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COMMENTARY

Caveolae – mechanosensitive membrane invaginations linked to actin filaments

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ABSTRACT

An essential property of the plasma membrane of mammalian cells is its plasticity, which is required for sensing and transmitting of signals, and for accommodating the tensional changes imposed by its environment or its own biomechanics. Caveolae are unique invaginated membrane nanodomains that play a major role in organizing signaling, lipid homeostasis and adaptation to membrane tension. Caveolae are frequently associated with stress fibers, a major regulator of membrane tension and cell shape. In this Commentary, we discuss recent studies that have provided new insights into the function of caveolae and have shown that trafficking and organization of caveolae are tightly regulated by stress-fiber regulators, providing a functional link between caveolae and stress fibers. Furthermore, the tension in the plasma membrane determines the curvature of caveolae because they flatten at high tension and invaginate at low tension, thus providing a tension-buffering system. Caveolae also regulate multiple cellular pathways, including RhoA-driven actomyosin contractility and other mechanosensitive pathways, suggesting that caveolae could couple mechanotransduction pathways to actin-controlled changes in tension through their association with stress fibers. Therefore, we argue here that the association of caveolae with stress fibers could provide an important strategy for cells to deal with mechanical stress.

KEY WORDS: Caveolae, Mechanotransduction, Stress fibers

Introduction

The organization of complex organisms requires specialization of cells into different shapes and functions, and part of this specialization occurs at the plasma membrane. In mammals, many cell types undergo constant shape changes during migration, mitosis or contraction. These processes are highly dependent on the forces that are generated by the actin cytoskeleton and other filaments. These forces are ultimately translated to the plasma membrane, which needs to adjust cell shape to new or evolving scenarios. The plasma membrane must therefore be highly plastic and able to sense, respond and adapt to the biochemical and physical clues that are imposed by its environment. The plasticity of the plasma membrane is in part accomplished by specialization into differentiated membrane domains. One such domain is the caveola, an invagination of the plasma membrane with a diameter of 60–80 nm and a characteristic omega (Ω) shape (Fig. 1).

Several decades after the initial description of caveolae in the early 1950s, caveolins were identified as the major components of caveolae (Rothberg et al., 1992; Tang et al., 1996; Way and Parton, 1996). Three genes encoding caveolin proteins exist in mammals: *CAV1*, *CAV2* and *CAV3*. Cav1 and Cav2 are expressed in all tissues except skeletal muscle, whereas Cav3 is expressed mostly in striated muscle (Parton and del Pozo, 2013). Absence of Cav1 or Cav3 results in loss of caveolae in the tissues that normally express them, strongly suggesting that these are essential caveolar components (Drab et al., 2001; Galbiati et al., 2001b). Cav1 also localizes outside caveolae, which expands the cellular roles of Cav1 (see Box 1).

Although Cav1 by itself can form caveolae-like structures in bacteria, other components are essential to obtain the shape and function of caveolae in mammalian cells (Walser et al., 2012). In the last five years, the cavin family (cavin1, cavin2, cavin3 and cavin4, also known as PTRF, SDPR, PRKCDBP and MURC) proteins have emerged as important factors in caveolar biology (Aboulaich et al., 2004; Ogata et al., 2008; Bastiani et al., 2009; Hansen and Nichols, 2010). Cav1, Cav2 and cavin1 form a complex that is essential for the formation of caveolae (Hill et al., 2008; Ludwig et al., 2013). Cavin1, in turn, interacts with either cavin2 or cavin3, but not with both at the same time (Ludwig et al., 2013; Gambin et al., 2014). Cavin2 has an important role in caveolae formation in certain tissues such as lung endothelium, whereas cavin3 appears to be important for caveolae formation in cell lines, but not in mice (Hansen et al., 2013; Hernandez et al., 2013). In addition to cavins, Pacsin2, an F-BAR protein involved in clathrin-mediated endocytosis, regulates caveolar shape (Hansen et al., 2011; Senju et al., 2011; Koch et al., 2012).

Caveolae are not uniformly distributed in different mammalian cell types; they are highly abundant in mechanically stressed cells, such as muscle cells, fibroblasts, endothelial cells and adipocytes, but are virtually absent in neurons and lymphocytes (Nassoy and Lamaze, 2012; Parton and del Pozo, 2013). In the last two decades, caveolae have been shown to be important for the regulation of many signaling cascades, including several mechanotransduction pathways (Parton and del Pozo, 2013). Caveolae also play a key role in lipid homeostasis, and the absence of caveolar components results in lipodystrophy in mouse models and humans (Pilch and Liu, 2011). Furthermore, caveolae have a role in buffering mechanical stress at the plasma membrane (Sinha et al., 2011), which could explain the occurrence of muscular dystrophies, myopathies and cardiopathies in mice and humans carrying mutations in caveolar components, as muscle cells are constantly experiencing mechanical stress at the plasma membrane (Galbiati et al., 2001a; Garg and Agarwal, 2008; Rajab et al., 2010). These topics have been reviewed elsewhere (Pilch and Liu, 2011; Nassoy and Lamaze, 2012; Parton and del Pozo, 2013), so in this Commentary, we focus on discussing the literature regarding a unique characteristic of caveolae, that is, its physical association and functional interplay with the actin cytoskeleton, particularly the stress fibers. As mechanically stressed cells adapt to the changing environment, especially from a mechanical point of view, cells ought to remodel their plasma membrane and actin cytoskeleton.

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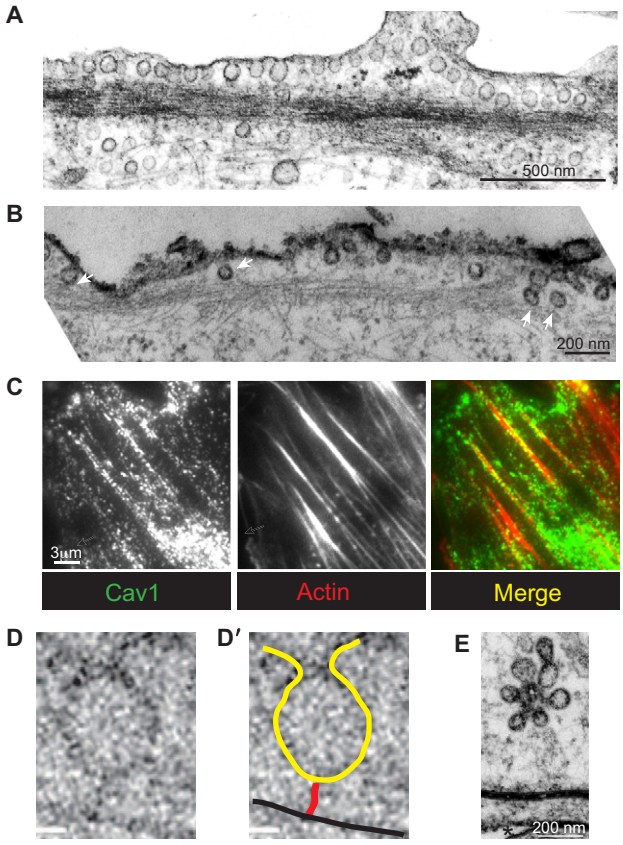

Fig. 1. Association of caveolae and Cav1 with stress fibers.
(A) Myofibroblasts analyzed by electron microscopy, showing numerous caveolae that are co-aligned with stress fibers. Reproduced from Valentich et al., 1997 with permission. (B) Electron microscopy image of mouse embryonic fibroblasts that have been fixed with glutaraldehyde in the presence of Ruthenium Red to label surface-connected structures (dark signal). Arrows mark areas where actin filaments are in close proximity to caveolae. (C) Cav1 co-aligns with stress fibers in NIH3T3 cells that were fixed and stained for Cav1 and actin with phalloidin–Rhodamine. The right panel shows a merged image, illustrating close colocalization. (D,D′) Electron microscopy image revealing a potential association between caveolae and actin filaments. The caveola (yellow), the potential linker (red) and the actin filaments (black) are highlighted in D′. Reproduced from Richter et al., 2008 with permission. Scale bars: 20 nm. (E) Electron microscopy image of a caveolar rosette. Human fibroblasts culture *in vitro* were detached from the ECM, fixed and processed for electron microscopy. Ruthenium Red labeling (dark signal) shows the connection of the rosette with the outside of the cell. The asterisk marks the outside of the cell.

