lajoie and Nabi, 2007). By contrast, innovative in vivo imaging approaches imply that caveolins have a key and highly active role in transcytosis (transport across a cell via endocytosis at one side and exocytosis at the other) of specific membrane proteins and ligands in endothelial cells (Oh et al., 2007). Caveolar budding is likely to be a highly regulated process, and so whether recruitment to caveolae serves to stabilize ligands at the plasma membrane or leads to their rapid uptake might well depend on cellular context. In addition, there is always the possibility that signaling pathways that are responsible for controlling caveolar budding are in some way misregulated in cultured cell lines.

The defined shape and size of caveolae suggests a well-controlled and precise molecular architecture (Stan, 2002). Until recently, however, caveolin proteins were the only candidates for structural components of caveolae. Independent biochemical purifications have identified the protein polymerase 1 transcript release factor (PTRF) as an additional caveolar component (Aboulaich et al., 2004; Vinten et al., 2005). A reduction in cellular levels of PTRF expression causes loss of morphologically defined caveolae and increased turnover of caveolin 1 (Hill et al., 2008; Liu and Pilch, 2008), and PTRF-knockout mice lack caveolae (Liu et al., 2008). PTRF colocalizes with caveolin 1 in the plasma membrane, but not with the biosynthetic pool of caveolin 1 in the Golgi complex (Hill et al., 2008), leading to the suggestion that the recruitment of PTRF to caveolin-1 microdomains or oligomers is important for the transition of these regions of the membrane from being flat to adopting the characteristic caveolar morphology. RNAi-mediated knockdown of PTRF causes the same phenotypes as caveolin-1 knockdown in zebrafish, and phenotypes of PTRF-knockout mice are similar to those of mice lacking caveolins, so PTRF is likely to be required for caveolar function as well as morphology (Hill et al., 2008; Liu et al., 2008).

Several intriguing questions are raised by these studies on PTRF. A truncation mutant of PTRF colocalizes well with microtubules (Liu and Pilch, 2008), implying that the protein has a microtubule-binding site and hinting at a link between caveolae and the microtubule cytoskeleton. There are four homologs of PTRF in mammals (see www.treefam.org, accession TF331031), and two of them [serum deprivation-response protein (SDPR) and SDPR-related gene product that binds to C-kinase (SRBC)] are also present in caveolae and are important for the recruitment of protein-kinase-C isoforms to these structures (Gustincich and Schneider, 1993; Gustincich et al., 1999; Mineo et al., 1998). The fourth homolog, muscle-restricted coiled-coil protein (MURC), is expressed only in muscle and has a similar subcellular distribution to caveolin 3, the muscle-specific isoform of caveolin (Ogata et al., 2008; Tagawa et al., 2008). It seems probable, then, that all four homologs associate with caveolae and caveolins, and participate in their function.

Caveolae without caveolins – flotillin-dependent endocytosis?

Of the three caveolin proteins in mammals, caveolin 1 and caveolin 2 are widely expressed and hetero-oligomerize with each other. Caveolin 3 is only expressed in muscle. Knockout of the gene for caveolin 1 in mice causes loss of most caveolin-2 expression, and loss of most morphologically defined caveolae (Drab et al., 2001; Razani et al., 2001; Zhao et al., 2002). Careful examination of endothelial cells in these mice, however, reveals residual caveolar structures, albeit with a slightly increased size (Zhao et al., 2002) (and see Fig. 2). Therefore, there is either an alternative non-caveolin-dependent mechanism for making caveolae, or caveolin 2 and caveolin 3 can somehow cause the formation of caveolae in these cells. Recent data from our laboratory favor the former explanation. The concomitant overexpression in HeLa cells of flotillin 1 and flotillin 2 [also called reggie 2 and reggie 1, respectively (Lang et al., 1998)], two proteins with a similar topology to caveolin 1, generates structures that look like caveolae (Frick et al., 2007; Glebov et al., 2006). Additionally, endocytosis of the GPI-linked protein Cripto (also known as CRGF), in complex with its ligand Nodal, takes place in flotillin-positive microdomains that look like caveolae by electron microscopy (Blanchet et al., 2008). The interpretation of these results is, however, not completely clear-cut, as a separate investigation did not find an increase in the number of caveolar structures in embryonic fibroblasts from caveolin-1-knockout mice upon expression of flotillin 1 and flotillin 2 (Kirkham et al., 2008).

Several observations suggest that flotillin 1 and flotillin 2 define a specific endocytic pathway. Co-assembly of both flotillins causes the formation of microdomains in the plasma membrane that appear (in time-lapse images of live cells) to bud into the cell, and loss of flotillin-1 expression causes a reduction in the rate of uptake of the GPI-linked protein CD59 (Frick et al., 2007; Glebov et al., 2006). Flotillin-positive microdomains and caveolin-positive caveolae share the ability to recognize and interact with antibody-induced clusters of GPI-linked proteins in the plasma membrane, hinting at some functional overlap between these structures (Stuermer et al., 2001), yet there is little or no colocalization between flotillins and caveolins in the plasma membrane (Fig. 3). It should be stressed that, although there is some consensus that flotillins define specific plasma-membrane microdomains, their dynamics and their contribution to total uptake of GPI-linked proteins are still under active investigation and debate (Langhorst et al., 2008).

The rate at which flotillin microdomains bud into the cell is, at least in common tissue-culture cells, likely to be much lower than the rate of budding of clathrin-coated pits (Glebov et al., 2006). This implies that budding of flotillin microdomains, similar to that of caveolin-containing caveolae, might be a highly regulated process, and that flotillin- or caveolin-positive vesicles are not likely to be abundant in the cytoplasm (Bauer and Pelkmans, 2006; Thomsen et al., 2002). Flotillins, similar to caveolins, have been reported to bind to Src-family kinases and, in both cases, mutation of specific tyrosine residues alters the subcellular distribution of these proteins (Neumann-Giesen et al., 2007; Stuermer et al., 2001; Sverdlov et al., 2007). It has recently been shown that both flotillin 1 and flotillin 2 are endocytosed in response to the expression of active Fyn kinase, and that mutation of the relevant tyrosine residues within these proteins results in the formation of flotillin microdomains that are not fully competent for internalization (Riento et al., 2009). Thus, budding of both flotillins and caveolins is likely to be regulated by Src-family kinases. Understanding of the signaling events that act upstream of these kinases in vivo remains limited.

