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COMMENTARY

Flotillins in intercellular adhesion – from cellular physiology to human diseases

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ABSTRACT

Flotillin 1 and 2 are ubiquitous and highly conserved proteins. They were initially discovered in 1997 as being associated with specific caveolin-independent cholesterol- and glycosphingolipid-enriched membrane microdomains and as being expressed during axon regeneration. Flotillins have a role in a large number of physiopathological processes, mainly through their function in membrane receptor clustering and in the regulation of clathrin-independent endocytosis. In this Commentary, we summarize the research performed so far on the role of flotillins in cell–cell adhesion. Recent studies have demonstrated that flotillins directly regulate the formation of cadherin complexes. Indeed, flotillin microdomains are required for the dynamic association and stabilization of cadherins at cell–cell junctions and also for cadherin signaling. Moreover, because flotillins regulate endocytosis and also the actin cytoskeleton, they could have an indirect role in the assembly and stabilization of cadherin complexes. Because it has also recently been shown that flotillins are overexpressed during neurodegenerative diseases and in human cancers, where their upregulation is associated with metastasis formation and poor prognosis, understanding to what extent flotillin upregulation participates in the development of such pathologies is thus of particular interest, as well as how, at the molecular level, it might affect cell adhesion processes.

KEY WORDS: Flotillin, Microdomain, Cadherin, Adhesion

Introduction

Adherens junctions, one of the four types of cell–cell junctions, form adhesive contacts that maintain the tissue architecture and, at the same time, facilitate cell movement. They are formed and remodeled repeatedly throughout development and adult life. Perturbation of this process is associated with cancer cell invasion and metastasis formation (Berx and van Roy, 2009; Gumbiner, 2005; Halbleib and Nelson, 2006; Harris and Tepass, 2010). Cell–cell junctions occur both in non-epithelial and epithelial tissues, where they are located just below tight junctions, and are composed of transmembrane proteins, the cadherins (see Box 1). The best-studied classical cadherins are type I and type II cadherins that are found in vertebrates and urochordates (Harris and Tepass, 2010). They undergo homophilic interactions with cadherins on adjacent cells through their extracellular domains and interact with catenins through their cytoplasmic tail. Cadherin complexes also associate with other proteins to form the cadherin adhesome, or 'cadhesome', which includes proteins that colocalize with cadherins, interact with cadherins or catenins and/or are involved in cadherin assembly or dynamics at cell–cell junctions (Zaidel-Bar, 2013). Our knowledge on the cadhesome has increased extensively over the past few years, as has the importance of the lipid membrane microenvironment in regulating the dynamic properties of the cadhesome (Causeret et al., 2005; Márquez et al., 2012; Taulet et al., 2009). Interestingly, flotillins, which recently emerged as being part of cadherin complexes, laterally assemble cholesterol- and sphingolipid-rich membrane microdomains, making them potential key players in organizing the membrane environment around cadherin complexes.

In this Commentary, we attempt to highlight the existing literature on the role of flotillins in intercellular adhesion. First, we present some background on flotillin proteins and their cellular distribution before portraying the relationship between flotillin structure and the mechanisms of flotillin heterooligomerization and association with the plasma membrane. We also discuss the role of flotillins in cadherin-mediated cell–cell adhesion, emphasizing both their direct and indirect roles in the assembly and stabilization of cadherin complexes. This will be illustrated in different pathological contexts where flotillins have recently been shown to be overexpressed.

Flotillin structure and cellular distribution

Flotillin expression

Flotillins 1 and 2 are two highly conserved membrane proteins that are strongly structurally and functionally related. They have a molecular mass of ~48 kDa and their amino acid sequences show about 50% identity (Rivera-Milla et al., 2006). Owing to their ability to heterooligomerize, they fulfill most of their cellular functions in a mutually dependent manner, notably by forming molecular scaffolds in cholesterol-rich membrane microdomains. Flotillins are currently considered to be protein markers of these membrane microdomains, isolated as lipid rafts (Bickel et al., 1997; Otto and Nichols, 2011). The name flotillin is now most commonly used, but, and this could be a source of confusion, flotillin 1 and 2, are also known as reggie 2 and 1, respectively, owing to their involvement in axon regeneration (Munderloh et al., 2009; Schulte et al., 1997).

Flotillin-related proteins are found in bacteria, fungi and plants (Hinderhofer et al., 2009; Morrow and Parton, 2005), but are absent from *Caenorhabditis elegans* and budding yeast. Flotillins have been detected in all vertebrates tested so far; here, they are encoded by two different genes that are highly conserved among species (e.g. 98% identity between the mouse and human flotillin 1 amino acid sequences). In mammals, flotillins are ubiquitously expressed but are enriched particularly in the nervous system, muscle and adipose tissue, and erythrocytes (Bickel et al., 1997; Volonte et al., 1999). Taken together, these observations suggest that flotillins have a fundamental role in cellular physiology, and