We propose that the association of caveolae with stress fibers ensures the coupling and communication between these two mechanosensors, which is needed to maintain the integrity of the plasma membrane and to guarantee proper signaling in response to physical cues.

Caveolae and the actin cytoskeleton

Association of caveolae with stress fibers

Many membranous structures, including the Golgi stacks, membrane ruffles, filopodia and mitochondria, are in some way linked to actin filaments (Boldogh and Pon, 2006; Chhabra and Higgs, 2007; Kondylis et al., 2007). Moreover, many processes of the endocytic system, such as endocytosis of clathrin-coated pits and vesicle movement and recycling, are highly dependent on actin (Chhabra and Higgs, 2007; Derivery et al., 2009). The pool of actin that contributes to membrane remodeling and movement required in these processes is controlled by the two main actin nucleators, the Arp2/3 complex and the formins (Chhabra and Higgs, 2007).

The association between caveolae and stress fibers was shown by early electron microscopy studies (Fig. 1A and B) (Röhlich and Allison, 1976; Singer, 1979; Rothberg et al., 1992; Valentich et al., 1997). The identification of Cav1 as the main component of caveolae led to the first immunostaining of caveolae, which confirmed the unique co-alignment of Cav1 with stress fibers (Rothberg et al., 1992) (Fig. 1C). This co-alignment of caveolae with stress fibers has been observed in many cell types, including fibroblasts (Röhlich and Allison, 1976; Singer, 1979; Rothberg et al., 1992), myofibroblasts (Valentich et al., 1997), epithelial cells (Mundy et al., 2002; Echarri et al., 2012) and muscle cells (Sharma et al., 2010). However, such a co-alignment is not observed in all cells that have prominent stress fibers. Why caveolae associate with certain stress fibers but not with others, and when and how this association is stimulated is unclear. Although other plasma membrane domains such as clathrin-coated pits are functionally linked to actin-regulatory factors (Girao et al., 2008), an alignment with stress fibers is not apparent in these other invaginations, suggesting that the association of caveolae with stress fibers fulfills a particular function and that differentiates them from clathrin-coated pits.

Several lines of evidence suggest that the proximity between caveolae and stress fibers reflects a direct association between both elements. Actin depolymerization by cytochalasin D induces a rapid movement of Cav1 spots, which track that of the depolymerizing actin fiber and finally concentrate with the resulting actin patches, pointing to a physical association between caveolae and actin filaments (Echarri et al., 2012; Stoeber et al., 2012). Electron microscopy studies have also indicated the presence of an electron-dense structure that potentially connects caveolae and actin filaments (Fig. 1D), suggesting that there is a linker protein or a protein complex that can bridge caveolae and stress fibers (Morone et al., 2006; Richter et al., 2008). Filamin A, an actin cross linker, has been proposed to regulate the physical association of caveolae with stress fibers, because (1) it directly interacts with Cav1 (Stahlhut and van Deurs, 2000; Sverdlov et al., 2009) and stress fibers (Stossel et al., 2001), and (2) closely localizes with caveolae,

Box 1. Functions of Cav1 beyond caveolae

Although it is clear that in the absence of caveolins there are no caveolae (Drab et al., 2001; Galbiati et al., 2001b), caveolins, or at least Cav1, have roles outside of caveolae. Cav1 is found in lipid droplets (Pol et al., 2004), peripheral adhesions (Nethe et al., 2010), the endomembrane system (Pelkmans et al., 2004; Botos et al., 2008; Muriel et al., 2011; Ritz et al., 2011) and even mitochondria (Fridolfsson et al., 2012), where caveolae are not present. In addition, Cav1 can form non-caveolar scaffolds at the plasma membrane (Lajoie et al., 2009). This implies that in loss-of-function studies that have targeted Cav1 and identified a phenotype associated with loss of Cav1, this phenotype could be due to the absence of caveolae or to the lack of Cav1 in its other functions. Although it is likely that the main role of Cav1 is to form caveolae and therefore most of the phenotypes associated with lack of Cav1 would indeed be owing to the loss of caveolae, this has not been formally proven. An additional problem is that, in the absence of Cav1, the level of cavin1 is also strongly reduced, as well as possibly that of other proteins such as filamin A, which complicates the interpretation of the data (Hill et al., 2008; Ravid et al., 2008). For these reasons throughout the review, we assign roles to caveolae when the respective study provided evidence pointing in that direction, otherwise we refer to a role for Cav1.

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Cav1 and stress fibers in spread cells, where prominent actin fibers are observed (Stahlhut and van Deurs, 2000). Silencing of filamin A increases the lateral motility of Cav1 and reduces its co-alignment with stress fibers. These phenotypes are dependent on the actin-binding domain of filamin A, further supporting the idea that filamin A anchors caveolae to stress fibers (Muriel et al., 2011) (Fig. 2).

Some evidence suggests that EHD2, a membrane remodeling ATPase that shares similarities with the dynamin superfamily (Daumke et al., 2007), might be also involved in regulating this association of caveolae with the actin cytoskeleton. Silencing of EHD2 increases Cav1 motility and prevents cytochalasin-D-induced Cav1 movement and clustering (Moren et al., 2012; Stoeber et al., 2012), suggesting that EHD2 might interfere with the association between caveolae and stress fibers. The mechanism of how EHD2 regulates caveolae motility and its association with stress fibers remains to be identified. Pacsin2, which binds to EHD2 (Moren et al., 2012), could link caveolae to actin as it has been recently shown to directly interact with actin (Kostan et al., 2014) (Fig. 2).

There is also evidence for an association of cavins with the actin cytoskeleton, and cavin1 has been suggested to be involved in linking caveolae to the actin cytoskeleton (Liu and Pilch, 2008). Analysis of detergent-resistant membranes (DRMs) purified after treatment of cells with latrunculin B, which prevents actin polymerization, has shown that although Cav1 recruitment to DRMs is unaltered, they contain significantly reduced amounts of cavin1 (Liu and Pilch, 2008). Moreover, silencing of cavin1, which also reduces the levels of Cav1, decreases the association of actin with DRMs (Liu and Pilch, 2008). It is therefore possible that more than one caveolar component forms links to the actin cytoskeleton.