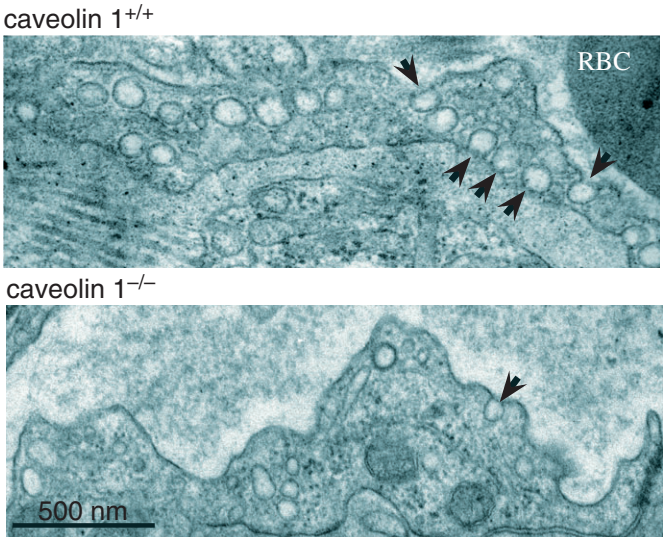

Fig. 2. Endothelial caveolae in wild-type and caveolin-1$^{-/-}$ mice. Endothelia that line blood vessels [containing red blood cells (RBCs)] were identified in sections of mouse lung studied by transmission electron microscopy. Caveolar structures that are open at the plasma membrane are indicated by arrows in the top panel, and a clathrin-coated pit is also visible in the caveolin-1$^{-/-}$ sample (arrow). These are unpublished data from our laboratory. A more detailed description of endothelial caveolae in caveolin-1$^{-/-}$ mice is given by Zhao et al. (Zhao et al., 2002).

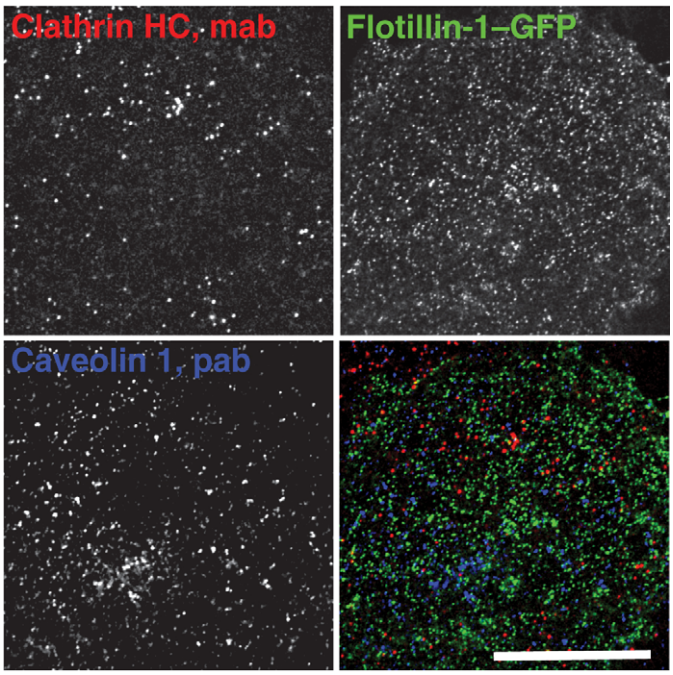

Fig. 3. Flotillins, caveolin 1 and clathrin all define different endocytic domains within the plasma membrane. A COS-7 cell expressing flotillin-1–GFP was fixed and stained with a monoclonal antibody (mab) against clathrin heavy chain (HC) and a polyclonal antibody (pab) against caveolin 1. Reproduced from Glebov et al. (Glebov et al., 2006), with permission. Scale bar: 20 nm.

Tubes and other structures

Ultrastructural data show that glycosphingolipids and other plasma-membrane components can be endocytosed in elongated, polymorphous structures that can be described as approximately tubular (Kirkham et al., 2005; Sabharanjak et al., 2002). Experiments showing that crosslinking of glycosphingolipids by bacterial toxins can induce tubulation (Romer et al., 2007), when coupled with previous observations of tubes involved in the uptake of GPI-linked proteins in the absence of crosslinking (Sabharanjak et al., 2002), imply that there might be more than one mechanism that underpins the formation of endocytic tubes.

The glycosphingolipid-binding B-subunit of Shiga toxin (STB) can be internalized through multiple pathways, and there is evidence that it up-regulates its own uptake in clathrin-coated pits (Lauvrak et al., 2006). Other data reveal that, in energy-depleted cells, STB induces pronounced tubulation of the plasma membrane (Romer et al., 2007). STB is a pentamer, and each subunit of the pentamer can bind to three molecules of a specific glycosphingolipid, Gb3 (Bast et al., 1999; Kitova et al., 2005). Thus, clustering of up to 15 copies of the glycosphingolipid appears to lead to the formation of membrane tubes through an unknown mechanism. These tubes do not colocalize with clathrin, and so might represent endocytic intermediates involved in the clathrin-independent internalization of STB that are unable to achieve scission from the plasma membrane in the absence of cellular energy sources such as ATP or GTP. The fact that STB also causes tubular deformations in liposomes (Romer et al., 2007) suggests that the reorganization of lipids alone is sufficient for this effect, although it remains possible that additional factors are recruited to, and help to form and stabilize, the tubes in cells. Dynamin is recruited to the STB tubes in energy-depleted cells, and it is therefore probable that dynamin would be involved in scission of these tubes in unperturbed cells, if indeed they form even without energy depletion. This implies a mechanism for the recognition of the tubes by appropriate cellular machinery. Whether a similar effect to that exerted by STB is seen with physiological rather than pathological ligands, and whether STB induces membrane tubulation in non-energy-depleted cells, remains to be ascertained, but the finding that the clustering of lipids is sufficient to induce morphological changes in the plasma membrane suggests a paradigm for the generation of endocytic transport intermediates that differs fundamentally from those offered by coated pits and caveolar structures.