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Box 1. The cadherin family

The cadherin family of cell–cell adhesion proteins contains more than 100 molecules with diverse protein structure and includes the classical cadherins, desmosomal cadherins, protocadherins and atypical cadherins (Fat, Dachsous, and Flamingo). Members of the cadherin family are defined by a common Ca^{2+}-binding extracellular cadherin (EC) domain, a 110-residue β-fold domain. Classical cadherins (referred here to here as cadherins), which are subdivided into type I and type II, are single transmembrane-spanning proteins with five EC domains in their extracellular region and a highly conserved cytoplasmic tail that interacts with the armadillo family members p120-catenin and β-catenin. Cadherin–β-catenin complexes are linked to the actin cytoskeleton through α-catenin, which directly binds to actin but, when stretched, they also bind to vinculin, another actin-binding protein. Type I cadherins promote strong cell–cell adhesion and have a conserved HAV tripeptide motif in their EC1 domain. They comprise six members, among which epithelial (E-), neuronal (N-) and placental (P-) cadherins are the most studied. Type II cadherins lack this motif and comprise members whose function is less known, except for vascular (VE)-cadherin. Cadherins form adherens junctions that have important functions in epithelial integrity and tissue morphogenesis, and their deregulation is linked to cancer and other diseases.

Desmosomal cadherins, desmogleins and desmocollins have a domain organization that is similar to cadherins, but are connected to the intermediate filament cytoskeleton through desmoplakin, which associates with the armadillo family members plakophilins and plakoglobin (also called γ-catenin), which also associates with classical cadherins. Desmosomes are formed by a uniform large desmosomal cadherin cluster, a structure that allows for strong cell–cell adhesion in tissues that are exposed to high mechanical stresses.

this has been supported by many studies describing flotillin involvement in cellular trafficking, signaling, cytoskeleton remodeling and adhesion. Although flotillins appear to be crucial for all these processes, their deficiency might be bypassed by compensatory mechanisms, as flotillin-knockout mice are viable and do not show any major phenotype (Banning et al., 2012; Bitsikas et al., 2014; Ludwig et al., 2010).

Cellular distribution of flotillins

The cellular distribution of flotillins is highly diverse and dynamic, depending on the cell type (Browman et al., 2007) and extracellular stimuli (Langhorst et al., 2008b; Neumann-Giesen et al., 2007; Riento et al., 2009). Flotillin 1 and 2 almost always colocalize in membrane compartments. They are present at the plasma membrane, where they accumulate at cell–cell contacts and in actin-driven mobile membranes, such as lamellipodia and ruffles (Browman et al., 2007; Guillaume et al., 2013). In leukocytes, flotillins are excluded from these structures and instead concentrate in the uropod where they participate in the regulation of the contractile actin network (Affentranger et al., 2011; Langhorst et al., 2007; Rossy et al., 2009). Flotillins can also be associated with many intracellular organelles, such as the Golgi (Langhorst et al., 2008b), endosomes (Gagescu et al., 2000), lysosomes, multi-vesicular bodies (Langhorst et al., 2008b), phagosomes (Dermine et al., 2001), lipid droplets and exosomes (de Gassart et al., 2003; Strauss et al., 2010). The mechanisms that regulate the dynamic partitioning of flotillin between the plasma membrane and intracellular compartments are still poorly understood. Flotillin 1 and flotillin 2 co-assemble to form microdomains that can induce the formation of intracellular vesicles and of plasma membrane invaginations, which are distinct from caveolae- and clathrin-coated pits (Frick et al., 2007; Glebov et al., 2006; Lang et al., 1998). These observations led to the hypothesis that flotillins might define a new, caveola- and clathrin-independent, endocytosis pathway (Otto and Nichols, 2011). So far, it has been shown that flotillin translocation from the plasma membrane into endosomes can be induced by epidermal growth factor (EGF) stimulation in HeLa cells (Neumann-Giesen et al., 2007; Riento et al., 2009). Moreover, flotillin 1 and flotillin 2 have to form heterooligomers in order to translocate into intracellular compartments (Babuke et al., 2009). Phosphorylation could also regulate flotillin endocytosis. In particular, Fyn phosphorylates flotillin 1 on Y160 and flotillin 2 on Y163 (Riento et al., 2009), and EGF-induced endocytosis of flotillins is blocked in cells that express a flotillin 2 Y163F mutant (Riento et al., 2009). However, other studies have reported that this effect is not due to the lack of phosphorylation on the Y163 residue in this flotillin 2 mutant but is instead due to the inability of this mutant to oligomerize with flotillin 1, demonstrating that flotillin-1–flotillin-2 heterooligomerization is required for their endocytosis (Babuke et al., 2009; Neumann-Giesen et al., 2007). Dynamin-2 appears to be involved in the endocytosis of flotillins (Meister et al., 2014). Moreover, flotillin trafficking between the plasma membrane and intracellular compartments could be sensitive to the amount of cholesterol. Indeed, in Oli-neu cells [oligodendroglial precursors O-2A immortalized by transfection with the t-neu tyrosine kinase (Jung et al., 1995)], it has been shown that an increase in cholesterol leads to the intracellular translocation of flotillins and, reciprocally, cholesterol depletion drives flotillins to the plasma membrane (Strauss et al., 2010). Therefore, although it is now clear that flotillin endocytosis is dependent on the formation of heterooligomers, the mechanisms that regulate flotillin endocytosis and its anterograde trafficking towards the plasma membrane need to be further investigated, as well as the roles of flotillin in intracellular compartments.

