The roles of flotillin microdomains – endocytosis and beyond

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Summary

Flotillins are membrane proteins that form microdomains in the plasma membrane of all mammalian cell types studied to date. They span the evolutionary spectrum, with proteins related to flotillins present in bacteria, fungi, plants and metazoans, which suggests that they perform important, and probably conserved, functions. Flotillins have been implicated in myriad processes that include endocytosis, signal transduction and regulation of the cortical cytoskeleton, yet the molecular mechanisms that underlie flotillin function in these different cases are still poorly understood. In this Commentary, we will provide an introduction to these intriguing proteins, summarise their proposed functions and discuss in greater detail some recent insights into the role of flotillin microdomains in endocytosis that have been provided by several independent studies. Finally, we will focus on the questions that are raised by these new experiments and their implications for future studies.

Key words: Cytoskeleton, Endocytosis, Flotillin, Microdomain

Introduction

Flotillin 1 and flotillin 2 are membrane-associated proteins that are thought to function in a number of cellular contexts, including signalling, endocytosis and interactions with the cytoskeleton. Despite being found almost ubiquitously across the evolutionary spectrum and, apparently, being expressed in all mammalian tissues, we are still some way away from understanding the molecular details of their various roles. In many respects the key and defining observation regarding the flotillins is their propensity to co-assemble into discrete microdomains in the plasma membrane. This means that flotillins have the potential to facilitate compartmentalisation and functional specialisation within the membrane. However, the lack of sequence features that directly suggest function, the diversity of tissues and cellular locations in which flotillins are detected and uncertainty as to which other proteins flotillins interact with, make understanding their fundamental roles a challenge.

A number of models have been proposed to explain how flotillins might function in different cellular processes, and it is possible that flotillins function in multiple ways in the same process. One view is that flotillin microdomains act, essentially, in a structural capacity, for example by providing molecular scaffolding for membrane rafts that act as signalling platforms or by demarcating sites for the delivery of specific cargo (Stuermer, 2011). An additional possibility is that flotillins act in a regulatory capacity, sensing changes in membrane properties, such as lipid composition or tension, and responding appropriately. A third model revolves around evidence that has highlighted that budding of flotillin microdomains from the plasma membrane defines a specific type of endocytic pathway (Glebov et al., 2006).

The goal of this Commentary is to discuss four very recent papers that, taken together, constitute an important step forwards in our understanding of flotillin function. First, a study from Wayne Lencer's laboratory has demonstrated that flotillins are required for cholera toxin trafficking and toxicity in both cultured cells and, importantly, in zebrafish (Saslowsky et al., 2010), which is the first demonstration that flotillins are directly involved in endocytosis in a model organism. Second, results from Ai Yamamoto and colleagues have shown that flotillins have a key role in endocytosis of glutamate and dopamine transporters in mammals (Cremona et al., 2011), which highlights specific and physiologically important substrates for flotillin-dependent endocytosis. Third, data from Song and colleagues imply that flotillins have a role in sensing membrane cholesterol and regulating uptake of the cholesterol transporter NPC1L1 (Ge et al., 2011). Finally, experiments published by our own group show that flotillins interact with the cortical cytoskeleton and are required for migration of neutrophils in vivo (Ludwig et al., 2010). In this Commentary, we attempt to integrate these new data with the existing body of literature on flotillin function. We will first provide some background about the discovery and properties of flotillin proteins. We will then summarise studies on the participation of flotillins in signalling pathways, a role that typically invokes their localisation to lipid rafts or microdomains, and also briefly discuss the role of flotillins in the regulation of the cytoskeleton. Finally, we will provide a more comprehensive overview of the role of flotillins in endocytosis.

Flotillin proteins

The flotillins were first identified as proteins that are upregulated during the regeneration of goldfish retinal ganglion cells after optic nerve injury (hence the earlier name reggie) (Schulte et al., 1997) and were later shown to be required for regeneration of retinal axons in zebrafish (Munderloh et al., 2009). They were also independently identified as components of detergent-resistant membrane fractions (from murine lung tissue) that float in density gradients (Bickel et al., 1997), which resulted in them being named flotillins. Both flotillin 1 and flotillin 2 have been found in all mammalian cell types studied to date, and are likely to be expressed ubiquitously (Bickel et al., 1997; Volonte et al., 1999;

von Philipsborn et al., 2005). Flotillins are topologically similar but unrelated in sequence to caveolins (Bauer and Pelkmans, 2006). In fact, they were thought to be present in caveolae (Volonte et al., 1999) or to substitute for caveolae in cell types or tissues, such as leukocytes, which lack detectable caveolin 1. Although this has not been ruled out, it is known, at least in cultured cells, that flotillins form membrane microdomains that are separate from caveolae (Frick et al., 2007; Lang et al., 1998; Stuermer et al., 2001). Proteins related to flotillins are found in bacteria (Lopez and Kolter, 2010), plants (Haney and Long, 2010), fungi and most metazoans, yet are curiously absent from *C. elegans* and yeast. They localise to both the plasma membrane and to late endosomes or lysosomes, and have also been detected in maturing phagosomes, early endosomes and exosomes (Babuke et al., 2009; Bared et al., 2004; Glebov et al., 2006; Okabayashi and Kimura, 2010; Staubach et al., 2009).