Separate experiments imply the presence of an additional mechanism for the generation of endocytic tubes. Both glycosphingolipids and GPI-linked proteins have been observed to be internalized in tubular intermediates, termed CLICs for clathrin-independent carriers (Kirkham et al., 2005; Sabharanjak et al., 2002). These intermediates are clearly formed even without crosslinking of glycosphingolipids and, at least as determined by overexpression of dominant-negative GTPase-inactive dynamin mutants, do not require dynamin activity for scission from the plasma membrane (Sabharanjak et al., 2002). CLIC formation is regulated by small GTPases, including Cdc42 and Arf1 (Kumari and Mayor, 2008). Identification of the effectors downstream of these GTPases, and elucidation of how such effectors bend the plasma membrane into tubules and then pinch off these tubules from the cell surface, will be a major step forward.

Recently, the first specific protein component of CLIC-like transport intermediates was identified – GRAF1, which provides a marker for tubular structures involved in uptake of glycosphingolipids, GPI-linked proteins and bulk fluid. A reduction of GRAF1 expression causes a marked decrease in uptake of these markers (Lundmark et al., 2008). GRAF1 contains distinct domains that suggest a direct role in membrane deformation and scission – a BAR domain (associated with the induction or sensing of membrane curvature), a pleckstrin homology (PH) domain {responsible for recruitment to the plasma membrane via binding to phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2]} and an SH3 domain [which has been shown to interact directly with dynamin (Lundmark et al., 2008)]. The findings that GRAF1 binds to dynamin, and that dynamin is likely to be involved in processing GRAF1-positive tubes during endocytosis (Lundmark et al., 2008), are currently difficult to reconcile with the previous reports of dynamin-independent CLIC formation and uptake of GPI-linked proteins (Sabharanjak et al., 2002). It might well be that more data on the roles of different dynamin-2 isoforms, differences in susceptibility of different dynamin-requiring processes to overexpression of dynamin dominant-negative mutants, or further subdivision of CLIC-like carriers into mechanistically different classes will resolve this issue.

Potential functions of clathrin-independent endocytosis

One can now state with some degree of confidence that mammalian cells possess at least four different ways of internalizing different regions of the plasma membrane. This raises two questions – why have multiple pathways for endocytosis evolved, and what is the specific function of individual endocytic pathways? As yet the literature does not provide firm answers to these questions, but there are some clues.

Distinct fates for internalized proteins?

Two important studies on the correlation between the mechanism of receptor internalization, signaling outputs and the rate of degradation of the receptor suggest that clathrin-independent endocytosis is linked to degradation of the relevant receptors (Di Guglielmo et al., 2003; Sigismund et al., 2008). In the case of the EGF receptor, stimulation with high concentrations of EGF resulted in clathrin-independent endocytosis and degradation, whereas, at lower concentrations, uptake via clathrin-coated pits and more prolonged intracellular signaling was observed (Sigismund et al., 2008). These experiments provide support for the general concept that different types of endocytosis have different functional consequences for proteins involved in intercellular signaling, but it still remains unclear what role specific mechanisms of clathrin-independent endocytosis play. Moreover, experiments in different cells argued that, even at high concentrations of EGF, internalization takes place predominantly via clathrin-coated pits (Kazazic et al., 2006), so it is not yet clear whether differential sorting of EGF receptors between different endocytic pathways is a general phenomenon.

Macropinocytosis is associated with membrane ruffling, and the extent to which this occurs in most cell types in vivo is unclear. At the higher concentrations of EGF used in the studies described above, a considerable amount of membrane ruffling and hence macropinocytosis has been observed, leading to the inference that macropinocytosis might result in the degradation of internalized EGF receptors (Liberali et al., 2008; Sigismund et al., 2008). Most physiological studies on macropinocytosis focus on its role in the internalization of major histocompatibility complex (MHC) proteins in dendritic cells, in which actin-rich protrusions and associated large endosomal structures are readily observed (Falcone et al.,

2006; Garrett et al., 2000). It will be important to determine whether PAK1 and CtBP are specifically recruited to these structures, as they are to the macropinosomes that are generated by EGF stimulation of common tissue-culture cells (Liberali et al., 2008).

Broader functions for caveolins and flotillins?

Caveolae are a striking and abundant feature of several cell types, including endothelial cells and adipocytes. It is not clear, however, whether budding from the plasma membrane is a ubiquitous feature of caveolar function in all contexts. Caveolae have been proposed to be important for many different processes, including lipid transport, and have been proposed to act as scaffolding or organizing platforms for signaling events, including nitric oxide synthase, endothelial (eNOS) activation and recruitment of protein-kinase-C isoforms to the plasma membrane (Garcia-Cardena et al., 1996; Mineo et al., 1998; Shaul et al., 1996). In these cases, budding from the membrane might have a regulatory role, but there is currently still speculation about this. There is evidence that caveolae have an important role in transcytosis across endothelial cells (Minshall et al., 2003; Oh et al., 2007), and this is a case in which budding from the membrane is obviously crucial. Intriguingly, transport of various markers from blood vessels to the surrounding tissues is not blocked in caveolin-1-knockout mice (Miyawaki-Shimizu et al., 2006; Schubert et al., 2002). This could be due to increased permeability of the tight junctions between endothelial cells, but compensatory vesicle trafficking and transcytosis cannot be ruled out.