Flotillin structure and assembly mechanisms

Flotillins are clearly involved in many cellular processes; however, the molecular mechanisms that underlie their different functions remain poorly understood. Both flotillins primarily consist of two parts: the N-terminal moiety that is mainly responsible for membrane interaction, and the C-terminal moiety that is important for flotillin oligomerization (Solis et al., 2007) (Fig. 1A).

The N-terminal part includes the SPFH (stomatin, prohibitin, flotillin, HflK/C) domain [also known as the prohibitin homology (PHB) domain] that spans from amino acids 5 to 183 in human flotillin 1, and from amino acids 7 to 183 in human flotillin 2 (Morrow et al., 2002). The features of the SPFH domain suggest that it could interact with cholesterol-rich membrane microdomains. Indeed, in flotillin 1, the SPFH domain is palmitoylated on C34, a post-translational modification that is crucial for flotillin 1 localization in lipid rafts (Morrow et al., 2002) and is important, but not essential, for its association with membranes. Flotillin 2 myristoylation on G2 of the unstructured motif upstream of the SPFH domain is essential for its membrane association, and crucial for the subsequent palmitoylation of C4 that enables flotillin 2 localization in lipid rafts (Neumann-Giesen et al., 2004). Flotillin 2 can also be palmitoylated on C19 and C20

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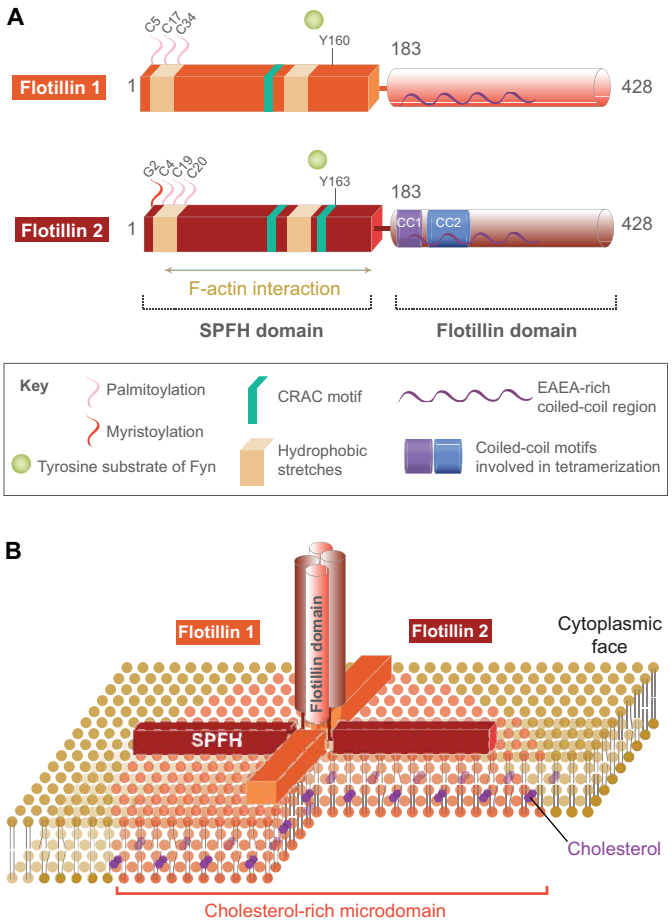

Fig. 1. Structural features of flotillins. (A) Linear representation of flotillin 1 and 2. The main functional motifs and residues identified are indicated. Only the coiled-coil (CC) motifs that have been shown experimentally to be involved in the association between flotillins monomers are represented, that is CC1 (amino acids 184–238) and CC2 (amino acids 239–321) in flotillin 2. (B) Schematic representation of a flotillin tetramer formed by coiled-coil interaction between the flotillin domains, and of its association with the plasma membrane in cholesterol-rich micro-domains through the SPFH domains.

but to a lesser extent (Neumann-Giesen et al., 2004). The SPFH domain also contains two hydrophobic stretches that could mediate its interaction with the inner leaflet of the plasma membrane (Rivera-Milla et al., 2006); however, this has never been demonstrated. The SPFH domain also contains putative cholesterol recognition amino acid consensus (CRAC) motifs (Roitbak et al., 2005) outside of the hydrophobic stretches, and these might participate in mediating the interaction of flotillins with membranes, and more specifically, with lipid rafts. Interestingly, a recent study in Oli-neu cells has shown that mutations of the CRAC motifs influence flotillin 2 localization by preventing its intracellular translocation (Strauss et al., 2010). However, it has not been tested whether this effect is due to the inability of this mutant to bind to cholesterol and to associate with lipid rafts. The SPFH domain might also contain some determinants that regulate flotillin heterooligomerization, although this function is mostly dependent on the C-terminal part that contains the flotillin domain (described below). For instance, it has been shown that the Y163 residue (located within the second CRAC motif of flotillin 2) is crucial for flotillin heterooligomerization, either per se (Babuke et al., 2009) or once phosphorylated (Riento et al., 2009). Although still speculative and never directly tested, the level of flotillin heterooligomerization could be influenced by the interaction of flotillin with cholesterol, notably through flotillin 2 Y163, possibly in its phosphorylated form.