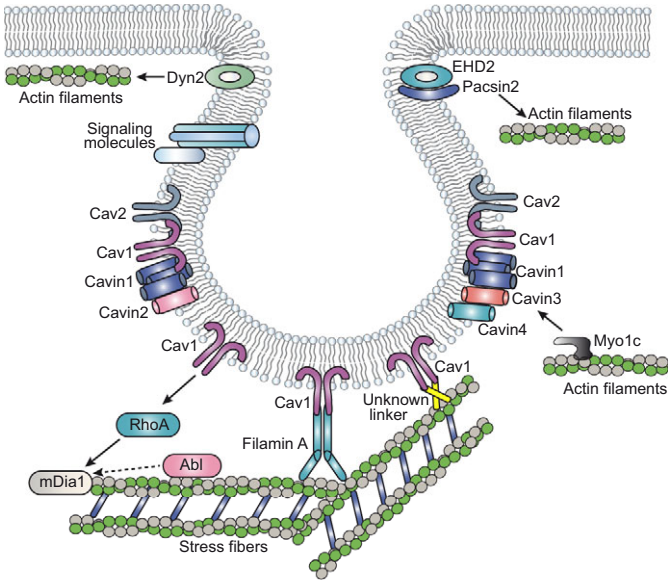

Fig. 2. Caveolae composition and their links to stress fibers and the actin cytoskeleton. The main molecules that shape caveolae (caveolins, cavins and pacsin2) and the proteins that regulate their dynamics (Dyn2, EHD2 and filamin A) are depicted. The caveolar molecules that have functional or physical association with the actin cytoskeleton, and therefore potentially mediate the physical and functional interaction between caveolae and actin fibers, are also indicated. Filamin A is depicted here as the main protein mediating a linkage with stress fibers, but other yet unidentified linkers might exist (indicated as unknown linker). Regulators of stress fibers (Abl kinases and mDia1) that impinge on caveolae organization and trafficking are shown next to RhoA, the main regulator of stress fibers, which is regulated by Cav1.

Recently, the motor protein myosin 1c (Myo1c) has also been suggested to have a role in mediating the association between caveolae and actin (Hernandez et al., 2013). Myo1c depletion induces a perinuclear accumulation of Cav1, which results in a decrease in caveolar density, a phenotype that is compatible with the described role of Myo1c in the recycling of lipid rafts (Brandstaetter et al., 2012). Interestingly, Myo1c interacts with Cavin3, and the two proteins share a similar subcellular distribution (Fig. 2) (Hernandez et al., 2013). However, whether Myo1c directly mediates the association between caveolae and stress fibers remains unclear. The width of the striped coat of the caveolar bulb, which is likely formed by Cav1 and cavins (Ludwig et al., 2013), changes upon exposure to myosin subfragment 1 (the myosin head fragment containing the ATPase domain and actin-binding region) or phalloidin, which stabilizes actin filaments, indicating that actin fibers could have a role in organization of the caveolae bulb (Izumi et al., 1988).

The actin cytoskeleton regulates the organization of caveolar domains

Caveolae are not always evenly or individually distributed at the plasma membrane and tend to cluster in some cell types or under certain conditions (Fig. 3). Early observations by electron microscopy revealed that caveolae-shaped invaginations in rat endothelium extend deep into the cytosplasm, raising the possibility that they represent pinched-off vesicles or vesicle clusters that are not connected with the plasma membrane. However, detailed analysis of these structures by serial sample sectioning and subsequent electron microscopy analysis revealed that they were interconnected with other neighboring vesicles and, ultimately, with the plasma membrane through a tubulo-vesicular structure (Bundgaard et al., 1983). Such structures were later on confirmed in other cell types, including muscle, adipocytes, macrophages, fibroblasts and hepatocarcinoma cells (Parton et al., 1997, 2002; Kiss et al., 2002; Kiss and Botos, 2009; Echarri et al., 2012); collectively, they are referred to as caveolar rosettes, but whether they have additional roles to those assigned to caveolae is currently unclear (Fig. 1E; Fig. 3).

The actin cytoskeleton appears to antagonize the formation of rosettes because treatment with cytochalasin D, which prevents actin polymerization, increases the abundance of caveolar rosettes as assessed by electron microscopy (Fujimoto et al., 1995; Mundy et al., 2002) (Fig. 3). Cytochalasin D also induces a strong clustering of Cav1 that can be detected by immunofluorescence, which reflects the formation of caveolae rosettes or the coalescence of individual caveolae (Mundy et al., 2002; Thomsen et al., 2002; Echarri et al., 2012; Stoeber et al., 2012). Similarly, caveolae clustering is induced by silencing of positive regulators of stress fibers, such as of the formin mDia1 (also known as DIAPH1) and Abl kinases (Echarri et al., 2012), whereas inducing an increase in stress fiber formation results in reduced numbers of caveolar rosettes (Echarri et al., 2012). In differentiated adipocytes, rosettes are highly abundant and this correlates with the absence of stress fibers in these cells (Novikoff et al., 1980; Parton et al., 2002), although it is worth noting that Cav1 is still located close to actin-rich structures (Kanzaki and Pessin, 2002).

In addition, caveolar rosettes or clusters can be induced by okadaic acid, a serine/threonine phosphatase inhibitor. Although the exact mechanism by which this treatment induces caveolar rosettes is unclear, it produces a strong cell rounding effect, which is compatible with a remodeling of stress fibers (Parton et al., 1994; Kiss and Botos, 2009). We have shown that the formation of

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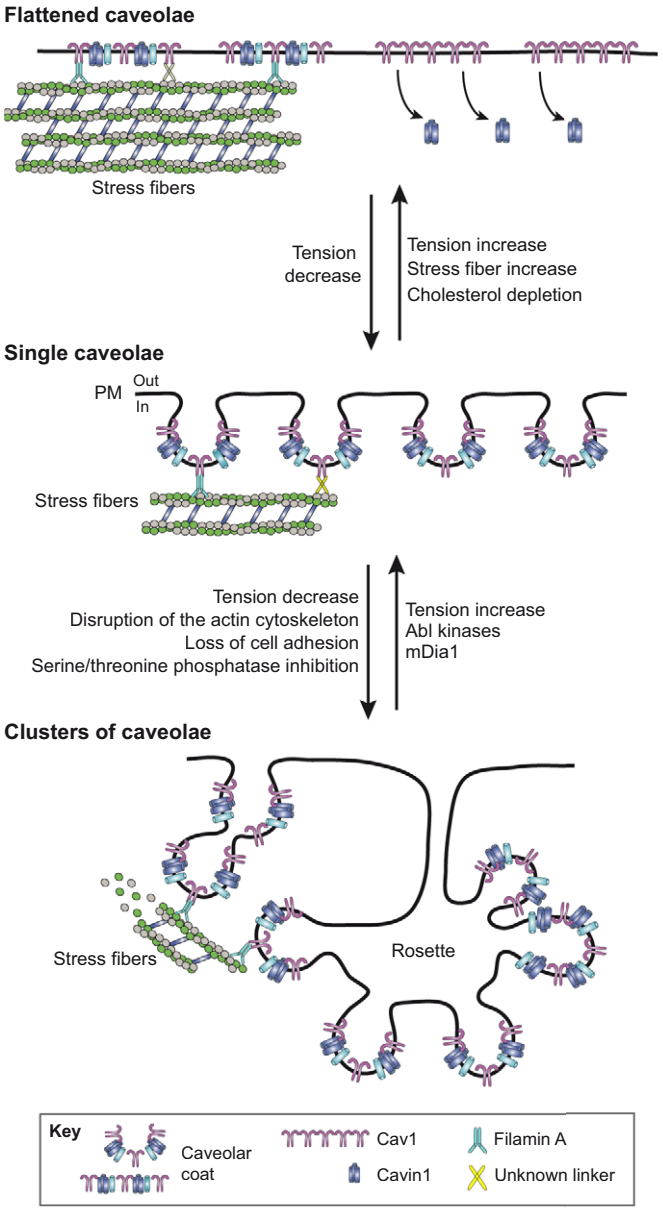