The flotillin family is composed of two highly homologous proteins, flotillin 1 (also called reggie 2) and flotillin 2 (also referred to as reggie 1) that share ~50% amino acid sequence identity (Fig. 1). They form part of a larger family of proteins that are characterised by the presence of a prohibitin homology (PHB) domain [also known as the stomatin, prohibitin, flotillin and HflK/C (SPFH) domain] (Browman et al., 2007; Liu et al., 2005a). PHB-domain-containing proteins have diverse functions in varied organisms, tissues and cellular locations, but share the common feature of behaving as integral membrane proteins that oligomerise to form microdomains. These microdomains demonstrate insolubility in cold non-ionic detergents and buoyancy on sucrose density gradients, both of which are classical hallmarks of lipid or membrane rafts. The PHB domain confers membrane association through the presence of acylation sites and putative hydrophobic hairpins that are thought to insert into the inner leaflet of the membrane (Morrow and Parton, 2005). Flotillin 2 is myristoylated on Gly2, and its major palmitoylation site is found at Cys4 (Neumann-Giesen et al., 2004). By contrast, flotillin 1 is not myristoylated but is palmitoylated on Cys34 (Morrow et al., 2002) (Fig. 1).

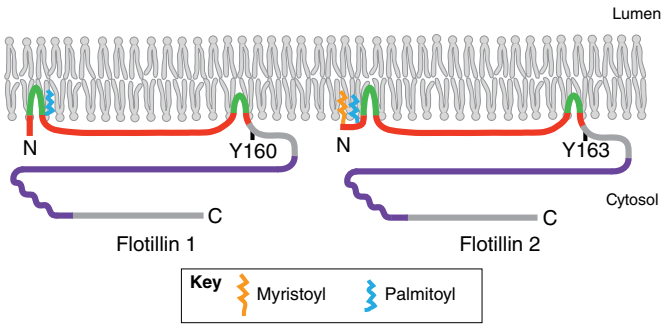


Fig. 1. Schematic representation of putative flotillin topology. Flotillins are tightly associated with the inner leaflet of the plasma membrane through the PHB domain in the N-terminal portion (N) of the protein (red), which contains two hydrophobic regions (green) that can form hairpins. Membrane association also occurs through myristoylation (orange) and palmitoylation (light blue). The tyrosine residues (Y160 and Y163 in flotillin 1 and flotillin 2, respectively) phosphorylated by Fyn kinase are also indicated. Flotillin 1 and flotillin 2 share a conserved flotillin domain (purple) that contains a predicted alpha-helical region (squiggle) that might be involved in oligomerisation. Unfortunately, little or no structural data are available for the flotillin proteins, either individually or in an oligomerised form, to provide insight into how the membrane microdomains are formed.

The PHB domain in the flotillins constitutes the N-terminus of the protein. The C-terminal portion is well conserved within the flotillin family but is not found in other proteins. It contains an alpha-helical region that mediates oligomerisation to form stable homo- and hetero-tetramers (Solis et al., 2007). Efficient association with membranes and incorporation into detergent-insoluble membrane microdomains requires both membrane association through the PHB domain and oligomerisation (Solis et al., 2007). The stability of both flotillin proteins is interdependent, so that the absence of one leads to a reduction in the protein level of the other (Babuke et al., 2009; Frick et al., 2007; Langhorst et al., 2008; Ludwig et al., 2010), presumably through proteasomal degradation (Solis et al., 2007).

Assembly of flotillin microdomains

Flotillin 1 and flotillin 2 bind to each other, and can be efficiently co-immunoprecipitated from cell extracts (Frick et al., 2007). In cells, they colocalise extensively in small plasma membrane puncta and thereby define specific microdomains or regions in the plasma membrane (Frick et al., 2007; Solis et al., 2007) (Fig. 2). Importantly, if either flotillin is overexpressed on its own this recruitment to microdomains is rapidly saturated (Box 1), but if both flotillins are overexpressed in the same cell additional flotillin microdomains are generated (Babuke et al., 2009; Frick et al., 2007). These results argue that the co-assembly of flotillin 1 and 2 is sufficient and necessary to create flotillin-positive microdomains of a defined size within the plasma membrane, and that these microdomains contain both proteins in approximately equal amounts (Frick et al., 2007). Unlike caveolae, the microdomains are laterally mobile within the membrane and appear to bud into the cell (Frick et al., 2007; Glebov et al., 2006). The distribution of flotillin microdomains, and their role in endocytosis, is likely to be controlled by tyrosine residue phosphorylation (Riento et al., 2009). Both flotillin 1 and flotillin 2 can be phosphorylated by Fyn and potentially other Src family kinases (Babuke et al., 2009; Neumann-Giesen et al., 2007; Riento et al., 2009). As discussed below in more detail, this post-translational modification is involved in regulating flotillin function during endocytosis.