The C-terminal part of flotillins (amino acids 180–353 in flotillin 1 and amino acids 184–353 in flotillin 2) includes the so-called flotillin domain that is only found in flotillin 1 and 2 and that is characterized by the presence of 25 heptad (i.e. 7 amino acids) tandem arrays rich in glutamate-alanine (EA), which are the typical structural units of coiled-coil structures. In flotillin 2, three coiled-coil (CC1–CC3) stretches have been predicted to exist and to be responsible for flotillin oligomerization (Rivera-Milla et al., 2006; Solis et al., 2007). Using a cross-linking approach in cells expressing different mutants of flotillins, only CC2 (amino acids 239–321) and, to a lesser extent, CC1 (amino acids 184–238) appeared to be important for tetramerization (Solis et al., 2007) (Fig. 1A). In cells, flotillins are mainly present as monomers and as highly stable hetero-tetramers that are composed of two flotillin 1 and two flotillin 2 molecules (Solis et al., 2007) (Fig. 1B). Hetero-tetramers appear to be the predominant form compared to homo-tetramers. This might be due to the fact that flotillin 1 is unstable when it is not associated with flotillin 2 (Guillaume et al., 2013; Langhorst et al., 2005). Membrane association, through the SPFH domain, could facilitate flotillin tetramerization, and flotillin-1–flotillin-2 hetero-tetramers might then assemble into larger complexes in order to fulfill their role as a scaffold. However, how the flotillin tetramers form large oligomeric platforms remains unclear as an involvement of the flotillin domain has not been demonstrated. Because the SPFH domains of stomatins, from which the SPFH domain has its name, interact directly to form dimers (Brand et al., 2012) or trimers (Kuwahara et al., 2009), one can hypothesize that flotillin tetramers also interact with each other through SPFH domains. Interestingly, the SPFH domain of flotillin 2 can also bind to F-actin without promoting F-actin polymerization (Langhorst et al., 2007). This interaction regulates the lateral motility of flotillin microdomains and could influence the formation of flotillin platforms and their scaffolding functions. Accordingly, actin-depolymerizing drugs increase the motility of flotillin microdomains (Affentranger et al., 2011; Langhorst et al., 2007; Rossy et al., 2009). In addition, protein adaptors from the vinexin and CAP/ponsin family, which are connected to the actin cytoskeleton and to the nectin–afadin and cadherin adhesive complexes, bind to flotillins through their sorbin homology (SoHo) domain (Kimura et al., 2001). These scaffolding proteins might therefore also participate in the assembly of flotillin hetero-tetramers.

Direct roles of flotillins in cadherin-mediated cell–cell adhesion

It is thought that flotillin hetero-tetramer oligomerization promotes the clustering of cholesterol-rich microdomains (for which flotillins exhibit a high affinity) to define a subpopulation of lipid rafts. Cadherin complexes have been shown to be part of membrane protein complexes that require a cholesterol-rich environment for their formation and function (Causeret et al., 2005; Taulet et al., 2009). Whether flotillins participate in the assembly of cadherin complexes, notably by creating a cholesterol-rich membrane environment has recently been investigated.

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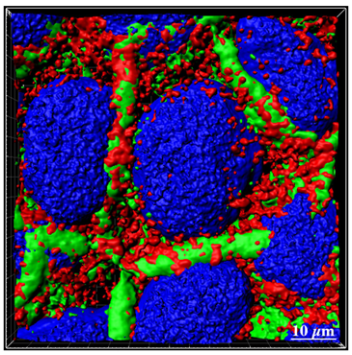

Fig. 2. Flotillins colocalize with E-cadherin at cell–cell junctions. The image shows E-cadherin (green) and flotillin 2 (red) expression in confluent epithelial Caco-2 cells (nuclei are in blue). As can be clearly seen, most of the flotillin colocalizes with E-cadherin at cell–cell junctions. The image stack was recorded using a Leica SP5 confocal microscope and images were visualized using the volume rendering command of the Imaris software.