Fig. 3. Plasticity in caveolar organization. Illustrated here are the different organizational forms of caveolae and the conditions that regulate the transition between them. Cell adhesion and actin cytoskeleton regulators, in addition to externally applied forces (osmotic swelling and stretching), control the different types or caveolar organization, such as caveolae flattening or caveolar rosettes. Flattening of caveolae is induced by an excess of stress fibers or by osmotic swelling, which is actin-independent and induces cavin1 disassembly from caveolae. Whether flattened caveolar domains are bound to stress fibers is unclear, but a linkage is proposed in the model shown here. Filamin A and unknown linkers are depicted as linkers between caveolae and stress fibers. Okadaic acid has been used as a serine/threonine phosphatase inhibitor and inducer of caveolar clustering (Parton et al., 1994; Kiss and Botos, 2009).

caveolae rosettes is also increased upon loss of cell adhesion, which induces a profound change in the organization of the actin cytoskeleton (del Pozo et al., 2005; Echarri et al., 2012) (Fig. 3). Such a correlation between loss of cell adhesion and rosette abundance has also been observed in adipocytes (Parton et al., 1997).

Caveolar rosettes are considered to confer additional flexibility to membranes that are under extensive stress, but specific studies are needed to verify this idea. Notably, rosettes are abundant in adipose tissue and muscle, which are also those tissues that show the most prominent phenotypes upon caveolae ablation in humans and mice (Parton and del Pozo, 2013). Consistent with this notion, Cav1 and rosettes are both highly upregulated during adipocyte differentiation (Scherer et al., 1994), which correlates with the accumulation of lipid droplets and thus an extensive increase in membrane area within the cell.

The formation of rosettes precedes the inward trafficking of Cav1 from the plasma membrane to the cytosol, suggesting that rosettes might be an intermediate stage during caveolae endocytosis (see below) (Echarri et al., 2012). Recently, endocytosis of caveolar clusters resembling rosettes has been implicated in the removal of plasma membrane wounds (Corrotte et al., 2013), suggesting that rosettes could represent a way to endocytose relatively big areas of the plasma membrane, which could help to regulate membrane integrity.

The plasticity of caveolae also depends on their curvature, which can range from the classical invaginated form to fully flattened (Fig. 3). Electron microscopy analysis suggests that flattened caveolae maintain the striated shape of their coat, which makes it possible to identify them as such (Prescott and Brightman, 1976; Nassoy and Lamaze, 2012). Cholesterol is required for caveolar curvature because reduced cholesterol levels result in flattening of caveolae (Rothberg et al., 1992).

The ability of caveolae to flatten was first shown in early studies of frog skeletal muscle cells under stretch and was proposed to contribute to reducing tension (Dulhunty and Franzini-Armstrong, 1975). Later on, flattening of caveolae has also been demonstrated in endothelial cells under stretch (Lee and Schmid-Schönbein, 1995). However, the number of caveolae does not change as a function of sarcomere length in rabbit myocardial cells (Levin and Page, 1980), raising the possibility that caveolae flattening might occur only *in vivo* in certain tissues. More recent studies have shown that mechanical stretching of cells or their osmotic swelling result in the flattening of about half of the caveolae (Kozera et al., 2009; Gervasio et al., 2011; Sinha et al., 2011). Caveolae flattening is also generated by an excess of stress fibers (Echarri et al., 2012), which results in an increase in membrane tension (Tamura et al., 2010; Echarri et al., 2012). Interestingly, actin does not appear to regulate caveolae flattening that is induced by osmotic swelling (Nassoy and Lamaze, 2012). This difference in actin-dependence could be explained by the existence of different caveolae pools or of different molecular mechanisms that induce caveolae flattening. Alternatively, actin polymerization per se might not be needed to flatten any caveolae, and the caveolae flattening that is induced by excessive stress fibers (Echarri et al., 2012) could be due to an indirect effect of an associated tension increase at the plasma membrane (Tamura et al., 2010) (Fig. 3).

The flattening of caveolae has been shown to protect muscle cells from rupture by reducing the tension at the plasma membrane (Sinha et al., 2011). Excess stress fibers increase tension at the plasma membrane (Tamura et al., 2010), with the associated risk of inducing membrane rupture, and is accompanied by an increase in the amount of Cav1 at the plasma membrane (Tamura et al., 2010; Wickström et al., 2010b; Echarri et al., 2012). This suggests that through their association with stress fibers, caveolae represent a buffering system to deal with the increase in tension without affecting plasma membrane integrity. Flattening of caveolae also could have effects on signaling pathways, as cavin1 is disengaged from the caveolar coat and moves to the cytosol upon osmotic swelling induced flattening, which will alter the biochemical properties of caveolae (Sinha et al., 2011).

Role of the actin cytoskeleton in caveolae endocytosis and Cav1 recycling

Endocytosis of caveolae has long been debated, but it is now becoming clear that a fraction of both individual and clustered caveolae is endocytosed (Kirkham et al., 2005). Cav1 has been used as an indirect measure of caveolae trafficking because an unambiguous identification of the caveolae 'pinching off' and their disconnection from the plasma membrane requires relatively complex electron microscopy protocols.

Caveolae endocytosis was first described to be dependent on the actin cytoskeleton (Parton et al., 1994). Later, studies using the SV40 virus, which enters through caveolae at least when caveolae are present in the cell (Damm et al., 2005), also showed that actin polymerization was required (Pelkmans et al., 2002). Similar to clathrin-mediated endocytosis, endocytosis of caveolae is also regulated by dynamin 2 (Dyn2) (Henley et al., 1998; Oh et al., 1998; Sharma et al., 2004; Le Lay et al., 2006; Muriel et al., 2011; Echarri et al., 2012), and Dyn2 has been shown to regulate actin polymerization directly (Gu et al., 2010) (Fig. 4).

The endocytic behavior of caveolae is highly dependent on adhesion of the cell to the extracellular matrix (ECM), as loss of this adhesion results in a relocalization of Cav1 from the plasma membrane to the perinuclear area (del Pozo et al., 2005; Muriel et al., 2011) (Fig. 4). This movement is dependent on Dyn2, actin filaments and actin regulatory proteins (Muriel et al., 2011; Echarri et al., 2012). Furthermore, filamin A, which cross-links stress fibers (Stossel et al., 2001), is required for the relocalisation of Cav1 to the perinuclear region (Muriel et al., 2011). Protein kinase Cα (PKCα), which phosphorylates filamin A, also regulates this route (Muriel et al., 2011) (Fig. 4). Similarly, silencing of stress fiber regulators, Abl kinases or mDia1, prevents caveolar endocytosis. In contrast, silencing of the Arp2/3 complex or of its activators, such as N-WASP or cortactin, does not affect the movement of Cav1 from the plasma membrane to the perinuclear area (Echarri et al., 2012). These studies highlight that stress fiber regulators determine caveolae endocytosis and that in contrast to clathrin-mediated endocytosis, Arp2/3-dependent actin polymerization is dispensable.