Flotillins have been associated with phagocytosis, control of the actin cytoskeleton, polarization of hematopoietic cells and other processes (Morrow and Parton, 2005), but a role for budding from the plasma membrane in these processes has yet to be well characterized. In the case of both flotillins and caveolins, identification of membrane components that are concentrated in these structures would provide further insights into their function (Oh et al., 2007).

A homolog of GRAF1, oligophrenin, is essential for the formation of dendritic spines and is often mutated in individuals with syndromic X-linked mental retardation (Govek et al., 2004; Zanni et al., 2005). Providing a molecular link between these phenotypes and endocytosis remains a problem for the future.

Conclusion

The main purpose of this Commentary has been to present advances in the mechanistic description of endocytic pathways, and to argue for a classification of these pathways on the basis of ultrastructure and specific molecular determinants. There is clearly still much to do before the level of knowledge of clathrin-independent endocytosis catches up with the current knowledge of clathrin-coated pits, in terms of understanding both the molecular details of membrane deformation and scission, and the function of different pathways in their physiological contexts.

C.G.H. is supported by MRC, Cowi Foundation, Ulla og Mogens Andersens Fond, Oticon Fonden, Julie Von Mullens Fond, Fuhrmann-Fonden, Krista og Viggo Petersen's Fond, Reinholdt W. Jorck og Hustrus Fond, Christian og Ottilia Brorsons Rejselegat for Yngre Videnskabsmœnd -og Kvinder, Henry Shaw's Legat. Deposited in PMC for release after 6 months.

References

Aboulaich, N., Vainonen, J. P., Stralfors, P. and Vener, A. V. (2004). Vectorial proteomics reveal targeting, phosphorylation and specific fragmentation of polymerase I and transcript release factor (PTRF) at the surface of caveolae in human adipocytes. *Biochem. J.* **383**, 237-248.

Amstutz, B., Gastaldelli, M., Kalin, S., Imelli, N., Boucke, K., Wandeler, E., Mercer, J., Hemmi, S. and Greber, U. F. (2008). Subversion of CtBP1-controlled macropinocytosis by human adenovirus serotype 3. *EMBO J.* **27**, 956-969.

Barnes, C. J., Vadlamudi, R. K., Mishra, S. K., Jacobson, R. H., Li, F. and Kumar, R. (2003). Functional inactivation of a transcriptional corepressor by a signaling kinase. *Nat. Struct. Biol.* **10**, 622-628.

Bashkirov, P. V., Akimov, S. A., Evseev, A. I., Schmid, S. L., Zimmerberg, J. and Frolov, V. A. (2008). GTPase cycle of dynamin is coupled to membrane squeeze and release, leading to spontaneous fission. *Cell* **135**, 1276-1286.

Bast, D. J., Banerjee, L., Clark, C., Read, R. J. and Brunton, J. L. (1999). The identification of three biologically relevant globotriaosyl ceramide receptor binding sites on the Verotoxin 1 B subunit. *Mol. Microbiol.* **32**, 953-960.

Bauer, M. and Pelkmans, L. (2006). A new paradigm for membrane-organizing and -shaping scaffolds. *FEBS Lett.* **580**, 5559-5564.

Benmerah, A., Lamaze, C., Begue, B., Schmid, S. L., Dautry-Varsat, A. and Cerf-Bensussan, N. (1998). AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J. Cell Biol.* **140**, 1055-1062.

Blanchet, M. H., Le Good, J. A., Mesnard, D., Oorschot, V., Baflast, S., Minchiotti, G., Klumperman, J. and Constam, D. B. (2008). Cripto recruits Furin and PACE4 and controls Nodal trafficking during proteolytic maturation. *EMBO J.* **4**, 4.

Bokoch, G. M. (2003). Biology of the p21-activated kinases. *Annu. Rev. Biochem.* **72**, 743-781.

Bonazzi, M., Spano, S., Turacchio, G., Cericola, C., Valente, C., Colanzi, A., Kweon, H. S., Hsu, V. W., Polishchuck, E. V., Polishchuck, R. S. et al. (2005). CtBP3/BARS drives membrane fission in dynamin-independent transport pathways. *Nat. Cell Biol.* **7**, 570-580.

Cao, H., Chen, J., Awoniyi, M., Henley, J. R. and McNiven, M. A. (2007). Dynamin 2 mediates fluid-phase micropinocytosis in epithelial cells. *J. Cell Sci.* **120**, 4167-4177.

Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P. and De Camilli, P. (1998). Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* **394**, 793-797.

Chen, M. S., Obar, R. A., Schroeder, C. C., Austin, T. W., Poodry, C. A., Wadsworth, S. C. and Vallee, R. B. (1991). Multiple forms of dynamin are encoded by shibire, a Drosophila gene involved in endocytosis. *Nature* **351**, 583-586.

Cheng, Z. J., Singh, R. D., Marks, D. L. and Pagano, R. E. (2006). Membrane microdomains, caveolae, and caveolar endocytosis of sphingolipids. *Mol. Membr. Biol.* **23**, 101-110.

Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol. Cell* **9**, 213-224.

Colanzi, A., Hidalgo Carcedo, C., Persico, A., Cericola, C., Turacchio, G., Bonazzi, M., Luini, A. and Corda, D. (2007). The Golgi mitotic checkpoint is controlled by BARS-dependent fission of the Golgi ribbon into separate stacks in G2. *EMBO J.* **26**, 2465-2476.

Conner, S. D. and Schmid, S. L. (2003). Regulated portals of entry into the cell. *Nature* **422**, 37-44.