Many studies have reported that flotillins are localized at cell–cell contact sites in different cell types (Babuke et al., 2009; Fernow et al., 2007; Liu et al., 2005; Málaga-Trillo et al., 2009; Morrow et al., 2002; Roitbak et al., 2005; Solis et al., 2007; Stuermer et al., 2001) (illustrated for Caco-2 cells in Fig. 2), as well as *in vivo* (von Philipsborn et al., 2005). However, the first demonstration for a role of flotillins in the regulation of cell–cell adhesion came from a study that used *Drosophila* mutants (Hoehne et al., 2005). The authors showed that the *Flotillin-2*-null mutant (in which flotillin 1 is also degraded) has no phenotypic defects, whereas overexpression of either flotillin 2 alone or both flotillin 1 and 2 perturbs the distribution of cell–cell adhesion molecules of the immunoglobulin (Ig) family (Rst and Kirre; NEPH or Kirrel in mammals) and their ligand Sns (nephrin in mammals) at cell boundaries (Hoehne et al., 2005). Subsequently, several research groups demonstrated, by using various cell lines, that flotillins are important regulators of cadherin-mediated adherens junctions, a major and conserved intercellular adhesive complex. For instance, a few years ago, it was shown that flotillin 1 is required for the recruitment of E-cadherin and p120-catenin (also known as catenin δ1) to cholesterol-rich membrane domains (Chartier et al., 2011), an important step for stabilization of the cadherin complex at cell–cell junctions (Causeret et al., 2005; Taulet et al., 2009). Then, in 2013, we and others reported that, in mesenchymal, as well as in normal and tumor epithelial cells, flotillins accumulate at cell–cell junctions, where they colocalize with cadherins and catenins (Guillaume et al., 2013; Kurrle et al., 2013). Although flotillins co-immunoprecipitate with N- and E-cadherin complexes, the interaction mechanism remains to be characterized. Knockdown experiments using specific flotillin 1 or 2 short hairpin RNAs (shRNAs) demonstrated that flotillins are major regulators of N- and E-cadherin stabilization at cell–cell junctions, both in mesenchymal and epithelial cells (Guillaume et al., 2013; Kurrle et al., 2013; Solis et al., 2012). Furthermore, flotillins are required for the formation of functional cell–cell junctions, which is necessary to allow cadherin-dependent signaling, which, in turn, controls myoblast differentiation and fusion, and epithelial cell–cell junction integrity and functionality (Guillaume et al., 2013), as well as enterocyte differentiation (Chartier et al., 2011). Our study also showed that flotillins have an important role in the scaffolding of cadherin complexes in cholesterol-rich plasma membrane domains containing the ganglioside GM1 (Guillaume et al., 2013) (Fig. 3). This might directly influence the function of cadherins because the binding strength of homophilic cadherin interactions is only low and, thus, in order to allow effective adhesion, a large number of cadherins have to cooperate through the formation of cadherin clusters (Hong et al., 2010; Truong Quang et al., 2013). Interestingly, afadin, which associates with the flotillin-1-binding protein CAP/ponsin, as well as with α-catenin and p120-catenin (Hoshino et al., 2005; Pokutta et al., 2002; Tachibana et al., 2000), strengthens cadherin-mediated adhesive clusters (Indra et al., 2014). Thus, scaffolding of the cadherin clusters by flotillins could be one of the mechanisms used by cells to regulate the size of cadherin clusters. Indeed, and as mentioned above, the clustering of cholesterol-rich microdomains that is promoted by flotillin oligomerization also regroups protein complexes that are present in the same microdomains, as we have shown for cadherins (Guillaume et al., 2013).

Finally, interactions between flotillin 1 or 2 and γ-catenin (also known as junction plakoglobin, JUP) have been demonstrated in immunoprecipitation experiments performed on MCF10A epithelial cells and *in vitro* by using purified proteins (Kurrle et al., 2013) (Fig. 3). Because γ-catenin is preferentially localized in desmosomes (see Box 1) (Nekrasova and Green, 2013), this interaction suggests that flotillins could also regulate desmosome assembly and/or function, an issue that, however, remains to be precisely addressed.

Indirect roles of flotillins in cell–cell adhesion regulation

Besides their direct involvement in the regulation of cadherin-mediated intercellular adhesion, flotillins could also have an indirect role in the assembly and stabilization of cadherin complexes, because they regulate endocytosis and are associated with, and might affect, the actin cytoskeleton dynamics.

Effect of flotillins on cadherin endocytosis

Cadherin-based cell–cell contacts are dynamic adhesive structures, and cadherin trafficking and turnover to and from the plasma membrane have an important role in their behavior (Baum and Georgiou, 2011; Le et al., 1999; Schill and Anderson, 2009; Yap et al., 2007). Cadherins are internalized from the plasma membrane through several routes, such as clathrin- or caveolae-mediated endocytosis, as well other internalization routes that are independent of both clathrin and caveolae, such as lipid-raft-mediated endocytosis and macropinocytosis (Harris and Tepass, 2010). As flotillins are involved in the endocytosis of various membrane proteins, they could also affect cadherin trafficking. Indeed, flotillins regulate the internalization of cargos that are endocytosed in a clathrin-independent manner, such as of the glycophosphatidylinositol (GPI)-anchor protein CD59 and the ganglioside GM1 (Aït-Slimane et al., 2009; Carcea et al., 2010; Frick et al., 2007; Glebov et al., 2006; Otto and Nichols, 2011; Saslowsky et al., 2010), but they also regulate cargos that are endocytosed in a clathrin-dependent manner, such as amyloid precursor protein (APP), dopamine transporter (DAT, also known as SLC6A3) and Niemann-Pick C1-like protein 1 (NPC1L1) (Sannerud et al., 2011; Cremona et al., 2011; Ge et al., 2011; Schneider et al., 2008). Although it has been hypothesized that flotillins might define an endocytic pathway that is independent of clathrin and caveolin (Frick et al., 2007; Glebov et al., 2006), it is still not entirely clear whether flotillins also regulate or