Some studies have shown that the apparent mobility of Cav1 inside the cell is increased upon exposure of cells to cytochalasin D (Mundy et al., 2002; Thomsen et al., 2002; Echarri et al., 2012). This, however, does not reflect increased endocytosis but instead a 'dragging' effect of depolymerizing actin fibers that are bound to caveolae (see above) (Echarri et al., 2012; Stoeber et al., 2012). Thus, the association of caveolae with stress fibers acts as anchoring sites that locally stabilize caveolae. At the same time, stress fiber regulators and actin polymerization are needed for caveolae endocytosis. These apparently contradictory functions could be explained by a two-step model, in which caveolae that are anchored to stress fibers remain static, whereas a reorganization of stress fibers that could be triggered by certain stimuli, for example reduced

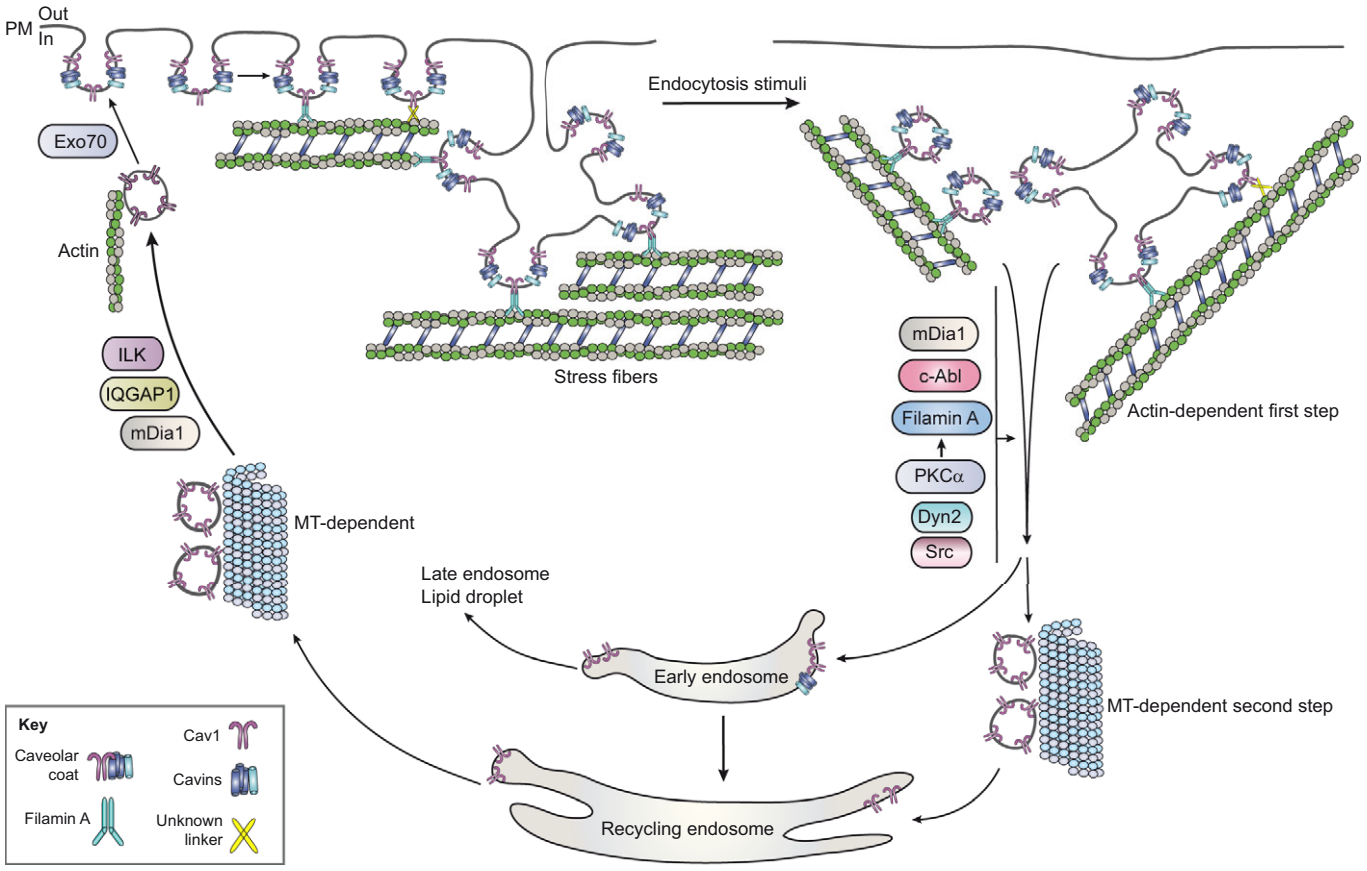

Fig. 4. Role of the actin cytoskeleton in caveolae trafficking. The proteins involved in endocytosis and recycling of caveolae are shown. Actin-dependent endocytosis of caveolae can be triggered by loss of cell adhesion, serine/threonine phosphatase inhibition or exposure to SV40 virus. This stage is regulated by several proteins (depicted). Subsequently, microtubules (MTs) are involved in sorting of Cav1-positive vesicles to different compartments. However, the motors that connect Cav1-positive vesicles to MTs are unknown. MTs are also involved in the initial phase of Cav1 recycling from the recycling endosome, together with ILK, IQGAP1 and mDia1, whereas actin is required to target Cav1 to the plasma membrane at a later stage, which also involves EXO70 (also known as EXOC7). Filamin A and unknown linkers are depicted as linkers between caveolae and stress fibers.

membrane tension, would pull caveolae away from the plasma membrane into the cytoplasm, resulting in their endocytosis. Trafficking of Cav1 from the plasma membrane to the perinuclear area is also dependent on microtubules, but at a later stage than the actin-regulated phase (Conrad et al., 1995; Mundy et al., 2002; Echarri et al., 2012) (Fig. 4).

Although loss of adhesion triggers caveolae endocytosis, re-adhesion induces a recycling of Cav1 and eventual formation of new caveolae (del Pozo et al., 2005). Regulators of stress fibers and/or microtubules have been implicated in Cav1 recycling. mDia1, together with the protein IQ motif containing GTPase activating protein 1 (IQGAP1), a scaffold molecule involved in cytoskeleton remodeling (Hedman et al., 2015), and integrin-linked kinase (ILK), which is also involved in cytoskeletal organization (Wickström et al., 2010a), regulate the transfer of Cav1 from microtubules to the cortical actin, and the disruption of any of these filaments prevents proper Cav1 recycling (Wickström et al., 2010b; Hertzog et al., 2012). Exo70, a subunit of the exocyst complex, has been also been implicated in the late stage of Cav1 recycling (Hertzog et al., 2012) (Fig. 4).

During mitosis Cav1 also changes its localization: during metaphase, Cav1 relocates from the plasma membrane to the endomembrane system, which is concomitant with cell rounding, and returns to the plasma membrane during cytokinesis, when the cell re-spreads (Boucrot et al., 2011). This again highlights the correlation between profound actin or membrane remodeling and endocytosis and recycling of caveolae. It is possible that changes in membrane tension that are associated with these adaptations in cell shape are the underlying reason for Cav1 trafficking. However, the broader implications of caveolae relocalization for signaling and lipid homeostasis remain to be determined.