Corda, D., Colanzi, A. and Luini, A. (2006). The multiple activities of CtBP/BARS proteins: the Golgi view. *Trends Cell Biol.* **16**, 167-173.

Damke, H., Baba, T., van der Bliek, A. M. and Schmid, S. L. (1995). Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. *J. Cell Biol.* **131**, 69-80.

Damm, E. M., Pelkmans, L., Kartenbeck, J., Mezzacasa, A., Kurzchalia, T. and Helenius, A. (2005). Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. *J. Cell Biol.* **168**, 477-488.

Dharmawardhane, S., Sanders, L. C., Martin, S. S., Daniels, R. H. and Bokoch, G. M. (1997). Localization of p21-activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells. *J. Cell Biol.* **138**, 1265-1278.

Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F. and Wrana, J. L. (2003). Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat. Cell Biol.* **5**, 410-421.

Doxsey, S. J., Brodsky, F. M., Blank, G. S. and Helenius, A. (1987). Inhibition of endocytosis by anti-clathrin antibodies. *Cell* **50**, 453-463.

Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F. C. et al. (2001). Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* **293**, 2449-2452.

D'Souza-Schorey, C., Li, G., Colombo, M. I. and Stahl, P. D. (1995). A regulatory role for ARF6 in receptor-mediated endocytosis. *Science* **267**, 1175-1178.

Edeling, M. A., Smith, C. and Owen, D. (2006). Life of a clathrin coat: insights from clathrin and AP structures. *Nat. Rev. Mol. Cell. Biol.* **7**, 32-44.

Ehrlich, M., Boll, W., Van Oijen, A., Hariharan, R., Chandran, K., Nibert, M. L. and Kirchhausen, T. (2004). Endocytosis by random initiation and stabilization of clathrin-coated pits. *Cell* **118**, 591-605.

Falcone, S., Cocucci, E., Podini, P., Kirchhausen, T., Clementi, E. and Meldolesi, J. (2006). Macropinocytosis: regulated coordination of endocytic and exocytic membrane traffic events. *J. Cell Sci.* **119**, 4758-4769.

Ford, M. G., Pearse, B. M., Higgins, M. K., Vallis, Y., Owen, D. J., Gibson, A., Hopkins, C. R., Evans, P. R. and McMahon, H. T. (2001). Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science* **291**, 1051-1055.

Frick, M., Bright, N. A., Riento, K., Bray, A., Merrified, C. and Nichols, B. J. (2007). Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. *Curr. Biol.* **17**, 1151-1156.