facilitate clathrin-dependent endocytosis by concentrating the cargos at the membrane. Indeed, although the knockdown of flotillins attenuates the binding of the clathrin adaptor AP2 and of clathrin to the transmembrane protein NPC1L1 to mediate its endocytosis (Ge et al., 2011), a colocalization of flotillins with clathrin could not be detected (Glebov et al., 2006). Flotillins are located at and define plasma membrane lipid micro-domains that are enriched in cholesterol and GM1 gangliosides (Bickel et al., 1997; Fernow et al., 2007; Frick et al., 2007; Stuermer et al., 2001), which participate in key steps of endocytosis (Johannes and Mayor, 2010). The interaction of flotillins with various membrane proteins appears to be dependent on cholesterol and, for instance, cadherins and APP are associated with gangliosides (Ge et al., 2011; Guillaume et al., 2013; Schneider et al., 2008; Yanagisawa et al., 1995). In neuroblastoma N2a cells and hippocampal neurons, APP clustering is promoted by flotillins and stimulates its subsequent endocytosis (Schneider et al., 2008). This suggests that APP internalization occurs in flotillin microdomains; however, in flotillin-1-null mouse embryonic fibroblasts, APP clustering is not significantly affected (Bitsikas et al., 2014). Moreover, flotillin clustering promotes the formation of a cholesterol-rich environment that favors endocytosis of NPC1L1 and of flotillin itself (Ge et al., 2011).

Despite these observations, a definitive role for flotillins in cadherin endocytosis remains to be demonstrated. Although flotillins clearly mediate the clustering or the stabilization of various proteins, such as APP (Schneider et al., 2008), EGF receptor (EGFR) (Amaddii et al., 2012) and the ligand-less EGFR family member ErbB2 (Pust et al., 2013) at the plasma membrane, under some circumstances, they could also induce their endocytosis. It will therefore be important to elucidate the molecular pathways that govern the functions of flotillin in either stabilization or endocytosis of plasma membrane proteins.

Flotillin-dependent regulation of cadherin recycling

In addition to their potential involvement in cadherin endocytosis, flotillins have also been shown to affect cadherin recycling, at least in human A431 carcinoma epidermoid cells (Solis et al., 2013). Here, E-cadherin has been detected in tubulovesicular recycling compartments that are positive for Rab11, SNX4 and flotillin 2 (Fig. 3), and E-cadherin recycling from the perinuclear vesicular compartments to the plasma membrane is impaired upon flotillin 2 knockdown. Although of potential interest, it is not known whether this flotillin-dependent cadherin recycling mechanism exists in other cell types. It is noteworthy that A431 cells overexpress EGFR and this could affect endocytosis and recycling of cadherin and flotillin (Bryant et al., 2007; Meister et al., 2014). Moreover, we never observed such a mechanism in the cell lines we tested.

Possible role of flotillins in Src-family-kinase-dependent regulation of cadherin complexes

Phosphorylation is also important for the regulation of adherens junction dynamics, although the exact events are still not very well known (Bertocchi et al., 2012; Pust et al., 2013). Src family kinases, which are recruited to cell–cell junctions upon cadherin binding, are regulators of cadherin-mediated adhesion and also affect flotillin function (Babuke et al., 2009; Bertocchi et al., 2012; Neumann-Giesen et al., 2007; Riento et al., 2009). In particular, Src co-immunoprecipitates with flotillin 2 in HeLa cells (Neumann-Giesen et al., 2007), and phosphorylation of flotillins by Fyn induces their endocytosis (Riento et al., 2009). In addition, Fyn induces E-cadherin internalization (Smyth et al., 2012) through an unknown mechanism, and it would thus be very interesting to determine whether flotillins indeed have a role in this process.

Possible links of flotillins to the actomyosin cytoskeleton

Rho GTPase activation and actin remodeling also need to be considered as possibly being affected by flotillins. As the actin