Flotillin microdomains as signalling platforms

Flotillins have been long considered markers of lipid rafts because they are detergent insoluble and float in sucrose density gradients. Consequently, flotillins, either on their own or in combination with the respective other flotillin, have been implicated in numerous signalling events and pathways that are thought to be organised in lipid rafts (Babuke and Tikkanen, 2007).

One of the earliest studies on the roles of flotillin microdomains argued that flotillins function in insulin receptor signalling: insulin stimulation leads to dissociation of a complex of the proto-oncogene CBL and CBL-associated protein (CAP, officially known as SORBS1) from the insulin receptor and translocation of the complex to lipid rafts, which is mediated by binding through the sorbin homology domain of CAP to flotillin 1 (Baumann et al., 2000; Liu et al., 2005b). This results in the activation of the small Rho GTPase RHOQ, translocation of the glucose transporter GLUT4 from intracellular stores to the plasma membrane, and glucose uptake (Baumann et al., 2000; Kimura et al., 2001).

Subsequent studies in various cell types have suggested that flotillin microdomains represent assembly sites for active signalling platforms that typically involve the activity of Src family kinases. In T cells, flotillins are polarised to one side of the cell even in quiescent cells, and this flotillin ‘cap’ marks the location of the

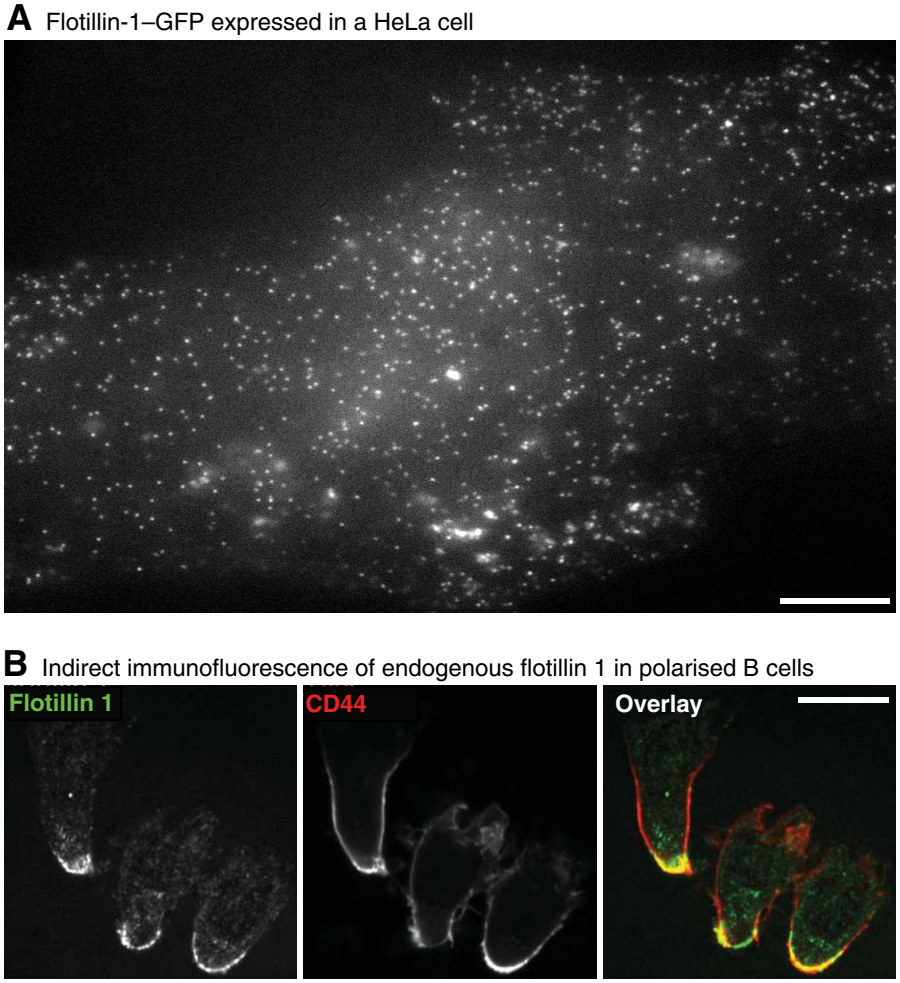


Fig. 2. Flotillin proteins form microdomains in the plasma membrane. (**A**) Epifluorescence image of flotillin-1–GFP expressed in a HeLa cell in which endogenous flotillin 1 has been depleted using siRNA. The highly punctate appearance of the flotillin microdomains is particularly noteworthy. (**B**) Endogenous flotillin 1 distribution in primary B cells that were isolated from mice, visualised by using confocal microscopy. The cells were stimulated with bacterial lipopolysaccharide, which causes them to polarise and results in a redistribution of flotillin to the uropod in the rear of the cell. The uropod is marked with an antibody detecting the cell surface glycoprotein CD44. Scale bars: 5 µm.