Gallop, J. L., Butler, P. J. and McMahon, H. T. (2005). Endophilin and CtBP/BARS are not acyl transferases in endocytosis or Golgi fission. *Nature* **438**, 675-678.
Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J. E. and Sessa, W. C. (1996). Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc. Natl. Acad. Sci. USA* **93**, 6448-6453.
Garrett, W. S., Chen, L. M., Kroschewski, R., Ebersold, M., Turley, S., Trombetta, S., Galan, J. E. and Mellman, I. (2000). Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* **102**, 325-334.
Glebov, O. O. and Nichols, B. J. (2004). Lipid raft proteins have a random distribution during localized activation of the T-cell receptor. *Nat. Cell Biol.* **6**, 238-243.
Glebov, O. O., Bright, N. A. and Nichols, B. J. (2006). Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat. Cell Biol.* **8**, 46-54.
Govek, E. E., Newey, S. E., Akerman, C. J., Cross, J. R., Van der Veken, L. and Van Aelst, L. (2004). The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis. *Nat. Neurosci.* **7**, 364-372.
Guha, A., Sriram, V., Krishnan, K. S. and Mayor, S. (2003). Shibire mutations reveal distinct dynamin-independent and -dependent endocytic pathways in primary cultures of Drosophila hemocytes. *J. Cell Sci.* **116**, 3373-3386.
Gustincich, S. and Schneider, C. (1993). Serum deprivation response gene is induced by serum starvation but not by contact inhibition. *Cell Growth Differ.* **4**, 753-760.
Gustincich, S., Vatta, P., Goruppi, S., Wolf, M., Saccone, S., Della Valle, G., Baggiolini, M. and Schneider, C. (1999). The human serum deprivation response gene (SDPR) maps to 2q32-q33 and codes for a phosphatidylserine-binding protein. *Genomics* **57**, 120-129.
Hansen, S. H., Sandvig, K. and van Deurs, B. (1992). Internalization efficiency of the transferrin receptor. *Exp. Cell Res.* **199**, 19-28.
Hao, M., Mukherjee, S. and Maxfield, F. R. (2001). Cholesterol depletion induces large scale domain segregation in living cell membranes. *Proc. Natl. Acad. Sci. USA* **98**, 13072-13077.
Henley, J. R., Krueger, E. W., Oswald, B. J. and McNiven, M. A. (1998). Dynamin-mediated internalization of caveolae. *J. Cell Biol.* **141**, 85-99.
Hidalgo Carcedo, C., Bonazzi, M., Spano, S., Turacchio, G., Colanzi, A., Luini, A. and Corda, D. (2004). Mitotic Golgi partitioning is driven by the membrane-fissioning protein CtBP3/BARS. *Science* **305**, 93-96.
Hill, M. M., Bastiani, M., Luetterforst, R., Kirkham, M., Kirkham, A., Nixon, S. J., Walser, P., Abankwa, D., Oorschot, V. M., Martin, S. et al. (2008). PTRF-Cavin, a conserved cytoplasmic protein required for caveola formation and function. *Cell* **132**, 113-124.
Izumi, G., Sakisaka, T., Baba, T., Tanaka, S., Morimoto, K. and Takai, Y. (2004). Endocytosis of E-cadherin regulated by Rac and Cdc42 small G proteins through IQGAP1 and actin filaments. *J. Cell Biol.* **166**, 237-248.
Karjalainen, M., Kakkonen, E., Upla, P., Paloranta, H., Kankaanpaa, P., Liberali, P., Renkema, G. H., Hyypia, T., Heino, J. and Marjomaki, V. (2008). A Raft-derived, Pak1-regulated entry participates in {alpha}2{beta}1 integrin-dependent sorting to caveosomes. *Mol. Biol. Cell* **19**, 2857-2869.
Kazazic, M., Roepstorff, K., Johannessen, L. E., Pedersen, N. M., van Deurs, B., Stang, E. and Madshus, I. H. (2006). EGF-induced activation of the EGF receptor does not trigger mobilization of caveolae. *Traffic* **7**, 1518-1527.
Kelly, B. T., McCoy, A. J., Spate, K., Miller, S. E., Evans, P. R., Honing, S. and Owen, D. J. (2008). A structural explanation for the binding of endocytic dileucine motifs by the AP2 complex. *Nature* **456**, 976-979.
Kenworthy, A. K., Nichols, B. J., Remmert, C. L., Hendrix, G. M., Kumar, M., Zimmerberg, J. and Lippincott-Schwartz, J. (2004). Dynamics of putative raft-associated proteins at the cell surface. *J. Cell Biol.* **165**, 735-746.
Kirkham, M., Fujita, A., Chadda, R., Nixon, S. J., Kurzchalia, T. V., Sharma, D. K., Pagano, R. E., Hancock, J. F., Mayor, S. and Parton, R. G. (2005). Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. *J. Cell Biol.* **168**, 465-476.
Kirkham, M., Nixon, S. J., Howes, M. T., Abi-Rached, L., Wakeham, D. E., Hanzal-Bayer, M., Ferguson, C., Hill, M. M., Fernandez-Rojo, M., Brown, D. A. et al. (2008). Evolutionary analysis and molecular dissection of caveola biogenesis. *J. Cell Sci.* **121**, 2075-2086.
Kitova, E. N., Daneshfar, R., Marcato, P., Mulvey, G. L., Armstrong, G. and Klassen, J. S. (2005). Stability of the homopentameric B subunits of shiga toxins 1 and 2 in solution and the gas phase as revealed by nanoelectrospray fourier transform ion cyclotron resonance mass spectrometry. *J. Am. Soc. Mass Spectrom.* **16**, 1957-1968.
Kumari, S. and Mayor, S. (2008). ARF1 is directly involved in dynamin-independent endocytosis. *Nat. Cell Biol.* **10**, 30-41.
Kwik, J., Boyle, S., Fooksman, D., Margolis, L., Sheetz, M. P. and Edidin, M. (2003). Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc. Natl. Acad. Sci. USA* **100**, 13964-13969.
Lajoie, P. and Nabi, I. R. (2007). Regulation of raft-dependent endocytosis. *J. Cell Mol. Med.* **11**, 644-653.
Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A. and Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol. Cell* **7**, 661-671.
Lang, D. M., Lommel, S., Jung, M., Ankerhold, R., Petrausch, B., Laessing, U., Wiechers, M. F., Plattner, H. and Stuermer, C. A. (1998). Identification of reggie-1 and reggie-2 as plasmamembrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. *J. Neurobiol.* **37**, 502-523.
Langhorst, M. F., Reuter, A., Jaeger, F. A., Wippich, F. M., Luxenhofer, G., Plattner, H. and Stuermer, C. A. (2008). Trafficking of the microdomain scaffolding protein reggie-1/flotillin-2. *Eur. J. Cell Biol.* **87**, 211-226.
Lauvrak, S. U., Walchli, S., Iversen, T. G., Slagsvold, H. H., Torgersen, M. L., Spilsberg, B. and Sandvig, K. (2006). Shiga toxin regulates its entry in a Syk-dependent manner. *Mol. Biol. Cell* **17**, 1096-1109.
Le, P. U., Guay, G., Altschuler, Y. and Nabi, I. R. (2002). Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. *J. Biol. Chem.* **277**, 3371-3379.
Liberali, P., Kakkonen, E., Turacchio, G., Valente, C., Spaar, A., Perinetti, G., Bockmann, R. A., Corda, D., Colanzi, A., Marjomaki, V. et al. (2008). The closure of Pak1-dependent macropinosomes requires the phosphorylation of CtBP1/BARS. *EMBO J.* **27**, 970-981.
Liu, L. and Pilch, P. F. (2008). A critical role of cavin (polymerase I and transcript release factor) in caveolae formation and organization. *J. Biol. Chem.* **283**, 4314-4322.
Liu, L., Brown, D., McKee, M., Lebrasseur, N. K., Yang, D., Albrecht, K. H., Ravid, K. and Pilch, P. F. (2008). Deletion of Cavin/PTRF causes global loss of caveolae, dyslipidemia, and glucose intolerance. *Cell Metab.* **8**, 310-317.
Lundmark, R., Doherty, G. J., Howes, M. T., Cortese, K., Vallis, Y., Parton, R. G. and McMahon, H. T. (2008). The GTPase-activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway. *Curr. Biol.* **18**, 1802-1808.
Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C. and Kirchhausen, T. (2006). Dynasore, a cell-permeable inhibitor of dynamin. *Dev. Cell* **10**, 839-850.
Marsh, M. and Helenius, A. (2006). Virus entry: open sesame. *Cell* **124**, 729-740.
Mayor, S. and Riezman, H. (2004). Sorting GPI-anchored proteins. *Nat. Rev. Mol. Cell. Biol.* **5**, 110-120.
Mayor, S. and Pagano, R. E. (2007). Pathways of clathrin-independent endocytosis. *Nat. Rev. Mol. Cell. Biol.* **8**, 603-612.
McNiven, M. A., Cao, H., Pitts, K. R. and Yoon, Y. (2000). The dynamin family of mechanoenzymes: pinching in new places. *Trends Biochem. Sci.* **25**, 115-120.
Mercer, J. and Helenius, A. (2008). Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science* **320**, 531-535.
Merrifield, C. J., Perrais, D. and Zenisek, D. (2005). Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. *Cell* **121**, 593-606.
Mineo, C., Ying, Y. S., Chapline, C., Jaken, S. and Anderson, R. G. (1998). Targeting of protein kinase Calpha to caveolae. *J. Cell Biol.* **141**, 601-610.
Minshall, R. D., Sessa, W. C., Stan, R. V., Anderson, R. G. and Malik, A. B. (2003). Caveolin regulation of endothelial function. *Am. J. Physiol. Lung Cell Mol. Physiol.* **285**, L1179-L1183.
Miyawaki-Shimizu, K., Predescu, D., Shimizu, J., Broman, M., Predescu, S. and Malik, A. B. (2006). siRNA-induced caveolin-1 knockdown in mice increases lung vascular permeability via the junctional pathway. *Am. J. Physiol. Lung Cell Mol. Physiol.* **290**, L405-L413.
Morrow, I. C. and Parton, R. G. (2005). Flotillins and the PHB domain protein family: rafts, worms and anaesthetics. *Traffic* **6**, 725-740.
Moya, M., Dautry-Varsat, A., Goud, B., Louvard, D. and Boquet, P. (1985). Inhibition of coated pit formation in Hep2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. *J. Cell Biol.* **101**, 548-559.
Munro, S. (2003). Lipid rafts: elusive or illusive? *Cell* **115**, 377-388.
Naslavsky, N., Weigert, R. and Donaldson, J. G. (2004). Characterization of a nonclathrin endocytic pathway: membrane cargo and lipid requirements. *Mol. Biol. Cell* **15**, 3542-3552.
Neumann-Giesen, C., Fernow, I., Amaddii, M. and Tikkanen, R. (2007). Role of EGF-induced tyrosine phosphorylation of reggie-1/flotillin-2 in cell spreading and signaling to the actin cytoskeleton. *J. Cell Sci.* **120**, 395-406.
Nichols, B. (2003a). Caveosomes and endocytosis of lipid rafts. *J. Cell Sci.* **116**, 4707-4714.
Nichols, B. J. (2003b). GM1-containing lipid rafts are depleted within clathrin-coated pits. *Curr. Biol.* **13**, 686-690.
Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D. and Lippincott-Schwartz, J. (2001). Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell Biol.* **153**, 529-541.
Ogata, T., Ueyama, T., Isodono, K., Tagawa, M., Takehara, N., Kawashima, T., Harada, K., Takahashi, T., Shioi, T., Matsubara, H. et al. (2008). MURC, a muscle-restricted coiled-coil protein that modulates the Rho/ROCK pathway, induces cardiac dysfunction and conduction disturbance. *Mol. Cell. Biol.* **28**, 3424-3436.
Oh, P., McIntosh, D. P. and Schnitzer, J. E. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J. Cell Biol.* **141**, 101-114.
Oh, P., Borgstrom, P., Witkiewicz, H., Li, Y., Borgstrom, B. J., Chrastina, A., Iwata, K., Zinn, K. R., Baldwin, R., Testa, J. E. et al. (2007). Live dynamic imaging of caveolae pumping targeted antibody rapidly and specifically across endothelium in the lung. *Nat. Biotechnol.* **25**, 327-337.
Palacios, F., Schweitzer, J. K., Boshans, R. L. and D'Souza-Schorey, C. (2002). ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. *Nat. Cell Biol.* **4**, 929-936.
Parton, R. G. (1994). Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. *J. Histochem. Cytochem.* **42**, 155-166.
Pelkmans, L. and Zerial, M. (2005). Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. *Nature* **436**, 128-133.
Pelkmans, L., Kartenbeck, J. and Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat. Cell Biol.* **3**, 473-483.
Pelkmans, L., Puntener, D. and Helenius, A. (2002). Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science* **296**, 535-539.