Caveolae and mechanotransduction

Caveolae are enriched in a remarkable amount of signaling molecules, including adaptor proteins, membrane receptors or small GTPases (Anderson, 1998). Together with their association with actin filaments and its ability to respond to tension, either imposed by mechanical stretching, osmotic swelling or actin-induced stretching, this positions caveolae as the optimal location to regulate mechanotransduction pathways (Fig. 5).

Cav1 regulates actin-related mechanosensitive pathways

The actin cytoskeleton is highly sensitive to mechanical forces and, specifically, stress fibers can sense and transmit mechanical inputs (Hayakawa et al., 2011; Burridge and Wittchen, 2013). This physical input can be transmitted to caveolae as discussed above and multiple lines of evidence suggest that such a regulation is mutual, that is, that Cav1 also regulates actin-regulatory pathways (del Pozo et al., 2005; Nevins and Thurmond, 2006; Grande-Garcia et al., 2007; Joshi et al., 2008; Peng et al., 2008) (Fig. 5).

RhoA signaling represents a major mechanosensitive pathway to control actomyosin contractility (Burridge and Wittchen, 2013), and the caveolar components Cav1 and cavin4 positively regulate RhoA activity (del Pozo et al., 2005; Grande-Garcia et al., 2007; Ogata et al., 2008). Cav1 is also required for the force-induced cytoskeletal reorganization that is mediated by RhoA (Peng et al., 2007). Interestingly, Cav1 and RhoA physically interact, and a pool of RhoA localizes to the same membrane fractions where Cav1 is concentrated (Kawamura et al., 2003); this could be the basis for the regulation of RhoA localization and activity (Gingras et al., 1998; Taggart et al., 2000; Samarakoon et al., 2011). However, other studies have shown that Cav1 negatively regulates the localization and activity of p190RhoGAP (also known as Arhgap35), which results in RhoA activation (Grande-Garcia et al., 2007; Goetz et al., 2011). Interestingly, silencing of Cav1 in endothelial cells decreases the intensity of fluorophore-labelled actin filaments, which could be explained by a reduction in RhoA activity (Wang et al., 2012). Although Cav1 clearly impacts on RhoA activity, which has been shown to be regulated by phosphorylation of Cav1 on tyrosine 14 (Grande-Garcia et al., 2007; Joshi et al., 2008), its remains unclear whether caveolae per se are important for RhoA activity. Cholesterol depletion, which disrupts caveolae (Rothberg et al., 1992), prevents the activation of RhoA by stretch (Kawamura et al., 2003). Although this result suggests that the caveolae could be important for RhoA activation, more studies are needed to confirm such a role. Moreover, the interplay is mutual as RhoA silencing reduces the alignment of Cav1 with stress fibers (Muriel et al., 2011). These links between caveolae and small GTPases, especially RhoA, indicate that caveolae likely provide a membrane domain to spatially restrict RhoA signaling. Similarly, it is reasonable to speculate that when cells spread and elongate, adhesion receptors and stress fibers must be able to sense the physical limits of the plasma membrane, and the association of caveolae with stress fibers could ensure the transmission of these limits, for example by flattening of caveolae, to stress fiber regulators, such as RhoA.

Contractility, a RhoA-dependent process, is fundamental to the function of muscle cells. TGFβ1-induced contractile phenotype markers such as smooth muscle actin are suppressed upon Cav1 silencing, suggesting that Cav1 has an important role in contractile signaling (Gosens et al., 2011). Indeed, as mentioned before, caveolae are highly abundant in smooth muscle cells and their abundance correlates with contractility (Thyberg et al., 1997; Halayko and Stelmack, 2005; Gosens et al., 2011). Several studies have shown that Cav1 is needed for proper cell contraction, such as in contraction of airway smooth muscle cells, which is dependent on muscarinic receptor agonist (Gosens et al., 2007), or in PKC-dependent contraction in vascular smooth muscle cells (Je et al., 2004). Similarly, urinary bladder contraction elicited by muscarinic receptor stimulation was reduced in Cav1-knockout mice (Lai et al., 2004). The muscarinic receptor localizes to the same membrane nanodomains as Cav1, suggesting that it associates with caveolae, which has been proposed to be a mechanism by which Cav1 controls muscarinic-receptor-mediated signaling (Gosens et al., 2007). In contrast, Cav1 depletion increased contraction in the ileum longitudinal muscle, indicating that different types of muscle might show different sensitivity to Cav1 depletion (Shakirova et al., 2006). Cell contraction and relaxation implies underlying changes in membrane tension (Gauthier et al., 2011), which would suggest that they have an effect on caveolae flattening. A study carried out on muscle strips showed that the number of caveolae, as analyzed by freeze-fracture electron microscopy, remained constant in response to contraction that is induced by either an acetylcholine receptor agonist or by stretching and relaxation, indicating that flattening of caveolae might not occur during muscle contraction and therefore changes in caveolar shape might not be involved in regulating contraction (Gabella and Blundell, 1978). However, this study did not analyze the amount of rosettes or caveolar clusters deep in the cytosol, and this pool of caveolae could be sensitive to stretching and relaxation.

Cav1 also regulates the activity of Rac1 and Cdc42 (Grande-Garcia et al., 2007). Cav1 modulates the targeting of Rac1 to the plasma membrane by decreasing the amount of Rac1-binding sites at the plasma membrane (del Pozo et al., 2005; Nethe et al., 2010). Cav1 also promotes Rac1 degradation, which also inhibits Rac1 signaling

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Journal of Cell Science (2015) 128, 2747-2758 doi:10.1242/jcs.153940