immunological synapse (Rajendran et al., 2003). In this context, flotillins might function in T cell receptor signalling because co-stimulation of T cells with anti-CD3 and anti-CD28 antibodies leads to recruitment of the signalling component linker for activation of T cells (LAT) to flotillin microdomains containing the Src family kinase Lck (Slaughter et al., 2003).

Ligation of the prion protein PrP, the Thy-1 cell surface antigen or other glycosylphosphatidylinositol (GPI)-anchored proteins with specific antibodies to induce crosslinking results in co-clustering of these crosslinked proteins with flotillin microdomains, as well as the recruitment of signalling molecules and activation of signalling pathways (Langhorst et al., 2006; Stuermer et al., 2001; Stuermer et al., 2004). Similarly, flotillin microdomains have a role in signal transduction pathways during mast cell degranulation, where crosslinking of the IgE receptor by antigen results in increased receptor association with flotillin 1 in membrane rafts as well as increased receptor phosphorylation by the Src family kinase Lyn (Kato et al., 2006).

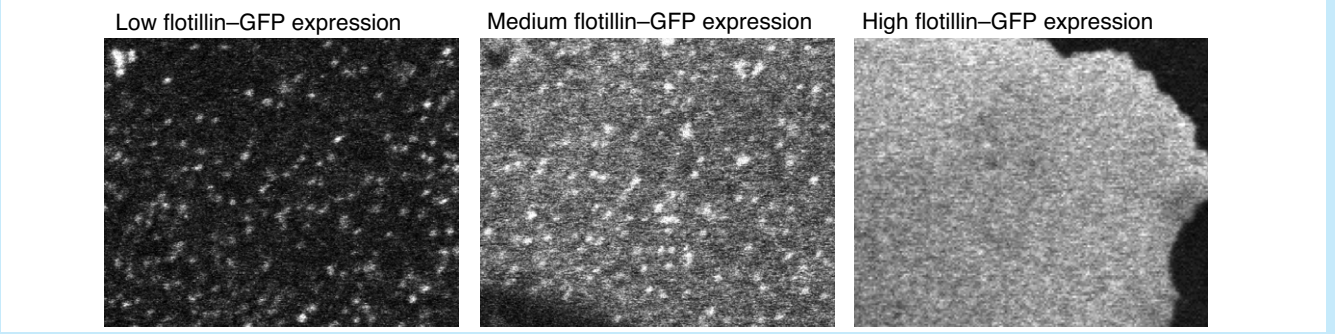
Finally, flotillins have been implicated in Gαq-mediated G-protein-coupled receptor (GPCR) signalling. Flotillins interact directly with Gαq in a manner that is independent of the nucleotide-binding state of the G-protein, and positively regulate Gαq signalling through effects on p38 MAPK (also known as mitogen-activated protein kinase 14) signalling and Src family kinase activity (Sugawara et al., 2007).

Although the studies mentioned above provide evidence that flotillins potentially regulate multiple signalling pathways, in all cases it remains unclear precisely what roles the flotillins have on a molecular level. These studies also raise an important technical issue. In some studies where detergent-resistant membranes (DRMs) are isolated and an increase in the association of a particular molecule with gradient fractions containing flotillins is found, this is taken as evidence that the molecule is directly associated with flotillin microdomains. However, precisely what incorporation of proteins into DRMs means in terms of protein organisation within the intact plasma membrane is debatable, and indeed has been much debated (Munro, 2003; Simons and Gerl, 2010). Without reiterating those arguments here, it is obviously important to take measures to increase the specificity of this biochemical approach and to obtain additional data from complementary experiments to confirm the association of various signalling molecules with flotillins in specific membrane domains.

Flotillin microdomains and cytoskeletal regulation

In the previous section, we discussed evidence for flotillin involvement in a number of signalling pathways, typically invoking the co-clustering or even physical interaction of flotillins with transmembrane receptors. The presence of flotillins on the inner plasma membrane places them at the interface between signalling

Box 1. Studying flotillin microdomains – technical challenges



Detergent resistance

Flotillins are highly resistant to solubilisation in cold non-ionic detergents such as Triton X-100 and might not be completely solubilised even in more potent detergent mixtures. This means that results from co-immunoprecipitation and fractionation experiments have to be interpreted with caution.

Overexpression of flotillins

Overexpression of flotillins rapidly saturates their recruitment to flotillin microdomains, which means that flotillin–GFP constructs only reflect the distribution of the endogenous proteins when expressed at very low levels. The figure shows flotillin-1–GFP at different expression levels and highlights how high expression levels can impact upon flotillin distribution.