Pryor, P. R., Jackson, L., Gray, S. R., Edeling, M. A., Thompson, A., Sanderson, C. M., Evans, P. R., Owen, D. J. and Luzio, J. P. (2008). Molecular basis for the sorting of the SNARE VAMP7 into endocytic clathrin-coated vesicles by the ArfGAP Hrb. *Cell* **134**, 817-827.

Pucadyil, T. J. and Schmid, S. L. (2008). Real-time visualization of dynamin-catalyzed membrane fission and vesicle release. *Cell* **135**, 1263-1275.

Puri, V., Watanabe, R., Singh, R. D., Dominguez, M., Brown, J. C., Wheatley, C. L., Marks, D. L. and Pagano, R. E. (2001). Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J. Cell Biol.* **154**, 535-547.

Razani, B., Engelman, J. A., Wang, X. B., Schubert, W., Zhang, X. L., Marks, C. B., Macaluso, F., Russell, R. G., Li, M., Pestell, R. G. et al. (2001). Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J. Biol. Chem.* **276**, 38121-38138.

Riento, K., Frick, M., Schafer, I. and Nichols, B. J. (2009). Endocytosis of flotillin-1 and flotillin-2 is regulated by Fyn kinase. *J. Cell Sci.* **122**, 912-918.

Rodal, S. K., Skretting, G., Garred, O., Vilhardt, F., van Deurs, B. and Sandvig, K. (1999). Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol. Biol. Cell* **10**, 961-974.

Rojek, J. M. and Kunz, S. (2008). Cell entry by human pathogenic arenaviruses. *Cell Microbiol.* **10**, 828-835.

Romer, W., Berland, L., Chambon, V., Gaus, K., Windschiegl, B., Tenza, D., Aly, M. R., Fraisier, V., Florent, J. C., Perrais, D. et al. (2007). Shiga toxin induces tubular membrane invaginations for its uptake into cells. *Nature* **450**, 670-675.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R. and Anderson, R. G. (1992). Caveolin, a protein component of caveolae membrane coats. *Cell* **68**, 673-682.