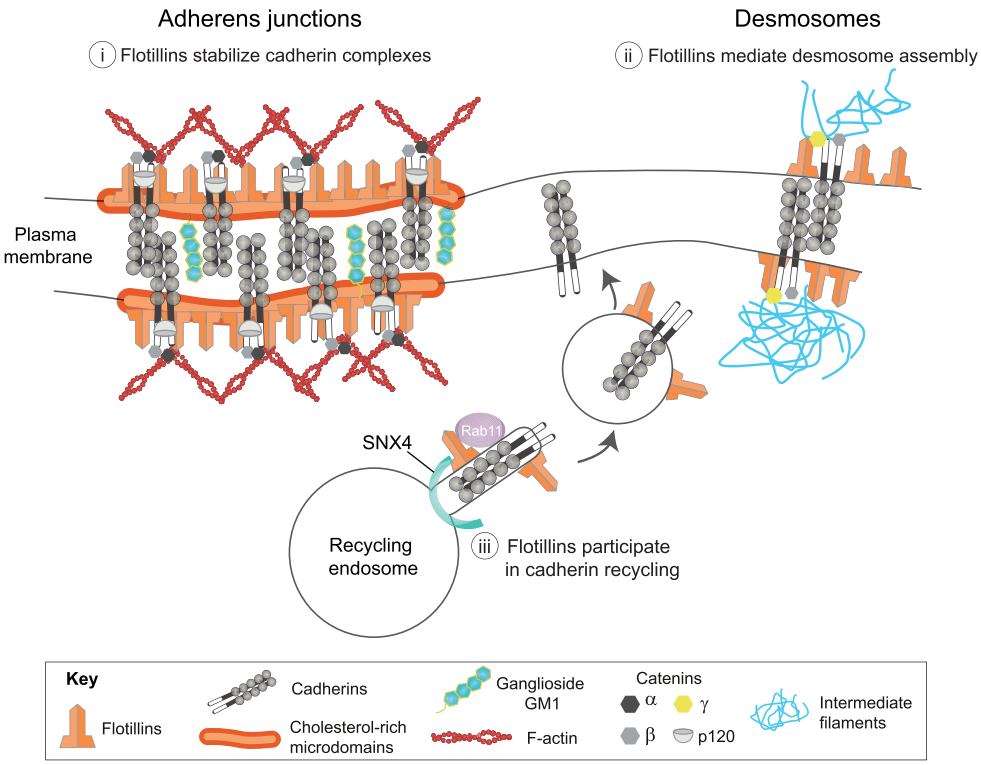

Fig. 3. Possible roles of flotillins in cadherin-mediated intercellular adhesion. Flotillins are involved in regulating cadherin-mediated intercellular adhesion at three levels: (i) localization of cadherin complexes in cholesterol-rich membrane microdomains and their stabilization at these, forming cell–cell contacts to allow the formation of mature adherens junctions; (ii) cadherin-mediated desmosome assembly through interaction with γ-catenin; and (iii) cadherin recycling via Rab11- and sorting-nexin 4 (SNX4)-positive tubulovesicular structures.

cytoskeleton plays a major role in cadherin cluster assembly, stability, movement and fission (Hong et al., 2013; Truong Quang et al., 2013), flotillins could participate in some of these steps. Indeed, flotillin 2 associates directly with F-actin through its N-terminal SPFH domain (Langhorst et al., 2007). F-actin modulates flotillin function in leukocytes because disruption of F-actin prevents chemoattractant-induced formation of large flotillin scaffolds at the cell rear (Affentranger et al., 2011; Langhorst et al., 2007; Rossy et al., 2009). Flotillins could also be involved in remodeling of the F-actin cytoskeleton and in Rho GTPase activation (Koch et al., 2013; Langhorst et al., 2008a; Munderloh et al., 2009; Neumann-Giesen et al., 2007). Moreover, in neutrophils, flotillin microdomains associate with the actin cortical cytoskeleton and regulate the activity of myosin II (Ludwig et al., 2010), a key mediator of cortical tension. Through these links with actomyosin-mediated contractility, flotillins could influence cadherin adhesive complexes. In this context, it is known that cadherins can transmit cell tension through interactions with the actin cytoskeleton (Lecuit and Lenne, 2007), and several reports support the idea that the composition of the E-cadherin complex might be regulated by tension (Ladoux et al., 2010; Leckband et al., 2011).

Disease relevance of flotillin-mediated roles at adhesions

The expression of flotillins is upregulated in human pathologies, such as cancer (Hazarika et al., 2004; Li et al., 2014; Rickman et al., 2008; Zhang et al., 2013; Zhu et al., 2013) and neurodegenerative diseases (Kokubo et al., 2000) (Fig. 4), which are pathologies in which cadherin-mediated adhesion has a prominent role, but only a little is known about the mechanisms underlying flotillin upregulation. Flotillin 2 is a target gene of p63 and p73, members of the p53 transcription factor family (Sasaki et al., 2008). Flotillin promoters are activated by mitogen-activated protein kinase (MAPK) signaling, retinoic acid treatment and expression of various transcription factors (Banning et al., 2012). Flotillin 1 expression is also negatively regulated at the post-transcriptional level by the microRNA (miR) miR-124, which targets the 3′ untranslated region (UTR) of flotillin 1 mRNA. In breast cancer cells, miR-124 expression is downregulated, resulting in increased flotillin 1 protein levels (Li et al., 2013). Moreover, the genomic region containing the flotillin 2 gene is amplified in breast cancer tumors (Berger et al., 2013). Flotillin upregulation is detected in several types of cancers and it is associated with poor patient survival prognosis and high risk of metastasis formation (Doherty et al., 2006; Hazarika et al., 2004; Li et al., 2013; Pust et al., 2013; Rickman et al., 2008; Wang et al., 2013; Zhang et al., 2013; Zhu et al., 2013). *In vivo* analysis of the role of flotillins during tumorigenesis using a transgenic mouse model of breast cancer has shown that flotillin 2 is not involved in primary tumor formation, but is an important regulator of mammary-tumor-derived lung metastasis (Berger et al., 2013). The contribution of cadherin to cancer initiation and progression has now been well demonstrated (for a review, see Berx and van Roy, 2009). Because flotillins are regulators of cadherins under physiological conditions, it will be important to elucidate whether and how they participate in cadherin function or perturbation during tumorigenesis. In particular, it will be interesting to determine whether flotillin overexpression influences the cross-talk between cadherins and EGFRs, because activation of signaling pathways downstream of EGFRs, particularly of Rho GTPases, which are key regulators of tumor cell migration and invasion (Vega and Ridley, 2008), induces cadherin destabilization (Fukata and Kaibuchi, 2001; Lanzetti et al., 2000). Interestingly, in breast and gastric tumors, flotillin 2 expression positively correlates with the expression of the EGFR ErbB2 (Wang et al., 2013; Zhu et al., 2013), and, in these cells, flotillin 2 has been shown to stabilize this receptor at the plasma membrane (Pust et al., 2013). Moreover, flotillin 1 is required for the activation of EGFR-dependent signaling, both with regard to EGFR phosphorylation and subsequent MAPK activation (Amaddii et al., 2012). Therefore, flotillin overexpression could induce the upregulation of EGFR signaling, thereby leading to disruption of cadherin-mediated intercellular adhesion. Reciprocally, cadherins also exert pro-oncogenic effects, notably in breast tumors, by participating in EGFR activation, either independent