Epitope accessibility

Indirect immunostaining of flotillins is very sensitive to fixation conditions. We have found that only methanol fixation gives a strong and specific signal with a variety of polyclonal and monoclonal antibodies.

receptors, intracellular proteins that transduce signals and the cytoskeleton, and there is a substantial body of evidence supporting a role for flotillins in interacting with and modulating the cytoskeleton in different contexts. One such context is the establishment of leukocyte polarity, which is crucial for the spatial segregation of proteins and lipids, and their associated functions, to distinct regions of the cell. This is especially important in cells that undergo chemotaxis, such as neutrophils, in which polymerising F-actin at the leading edge is segregated from contractile actomyosin assemblies at the back of the cell. Following stimulation with chemoattractants, flotillin microdomains become rapidly redistributed to the uropod, a contractile structure at the back of migrating white blood cells (Fig. 2) (Sanchez-Madrid and Serrador, 2009). This suggests that these microdomains have some functional role in polarisation and/or chemotaxis (Rajendran et al., 2009; Rossy et al., 2009). We tested this hypothesis using flotillin-1-knockout mice and demonstrated that flotillin microdomains are indeed important for neutrophil recruitment to chemoattractants in vivo (Ludwig et al., 2010). Data from biochemical and imaging experiments imply that there is a connection between flotillin microdomains and actin-associated proteins, such as myosin IIA and spectrin, during this process. Furthermore, primary neutrophils isolated from flotillin-1-knockout mice show defects in myosin IIA activity, uropod formation, and migration through a resistive environment in vitro (Ludwig et al., 2010). Because flotillins have a similar distribution in T-cells to that in neutrophils, flotillins might have a general role in regulating leukocyte motility (Affentranger et al., 2011).

The data on flotillins and neutrophil migration fits well with several previous experiments that have implied a connection between flotillins and the cortical cytoskeleton (Neumann-Giesen et al., 2007). Overexpression of flotillins in tissue culture cells is sufficient to generate actin-rich cell protrusions or spikes (Hazarika et al., 1999; Neumann-Giesen et al., 2004). In addition, flotillins have been implicated in actin cytoskeleton regulation in the context of the lipid raft-mediated signalling platforms discussed above and have been suggested to bind to actin (Langhorst et al., 2007).

Flotillins have also been implicated in cell–cell adhesion: in zebrafish embryos, the PrP protein co-clusters with flotillins at cell contact sites, which results in the activation of Src family kinases, recruitment of adhesion molecules, such as E-cadherin, which is crucial for morphogenetic cell movements, and reorganisation of the actin cytoskeleton (Malaga-Trillo et al., 2009). As mentioned above, flotillins also cluster with antibody-patched cell adhesion molecules like Thy1 and F3 in neurons (Lang et al., 1998). In hippocampal neurons, flotillin 1 promotes formation of glutamatergic synapses (Swanwick et al., 2010b) and interacts with synaptic adhesion-like molecules (SALMs) to regulate neurite outgrowth by coordinating adhesion, exocytosis and actin cytoskeleton dynamics (Swanwick et al., 2010a).

Putting all this data together, there is now a considerable body of evidence showing that flotillins have an important role in regulating cortical actin, possibly through changing the phosphorylation of myosin IIA (Ludwig et al., 2010). Although this provides welcome progress, the specific molecular connections between the proteins remain largely obscure, and the nature of the interactions between flotillin microdomains in the plasma membrane and the underlying cytoskeletal network remains something of a ‘black box’. Further biochemical and cell biological experiments are clearly needed to resolve this issue.

Flotillin microdomains and endocytosis

A growing body of evidence links flotillin microdomains to clathrin-independent endocytosis. In the absence of a more detailed understanding of the mechanisms involved it is still unclear how this relates to the regulation of cytoskeletal dynamics described above. However, the many direct and indirect links between the actin cytoskeleton and endocytic processes raises the possibility

that there are direct mechanistic links. Here, we describe what is known about flotillins and endocytosis, focussing on recent studies.

The first data that directly implicated flotillins in endocytosis came from studies on the uptake of the GPI-anchored protein CD59 and the receptor for cholera toxin, the glycosphingolipid GM1 (monosialotetrahexosylganglioside, see below for additional detail). Knockdown of flotillin 1 in combination with the inhibition of dynamin reduce the internalisation of both CD59 and the cholera toxin B subunit (CTxB), which strongly argues for a role of flotillin 1 in the endocytosis of these two cargoes. Additional evidence for flotillins being involved in the endocytic process came from the dynamics of flotillin microdomains, which appear to bud into the cell during endocytosis (Glebov et al., 2006), and from data that showed the co-localisation of flotillins with endocytic cargoes at early time-points after internalisation (Glebov et al., 2006). Subsequently, microdomains composed of co-assembled flotillin 1 and flotillin 2 were shown to colocalise with antibody-patched GPI-anchored proteins (Frick et al., 2007) and correlative light and electron microscopy showed that flotillin microdomains coincide with membrane invaginations that are distinct from caveolae (Frick et al., 2007). Furthermore, phosphorylation of the flotillin proteins by Fyn leads to internalisation of the microdomains and a redistribution of both flotillin proteins from the plasma membrane to late endosomes and lysosomes (Babuke et al., 2009; Riento et al., 2009).