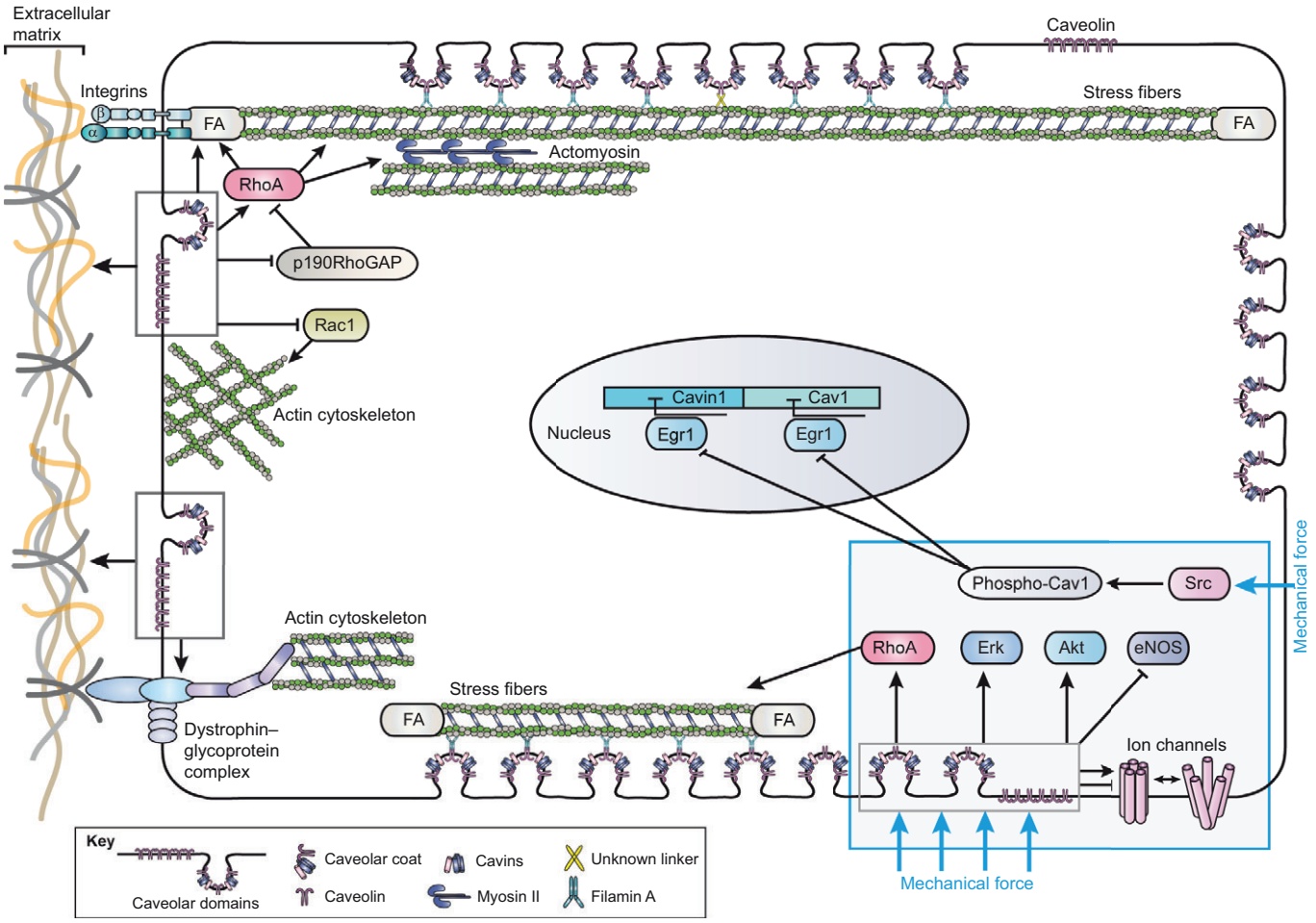

Fig. 5. Overview of cellular pathways regulated by caveolar domains. Caveolar domains, comprising Cav1-containing domains, regulate cytoskeletal and mechanosensitive pathways, as well as reorganization of the extracellular matrix. Caveolar domains regulate RhoA activity through two possible mechanisms, by inhibiting p190RhoGAP, which results in RhoA activation, or by directly affecting RhoA localization within the plasma membrane, leading to its activation. Control over the RhoA pathway regulates the amount of stress fibers, actomyosin contractility and focal adhesion (FA) maturation, which is also responsible for the remodeling of the ECM that is observed in Cav1-expressing cells and not in Cav1-deficient cells. The functional relationship between caveolar domains and the dystrophin–glycoprotein complex is shown (see text for details). Caveolar domains might also regulate the ECM remodeling by other yet unknown pathways. Caveolar domains inhibit Rac1 activity by mediating internalization of plasma membrane domains that contain the membrane lipids to which Rac1 binds at the plasma membrane, resulting in downregulation of many Rac1-driven signaling pathways (e.g. migration, cell cycle progression) (reviewed in del Pozo and Schwartz, 2007). Mechanosensitive pathways that are regulated by caveolar domains are highlighted in the blue box. Mechanical force leads to phosphorylation of Cav1 (phospho-Cav1) in a Src-dependent manner, which inhibits Egr1, therefore overcoming the inhibitory effect of Egr1 on Cav1 and cavin1 promoters; this results in expression of Cav1 and cavin1 and leads to an increased number of caveolae. Mechanical forces activate RhoA, Erk, Akt and eNOS, and caveolar domains play a role in this activation. Caveolar domains regulate many ion channels and both positive and negative effects on their activity have been reported (reviewed in Balijepalli and Kamp, 2008). In the case of the chloride channel $I_{Cl,swell}$, caveolae play a mechanoprotective role and reduce its activation upon osmotic swelling (see text).

(Nethe et al., 2010). As Rac1 and RhoA regulate focal adhesions (Burridge and Wennerberg, 2004), there is also a functional interplay between Cav1 and focal adhesions (discussed in the next section).

Cav1 is functionally linked to the extracellular matrix

The ECM is connected to stress fibers through focal adhesions, modular structures that include the main adhesion receptors, the integrins, and actin-binding linkers, such as filamin A or talin (Brakebusch and Fässler, 2003). Focal adhesions are highly dependent on stress fibers and have a major role in mechanotransduction (Geiger et al., 2009).

Several studies suggest that Cav1 regulates focal adhesion turnover. Cav1 increases the stability of nascent focal contacts at cell edges (Grande-Garcia et al., 2007; Urra et al., 2012), likely through regulating RhoA, as the activity of this GTPase is required for the maturation of nascent focal contacts into focal adhesions. In mature focal adhesions, localization of phosphorylated Cav1 (phospho-Cav1) facilitates the association of focal adhesion kinase (FAK) with focal adhesions, as well as focal adhesion disassembly (Goetz et al., 2008). In addition, a pool of Cav1 also localizes, at least under specific conditions, to peripheral adhesions which could facilitate a direct regulation of adhesive components (Nethe et al., 2010). Similarly, caveolae-dependent endocytosis of integrin, and the association between Cav1 and filamin A could influence focal adhesion dynamics (Stahlhut and van Deurs, 2000; Shi and Sottile, 2008; Bass et al., 2011). Furthermore, the association of Cav1 and caveolae with stress fibers could also affect focal adhesion turnover because Rho-driven actomyosin contraction is required for the maturation of focal adhesions (Hanein and Horwitz, 2012). However, additional studies are required to better understand how Cav1 and/or caveolae and their association with stress fibers regulate the different types of focal adhesions.

In skeletal muscle cells where caveolae are highly abundant, a strong mechanical link between the ECM and the actin cytoskeleton is mediated by the dystrophin–glycoprotein complex (DGC) (Lapidos et al., 2004). In this complex, dystrophin tethers the actin cytoskeleton to β-dystroglycan, the core transmembrane unit of the DGC. β-dystroglycan binds to α-dystroglycan, which acts as a receptor for laminin at the basal lamina (Lapidos et al., 2004).

Several studies have linked the DGC and Cav3 (Fig. 5). Although biochemical analysis has shown that Cav3 is not an integral component of the DGC (Crosbie et al., 1998), other studies have provided evidence that support a functional relationship between the DGC and caveolae. DGC distribution in the plasma membrane is altered in Cav3-knockout mice, which present a mild myopathy (Galbiati et al., 2001b), and, conversely, the distribution of Cav3 is affected in mice lacking dystroglycan or dystrophin (Cote et al., 2002). Notably, near-complete loss of α-dystroglycan expression has been reported in a patient with limb girdle muscular dystrophy (LGMD) who carried a dominant-negative mutation in Cav3, again suggesting that there is a functional relationship (Herrmann et al., 2000). A transgenic mouse overexpressing sarcospan, a protein component of the DGC, induces elevated levels of many DGC proteins and also Cav3 (Peter et al., 2007). Dystrophin localizes to caveolae-enriched areas in smooth muscle cells (North et al., 1993) and can be detected in Cav3 immunoprecipitates (Doyle et al., 2000). Notably, independent studies have shown that Cav3 and Cav1 bind to β-dystroglycan (Sotgia et al., 2000; Sharma et al., 2010). Muscle-specific filamin A has been shown to interact with sarcoglycans, a family of dystrophin-associated glycoproteins, indicating that filamin A could link Cav3 to the DGC (Thompson et al., 2000).