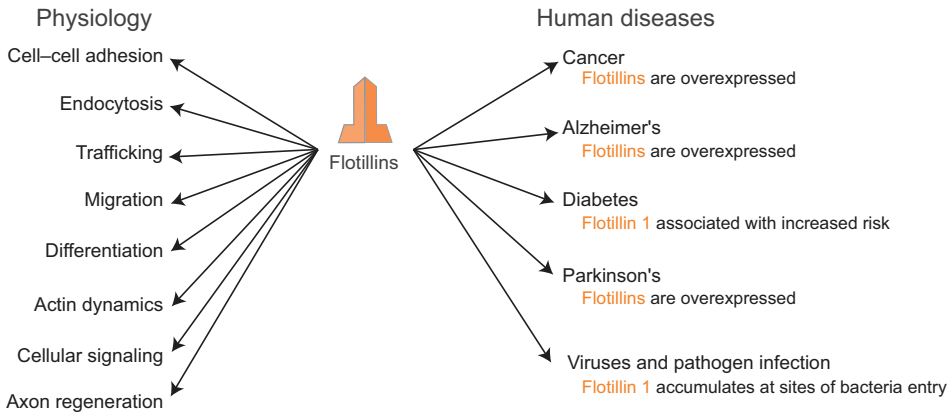

Fig. 4. Functions of flotillins in cellular physiology and human diseases. Flotillins have important roles in the clustering of various plasma membrane receptors and in membrane dynamics; they also participate in various vital cellular processes as indicated. Flotillins are overexpressed in cancers, neurodegenerative deseases, and flotillin 1 expression is associated with risk of type 2 diabetes (Galazis et al., 2013). Overexpression of flotillins in tumors cells is associated with metastasis formation but the molecular mechanisms involved remain to be determined. Flotillins associate with BACE1/β-secretase and APP, two major factors involved in the production of amyloid-β peptide and of senile plaque formation, and appear to regulate their trafficking (John et al., 2014) or clustering (Schneider et al., 2008), respectively. Flotillin 1 also accumulates at sites of bacteria and virus entry (Korhonen et al., 2012; Li et al., 2008), and receptors of HIV in macrophages are located in membrane domains that are enriched in flotillin 1 (Carter et al., 2009).

of or in cooperation with EGF (Andl and Rustgi, 2005; Brouxhon et al., 2013; Perrais et al., 2007; Qian et al., 2004). Thus, as flotillins stabilize ErbB2 and cadherins at the plasma membrane, their upregulation could also increase the activation of oncogenic signaling downstream of EGFR that is mediated by cadherin, another issue that will be important to address in future studies.

In neuronal cells, flotillins might modulate cadherin-mediated cell–cell adhesion, which is important for synapse organization and function, neuron regeneration, dendritic spine morphogenesis and in the context of Alzheimer's disease (Takeichi, 2007; Togashi et al., 2002; Uemura et al., 2007). In this neurodegenerative disease, N-cadherin and flotillins contribute to the production of amyloid-β peptide, the major component of senile plaques (Andreyeva et al., 2012) (Bitsikas et al., 2014). Both N-cadherin and flotillins facilitate dimerization of the amyloid-β peptide precursor APP and its subsequent endocytosis, thereby allowing its cleavage in the acidic endosomal compartment, which increases generation of the amyloid-β peptide (Asada-Utsugi et al., 2011; Schneider et al., 2008).

Finally, both flotillins and cadherin complexes accumulate at cell–cell contact sites, where axonal processes take place, and at synapses, where they regulate axon regeneration and synapse formation (Blackmore and Letourneau, 2006; Ishiyama et al., 2010; Koch et al., 2013; Langhorst et al., 2008a; Munderloh et al., 2009). However, the role of flotillins in assembly, stabilization, dynamic recycling and function of cadherin complexes in neural cells in particular needs to be further defined.

Conclusions

Although it has been known for a long time that flotillins accumulate at cell–cell contact sites, their roles there have only recently begun to be elucidated. Flotillins regulate cell–cell adhesion by acting to stabilize of cadherin complexes at plasma membrane microdomains, thereby allowing the formation of cell–cell contact sites. Because these effects of flotillins were established using various cell lines in culture, and the loss of flotillins does not appear to perturb the development of mouse and *Drosophila* embryos, the use of other animal models might be informative, particularly in view of flotillin overexpression in human pathologies, such as cancer and Alzheimer's disease (Fig. 4). It is thus important to develop new strategies to analyze the effect of flotillin overexpression on cell adhesion processes in order to better understand their contribution to these pathological conditions. In particular, how these proteins regulate membrane lipid organization and dynamics at cell–cell contacts and how their overexpression influence these parameters need to be investigated in greater detail. To that end, the generation of knock-in mice or the use of other animal models that mimic these diseases to allow monitoring the effect of flotillin overexpression will be of great interest in the field.

Competing interests

The authors declare no competing interests.

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