A number of studies have confirmed the link between flotillins and endocytosis (Hansen and Nichols, 2009). The basolateral internalisation of CTxB and apically-destined GPI-linked proteins in the polarised HepG2 hepatocyte cell line has been shown to depend on dynamin and flotillin 2, but not clathrin, caveolin 1, CDC42 or EPS15 (Ait-Slimane et al., 2009). Flotillins have also been shown to be important for the processing and maturation of the secreted factor Nodal by influencing the trafficking of the GPI-anchored Nodal co-receptor Cripto (officially known as TDGF1) (Blanchet et al., 2008). In this scenario, cell surface Cripto binds both Nodal and the enzymes that process Nodal, and is then internalised through flotillin microdomains to a class of endosomes that are distinct from those containing the transferrin receptor. Flotillin 1 also defines a dynamin-independent endocytic pathway that internalises the guidance cue semaphorin 3A and its receptor, which in turn regulates LIM domain kinase (LIMK1) activity, actin cytoskeleton dynamics and adhesion in cortical neurons (Carcea et al., 2010).

In addition, cationic molecules like lipids, polyamines and cell-penetrating peptides enter cells through heparan sulphate proteoglycans, and these molecules are endocytosed through a clathrin- and caveolin-independent, but flotillin-1- and dynamin-2-dependent route (Payne et al., 2007). A more recent study confirmed the finding that cationic polyplexes could be internalised through a flotillin-mediated endocytic route (Vercauteren et al., 2011).

Flotillins and endocytosis in vivo

All of the studies on flotillins and endocytosis referred to above have been carried out in mammalian cell-based systems, which are not ideally suited to discover functions that rely upon tissue contexts or intercellular interaction. A paper from Lencer and colleagues has recently established a role for flotillins in cholera intoxication of zebrafish, and this has opened the way for further experiments on flotillins in this model organism. Cholera toxin enters the cytosol of host cells through a mechanism whereby the cholera toxin subunit B (CTxB) binds to its receptor, the ganglioside GM1, at the cell surface. This causes internalisation of the toxin and its retrograde transport from endosomes, through the trans-Golgi network and finally into the endoplasmic reticulum (ER). In the ER, the A subunit of the toxin (CTxA) is retrotranslocated into the cytoplasm to exert its toxic effects. Lencer and colleagues found that flotillins are required for cholera intoxication of both zebrafish embryos and mammalian cells (Saslowsky et al., 2010). They showed that CTxB colocalises with flotillins on endosomes and at the plasma membrane in COS1 cells, but that flotillin knockdown affected neither the total amount of toxin bound to cells nor the amount internalised. However, flotillin knockdown did prevent intoxication of both zebrafish and tissue culture cells, which clearly argues for a requirement for flotillins in the transport of the toxin from the plasma membrane to the ER. The apparent paradox between a lack of effect of the flotillin knockout on total toxin uptake and a requirement for these proteins for delivery to the ER can readily be reconciled by the existence of multiple endocytic routes for cholera toxin (Massol et al., 2004), with only the flotillin-dependent one being directly coupled to trafficking to the ER.

Intracellular trafficking, but not total uptake, of Shiga toxin and ricin is also dependent upon flotillins, which raises the additional possibility that flotillins are involved in intracellular sorting of endosomal membranes during endocytosis. However, in the case of Shiga toxin and ricin, flotillin depletion intriguingly enhances toxicity (Pust et al., 2010). It is possible that flotillin-dependent trafficking of Shiga toxin and ricin provides a protective mechanism for cells by diverting these toxins away from a toxicity-producing pathway. This intriguing difference between the intracellular fates of the different types of toxin might be due to different sorting of their receptors, or differences between the cell types used for these studies.

Flotillins and endocytosis: open questions

Despite the evident connection between flotillins and endocytosis, a number of questions remain. Although overexpression of flotillins generates membrane invaginations that are reminiscent of caveolae in HeLa cells (Frick et al., 2007), this is not the case in mouse embryonic fibroblasts that lack caveolin 1 (Kirkham et al., 2008), so it is impossible to completely rule out a connection between flotillins and caveolae. In addition, in the examples discussed above, flotillin-mediated endocytosis has been demonstrated to be either dependent on or independent of dynamin. The absence of a detailed picture of the precise molecular functions of flotillins during endocytosis means that it is not clear whether this incongruity reflects different experimental conditions and approaches, varying dynamin requirements for different cargoes or cell types, or whether flotillins could be adaptors for other modes of endocytosis that differ in their dependence on dynamin action (see Fig. 3 for models of the role of flotillin microdomains in endocytosis).