Roux, A. and Antonny, B. (2008). The long and short of membrane fission. *Cell* **135**, 1163-1165.

Sabharanjak, S., Sharma, P., Parton, R. G. and Mayor, S. (2002). GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev. Cell* **2**, 411-423.

Sandvig, K. and van Deurs, B. (1990). Selective modulation of the endocytic uptake of ricin and fluid phase markers without alteration in transferrin endocytosis. *J. Biol. Chem.* **265**, 6382-6388.

Sandvig, K., Torgersen, M. L., Raa, H. A. and van Deurs, B. (2008). Clathrin-independent endocytosis: from nonexisting to an extreme degree of complexity. *Histochem. Cell Biol.* **129**, 267-276.

Schmid, E. M. and McMahon, H. T. (2007). Integrating molecular and network biology to decode endocytosis. *Nature* **448**, 883-888.

Schubert, W., Frank, P. G., Woodman, S. E., Hyogo, H., Cohen, D. E., Chow, C. W. and Lisanti, M. P. (2002). Microvascular hyperpermeability in caveolin-1 (–/–) knock-out mice. Treatment with a specific nitric-oxide synthase inhibitor, L-NAME, restores normal microvascular permeability in Cav-1 null mice. *J. Biol. Chem.* **277**, 40091-40098.

Sharma, D. K., Brown, J. C., Choudhury, A., Peterson, T. E., Holicky, E., Marks, D. L., Simari, R., Parton, R. G. and Pagano, R. E. (2004a). Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. *Mol. Biol. Cell* **15**, 3114-3122.

Sharma, P., Varma, R., Sarasij, R. C., Ira Gousset, K., Krishnamoorthy, G., Rao, M. and Mayor, S. (2004b). Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell* **116**, 577-589.

Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G. and Michel, T. (1996). Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae. *J. Biol. Chem.* **271**, 6518-6522.

Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S. and Di Fiore, P. P. (2008). Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev. Cell* **15**, 209-219.

Smith, A. E. and Helenius, A. (2004). How viruses enter animal cells. *Science* **304**, 237-242.

Stagg, S. M., LaPointe, P. and Balch, W. E. (2007). Structural design of cage and coat scaffolds that direct membrane traffic. *Curr. Opin. Struct. Biol.* **17**, 221-228.

Stan, R. V. (2002). Structure and function of endothelial caveolae. *Microsc. Res. Tech.* **57**, 350-364.

Stuermer, C. A., Lang, D. M., Kirsch, F., Wiechers, M., Deininger, S. O. and Plattner, H. (2001). Glycosylphosphatidyl inositol-anchored proteins and fyn kinase assemble in noncaveolar plasma membrane microdomains defined by reggie-1 and -2. *Mol. Biol. Cell* **12**, 3031-3045.

Sverdlov, M., Shajahan, A. N. and Minshall, R. D. (2007). Tyrosine phosphorylation-dependence of caveolae-mediated endocytosis. *J. Cell Mol. Med.* **11**, 1239-1250.

Swanson, J. A. and Watts, C. (1995). Macropinocytosis. *Trends Cell Biol.* **5**, 424-428.

Tagawa, M., Ueyama, T., Ogata, T., Takehara, N., Nakajima, N., Isodono, K., Asada, S., Takahashi, T., Matsubara, H. and Oh, H. (2008). MURC, a muscle-restricted coiled-coil protein, is involved in the regulation of skeletal myogenesis. *Am. J. Physiol. Cell Physiol.* **295**, C490-C498.

Thomsen, P., Roepstorff, K., Stahlhut, M. and van Deurs, B. (2002). Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. *Mol. Biol. Cell* **13**, 238-250.

van der Bliek, A. M. and Meyerowitz, E. M. (1991). Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. *Nature* **351**, 411-414.

van der Bliek, A. M., Redelmeier, T. E., Damke, H., Tisdale, E. J., Meyerowitz, E. M. and Schmid, S. L. (1993). Mutations in human dynamin block an intermediate stage in coated vesicle formation. *J. Cell Biol.* **122**, 553-563.

Vinten, J., Johnsen, A. H., Roepstorff, P., Harpoth, J. and Tranum-Jensen, J. (2005). Identification of a major protein on the cytosolic face of caveolae. *Biochim. Biophys. Acta* **1717**, 34-40.

Watts, C. and Marsh, M. (1992). Endocytosis: what goes in and how? *J. Cell Sci.* **103**, 1-8.

Weigert, R., Silletta, M. G., Spano, S., Turacchio, G., Cericola, C., Colanzi, A., Senatore, S., Mancini, R., Polishchuk, E. V., Salmona, M. et al. (1999). CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. *Nature* **402**, 429-433.

Yang, J. S., Lee, S. Y., Spano, S., Gad, H., Zhang, L., Nie, Z., Bonazzi, M., Corda, D., Luini, A. and Hsu, V. W. (2005). A role for BARS at the fission step of COPI vesicle formation from Golgi membrane. *EMBO J.* **24**, 4133-4143.

Zanni, G., Saillour, Y., Nagara, M., Billuart, P., Castelnau, L., Moraine, C., Faivre, L., Bertini, E., Durr, A., Guichet, A. et al. (2005). Oligophrenin 1 mutations frequently cause X-linked mental retardation with cerebellar hypoplasia. *Neurology* **65**, 1364-1369.

Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J. and Bokoch, G. M. (1995). Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J. Biol. Chem.* **270**, 23934-23936.

Zhao, Y. Y., Liu, Y., Stan, R. V., Fan, L., Gu, Y., Dalton, N., Chu, P. H., Peterson, K., Ross, J., Jr and Chien, K. R. (2002). Defects in caveolin-1 cause dilated cardiomyopathy and pulmonary hypertension in knockout mice. *Proc. Natl. Acad. Sci. USA* **99**, 11375-11380.

Journal of Cell Science