These studies indicate that caveolins are frequently, functionally and physically connected with cellular elements that support and sense mechanical force (Fig. 5). These pathways also regulate the organization of the ECM, which is also controlled by Cav1 through Rho and actomyosin-contraction-driven ECM remodeling, which also favors metastasis (Goetz et al., 2011). Therefore, the link between Cav1 and force-regulated pathways has important physiological and pathological implications.

Caveolae regulate other mechanotransduction pathways

Mechanical forces control multiple pathways and many studies in the caveolae field have addressed the interplay between caveolae and/or Cav1 and mechanosensitive pathways, including mechanosensitive ion channels, Akt and ERK family proteins, and endothelial nitric oxide synthase (eNOS; also known as NOS3).

Several studies have provided compelling evidence that Cav1 regulates mechanosensitive ion channels (Fig. 5) (Balijepalli and Kamp, 2008; Kozera et al., 2009; Huang et al., 2013). Furthermore, Cav3 deficiency can lead to long QT syndrome, a frequent consequence of defective ion channel functioning (Moss and Kass, 2005). In addition, many ion channels localize to caveolae and several cardiac arrhythmias are related to caveolae malfunction (Balijepalli and Kamp, 2008). Interestingly, stretch-activated Ca^{2+} influx through stretch-activated ion channels, some of which are localized in caveolae and/or regulated by caveolins (Gervasio et al., 2008), depends on an intact actin cytoskeleton, which raises the possibility that the association of caveolae with stress fibers might fine-tune these membrane channels (Hayakawa et al., 2008; Ito et al., 2010). A specific role for caveolae, mediated by their ability to flatten out, has recently been demonstrated in the regulation of osmotic swelling activated chloride channel $I_{Cl,swell}$. Activation of this channel was increased in the absence of caveolae, suggesting that caveolae can buffer the effect of mechanical force on specific membrane components (Kozera et al., 2009).

Caveolae are highly abundant in vascular smooth muscle cells; here, stretch-induced Akt activation, and cell-cycle progression is Cav1-dependent (Sedding et al., 2005). Furthermore, Cav1 is also necessary for both rapid and long-term mechanotransduction in intact blood vessels (Yu et al., 2006). The levels of Cav1 and caveolae are regulated by stretching in an actin-dependent manner. Mechanistically, stretch-induced phosphorylation of Cav1 tyrosine 14 triggers a cascade that depends on PKC and RhoA, and that leads to the inhibition of the transcription factor Egr1, which normally represses the expression of Cav1 and cavin1, essential components for the formation of caveolae. Thus, this pathway represents a positive-feedback loop that, upon stretching and Cav1 phosphorylation, results in the formation of additional caveolae. Likely, this pathway ensures a protective mechanism (i.e. an increase in caveolae) to deal with the tension increase at the plasma membrane upon stretch (Joshi et al., 2012) (Fig. 5).

Caveolae are also abundant in endothelial cells that constantly experience fluid force. Shear stress induces a relocation of caveolae to the trailing edge of the cell (Isshiki et al., 2002) and triggers an increase in caveolae density at the luminal face (Boyd et al., 2003). Shear stress activates several pathways that lead to Src-dependent Cav1 phosphorylation (Radel and Rizzo, 2005) or Cav1-dependent ERK activation (Park et al., 2000). Phosphorylation of Cav1 tyrosine 14 upon experiencing shear forces (Radel and Rizzo, 2005) could explain the increase in caveolae observed upon shear stress (Boyd et al., 2003; Joshi et al., 2012), as Cav1 phosphorylation results in transcriptional upregulation of Cav1 and cavin1 (see above) (Joshi et al., 2012) (Fig. 5). Cav1 interacts with eNOS, which inhibits its activity, and shear stress reduces this interaction, thereby resulting in eNOS activation. As eNOS controls the production of nitric oxide, a major player in vascular tone regulation, Cav1 could partially influence vascular tone through binding to eNOS (Rizzo et al., 1998; Balligand et al., 2009). Interestingly, actin and eNOS co-immunoprecipitate in purified caveolar fractions, suggesting that actin-linked caveolar domains might be involved in the regulation of eNOS (Su et al., 2003).

Stress fibers are highly sensitive to shear stress (Malek and Izumo, 1996), and actin reorganization correlates with a change in Cav1 distribution and an increase in its expression (Zeng and Tarbell, 2014). As expected, shear-stress-induced stress fiber formation is dependent on both Cav1 (Yang et al., 2011) and RhoA (Tzima et al., 2002). Interestingly, *in vivo*, the number of caveolae is reduced upon the increase of the transmural tension in endothelial cells, probably due to a flattening effect (Lee and Schmid-Schönbein, 1995). Taken together, these studies suggest that mechanical forces reorganize caveolae *in vivo*, which could facilitate the regulation of the actin cytoskeleton and other mechanosensitive pathways (Fig. 5).

Conclusions and perspectives

The fact that both caveolae and stress fibers are sensitive to force and regulate tension at the plasma membrane raises the possibility that, conceptually, regulation of tension is the main reason for the association between caveolae and stress fibers. The current literature provides two non-mutually exclusive models that could provide the molecular basis for this association; first, Cav1 and/or caveolae could associate with stress fibers to locally regulate signaling associated with stress fibers, mostly RhoA signaling. Second, the association of caveolae with stress fibers would help to buffer tension in areas of the plasma membrane where tension changes are likely to occur through

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the action of stress fibers. This would prevent membrane rupture by ensuring that the physical limits of the plasma membrane are recognized and not exceeded. In this scenario, it is possible that the flattening of caveolae, besides buffering membrane tension, could also impinge on RhoA signaling. Caveolae flattening results in the release of cavin1 from caveolar Cav1 (Sinha et al., 2011), which raises the question of what is the effect of disengaged caveolar components on RhoA or other signaling pathways.

Despite the accumulated information regarding the interplay between caveolae and stress fibers, we still do not understand the exact mechanisms by which caveolae are linked to stress fibers. Similarly, the timing of caveolae association with stress fibers and the pool of fibers they associate with remains unknown. Furthermore, it is important to understand which of the actin-related functions of Cav1, that is, activation of RhoA, focal adhesion stability, functional association with the DGC or with filamin A, or integrin endocytosis, are direct consequences of the association of caveolae with stress fibers. It is probably not a coincidence that caveolae associate with stress fibers, which themselves are linked to pathways or cellular components that are also regulated by caveolae.

In physiological terms, future studies will need to address the extent to which actin filaments control the organization of caveolar domains and how caveolae regulate actin filaments in response to different types of mechanical force and other stimuli *in vivo*. Answers to these questions will not only shed light into the biological mechanisms underlying this association, but might also be exploited in pathological conditions arising from them, such as cancer metastasis and myopathies.

Acknowledgements
Simon Bartlett (CNIC) provided editorial assistance. We apologize to those authors whose work could not be cited due to space limitations.

Competing interests
The authors declare no competing or financial interests.

Funding
Our work was supported by grants from the Spanish Ministry of Economy and Competitiveness (MINECO) [grant number SAF2011-25047 and CONSOLIDER CSD2009-00016 to M.A.D.P.] and from Fundació la Marató TV3 (674/C/2013) to M.A.D.P. The Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC) is supported by MINECO and the Pro-CNIC Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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