The model of flotillins as specialised adaptors for other endocytic pathways has been proposed for the internalisation of cargoes such as amyloid precursor protein (APP) (Schneider et al., 2008). Initial observations that linked flotillins to APP include flotillin 1 staining of β-amyloid plaques in brain sections from patients with Alzheimer's disease and the finding that Aβ overproduction leads to the accumulation of Aβ in flotillin-1-positive endosomes (Rajendran et al., 2007). The intracellular domain of APP has been shown to interact with flotillin 1 (Chen et al., 2006). Further studies showed that, both in neuronal cells and primary hippocampal neurons, flotillin and membrane cholesterol enhance

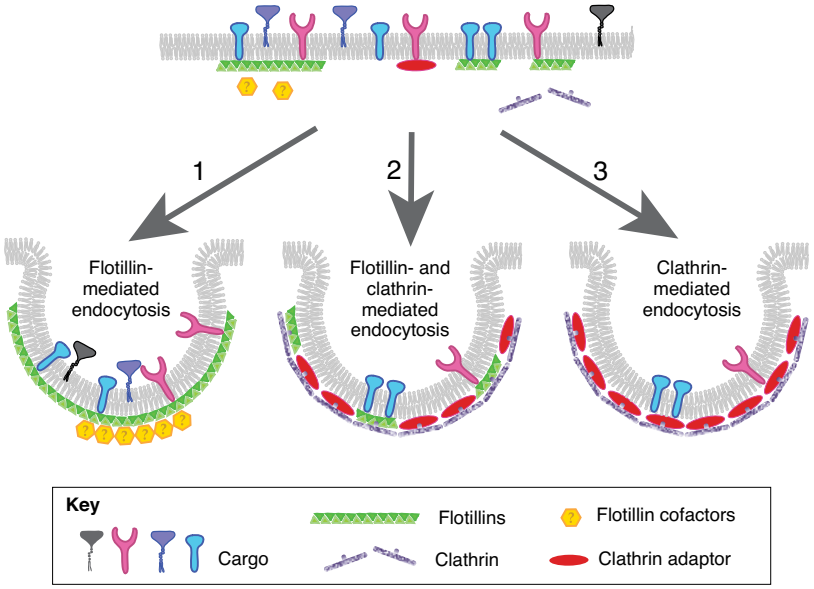


Fig. 3. Models of flotillin function in endocytosis. Although there is now a considerable body of evidence supporting a role for flotillins in endocytosis, the mechanistic details of how they act are still not clear. Recent data suggests that endocytosis of the membrane proteins NPC1L1, APP and DAT (SLC6A3), all previously believed to be internalised through clathrin-coated pits, is dependent on flotillin (Cremona et al., 2011; Ge et al., 2011; Schneider et al., 2008). This can be explained in different ways as depicted here. Either the proteins (cargo) could be internalised through separate pathways, one of which is flotillin dependent (1) and one of which is clathrin dependent (3), or flotillins could somehow act as adaptors for clathrin-mediated endocytosis of some cargoes (2). There is no direct evidence in support of this latter possibility, and the lack of colocalisation between flotillins and clathrin argues against it. However, owing to the lack of conclusive experimental evidence it is probably wise to be cautious in advancing one model over another. It should also be noted that flotillins might act on endocytic trafficking pathways after internalisation from the plasma membrane (Pust et al., 2010).

APP endocytosis, whereas flotillin knockdown reduces APP endocytosis and amyloidogenic processing (Schneider et al., 2008). Because there is a substantial body of data showing that APP is internalised through clathrin-coated pits, the authors of that study suggested that flotillins residing in cholesterol-rich microdomains serve to cluster cell surface APP and somehow render these clusters competent for internalisation in coated pits (Schneider et al., 2008).

New insights and new cargoes

A recent paper on flotillin-dependent endocytosis in a different context provides some support for the model of flotillins as mediators of recruitment to coated pits. Dietary cholesterol absorption in mammals is mediated by NPC1L1, a polytopic transmembrane protein that is expressed in intestinal and liver cells (Altmann et al., 2004; Davis et al., 2004). NPC1L1 is trafficked to the plasma membrane when cholesterol is depleted and is internalised through clathrin-mediated endocytosis when it is replenished (Ge et al., 2008). Song and colleagues have shown that flotillins are important in NPC1L1-mediated cholesterol uptake, both in vitro (in a model hepatic cell line) and in vivo (in mice) (Ge et al., 2011). They showed that flotillins and NPC1L1 interact and, at least when overexpressed, are trafficked along the same pathway between the plasma membrane and endosomes during cholesterol depletion and replenishment. In addition, flotillin knockdown diminishes cholesterol and NPC1L1 internalisation upon cholesterol replenishment and diminishes the interaction of NPC1L1 with clathrin and AP2, which suggests that flotillins have a role upstream of these events. From these data, the authors suggest a model whereby cholesterol-rich flotillin microdomains are recognised by NPC1L1 [perhaps by binding directly to cholesterol (Zhang et al., 2011)], which recruits them to clathrin-coated pits for endocytosis.

An additional study provides new evidence for the importance of flotillin microdomains during endocytosis and identifies new protein cargoes that might be internalised in a flotillin-dependent fashion. Yamamoto and colleagues studied endocytosis of two neurotransmitter transporters, the dopamine transporter [DAT, officially known as solute carrier family 6, member 3 (SLC6A3)] and the glial glutamate transporter (EEAT2, also known as GLT1 and SLC1A2), which modulate the strength of neurotransmission by regulating neurotransmitter levels at synapses (Cremona et al., 2011). Intracellular signalling pathways can modulate the plasma membrane levels of these transporters, which results in transporter internalisation and trafficking to recycling compartments or lysosomes (Melikian, 2004). Protein kinase C (PKC)-triggered internalisation of DAT and EEAT2 is dependent on flotillin 1, and, in particular, PKC phosphorylation of Ser315 on flotillin 1 is required for DAT internalisation. These findings were confirmed in murine primary dopaminergic neuronal cultures in which flotillin 1 was silenced with small hairpin RNA (Cremona et al., 2011). In addition, the amphetamine-mediated efflux of dopamine through DAT was inhibited upon flotillin 1 knockdown in this system, which indicates that flotillin-mediated DAT localisation is also important for this process.

Like APP and NPC1L1, DAT has been reported to be internalised through clathrin-coated pits (Sorkina et al., 2005). Therefore, the results described above again cannot rule out the possibility that flotillin microdomains act to increase the efficiency of clathrin-mediated endocytosis by concentrating specific molecules [either APP (Schneider et al., 2008), NPC1L1 (Ge et al., 2011) or DAT (Cremona et al., 2011)] in specific parts of the plasma membrane. However, there is little, if any, colocalisation between flotillins and clathrin (Glebov et al., 2006), and all of the relevant experiments have been performed using incomplete loss-of-function approaches such as using short interfering RNA (siRNA). It is possible, or even likely, that there are multiple pathways for endocytosis of any one of the cargoes, complicating analysis, particularly as there is no direct evidence as yet that APP, NPC1L1 or DAT are found exclusively (or even predominantly) within flotillin microdomains in the plasma membrane. The model of flotillin microdomains as facilitators of clathrin-mediated endocytosis predicts that complexes between flotillin and APP, NPC1L1 or DAT, respectively, must coincide spatially and temporally with clathrin-coated pits before internalisation (Fig. 3). Thus, it would be useful to demonstrate in future experiments that APP or NPC1L1 are transferred from flotillin microdomains into clathrin-coated pits, for example, by showing transient colocalisation of the three molecules at the plasma membrane.

Perspectives

It is becoming apparent that flotillin microdomains have different functions in a multitude of cell types and tissues, but two major themes are emerging. First, flotillin microdomains, either directly or indirectly, are intricately associated with the cortical cytoskeleton. Flotillin microdomain dynamics depend on the actin cytoskeleton, and flotillins regulate the activity of cytoskeletal proteins like myosin II. Second, several recent studies provide more concrete evidence that flotillins are directly involved in endocytosis. In addition, the role of flotillins as signalling platforms has also become better established, and it is possible that, in this context, the flotillins perform their signalling role through endocytosis and their interactions with the cytoskeleton.

The major challenge in the study of flotillin proteins is still the need to understand the molecular mechanism of their action in endogenous cellular and tissue contexts. Is there a single unifying mechanism of action or can these microdomains function in fundamentally different ways under different conditions? Future studies could address these types of question by using a plethora of tools to assess different aspects of the problem. Studying flotillins, like caveolins, can be challenging because of the particular biophysical and biochemical properties of these proteins (Box 1). This class of proteins associates tightly with the inner leaflet of the plasma membrane, which precludes the use of classical membrane-impermeant tools, such as antibodies, to study their endocytosis and dynamics. Additionally, they form homo- and hetero-oligomers that are resistant to detergent solubilisation, and consequently it has proven difficult to produce recombinant proteins for biochemical studies, for example, to demonstrate direct binding of flotillins to potential binding partners. This resistance to solubilisation with detergents means that care needs to be taken to ensure that artefactual association of flotillins and membrane proteins is ruled out by careful controls for specificity and by complementary experimental approaches. Despite these challenges, progress is being made in studying the roles of flotillins, and we envisage substantial advances in our understanding of flotillin function now that flotillin-knockout mice are available and zebrafish can be used as an additional model system to study the functions of flotillin in different cellular processes.

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