The multiple faces of caveolae

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Abstract | Caveolae are a highly abundant but enigmatic feature of mammalian cells. They form remarkably stable membrane domains at the plasma membrane but can also function as carriers in the exocytic and endocytic pathways. The apparently diverse functions of caveolae, including mechanosensing and lipid regulation, might be linked to their ability to respond to plasma membrane changes, a property that is dependent on their specialized lipid composition and biophysical properties.

Stomatal diaphragm
A specialized structure at the neck of caveolae in certain endothelial cells that consists of a central density and radial spikes, and is generated by the transmembrane protein PV1.

Lipid rafts
Small, heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that are formed by lipid–lipid interactions that compartmentalize cellular processes. Small lipid rafts can be stabilized to form larger platforms through protein–protein and protein–lipid interactions.

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Caveolae were first identified by electron microscopy[1,2] and are defined as pits of 60–80-nm diameter in the plasma membrane (FIG. 1). Caveolae have a characteristic flask shape and no obvious coat[3], as is seen, for example, on the cytoplasmic surface of clathrin-coated pits. In some tissues, caveolae have a unique specialized structure at the neck of the flask, known as the stomatal diaphragm[4].

The incredible abundance of caveolae in specific cell types, such as smooth-muscle cells, fibroblasts (BOX 1; FIG. 1), endothelial cells and adipocytes has long puzzled researchers who are seeking to understand their cellular functions. Caveolae have been implicated in endocytosis, transcytosis, calcium signalling and numerous other signal transduction events[5–7]. They have also been exploited by pathogens, both as direct portals for endocytic entry[8] and to facilitate the entry of pathogens that are larger than a single caveola[9]. Caveolae, and the main membrane proteins of caveolae, caveolins, have also been linked to disease; mutations in caveolins have been found in breast cancer and in limb girdle muscular dystrophy (BOX 2).

What might the function of this specialized surface domain be? In the past, signalling has been proposed as the primary function of caveolae. Although caveolae are certainly involved in specific signalling events, as will be discussed below, a more general role in signalling remains controversial and must be reinvestigated with the functional systems that are now available. The ability to experimentally manipulate caveolins, which are essential for caveola formation[10–12], has allowed researchers to examine the effect of caveola deficiency in specific cell types in tissue culture and *in vivo*. These studies are providing fascinating new insights into the functions of caveolae.

Features and properties of caveolins

Caveolin-1 (CAV1) and CAV2 are abundant in caveola-rich non-muscle cells, whereas CAV3 is found in skeletal muscle and in some smooth-muscle cells[13,14]. Ablation of CAV1 and CAV3 causes loss of caveolae from those specific cell types[11,12]. By contrast, loss of CAV2 has no apparent effect on caveola formation *in vivo*[15] but might contribute to caveola formation in certain cell types[16,17]. All three caveolins show an unusual topology with N and C termini in the cytoplasm and a long putative hairpin intramembrane domain. Caveola formation by CAV1 and CAV3 involves oligomerization and association with cholesterol-rich lipid-raft domains. CAV1 binds to 1–2 cholesterol molecules[18] and is also palmitoylated in the C-terminal region[19]. Cholesterol depletion disrupts the structure of caveolae[20].

The main structural features of caveolin are summarized in FIG. 1. An estimated 144 molecules of caveolin are present in a single caveolar structure[21]. The relative amount of cholesterol in caveolae might be more than 100 times higher than this, estimated as 20,000 molecules in immuno-isolated caveolae[22]. Some glycosphingolipids (for example, GM1 and GM3) and sphingomyelin are also enriched in caveolae relative to the bulk plasma membrane. Importantly, the density of lipids was found to be higher in the caveolae than in the plasma membrane fraction from which the caveolae were isolated[22]. Both the lipid composition and the packing of the lipid bilayer conform to the parameters that would be expected from a clustered liquid-ordered lipid-raft domain[23–25]. In summary, caveolae represent a specialized, morphologically distinct sphingolipid-cholesterol microdomain, which is stabilized by the caveolin protein.

Trafficking of caveolins

The remarkable ability of exogeneously expressed CAV1 or CAV3 to generate caveolae has been shown in many experimental systems[10,26] (for a review addressing mechanisms of caveola formation, see REF. 27). An understanding of caveolin trafficking and caveola formation is crucial to understanding the possible role of caveolins and caveolae.

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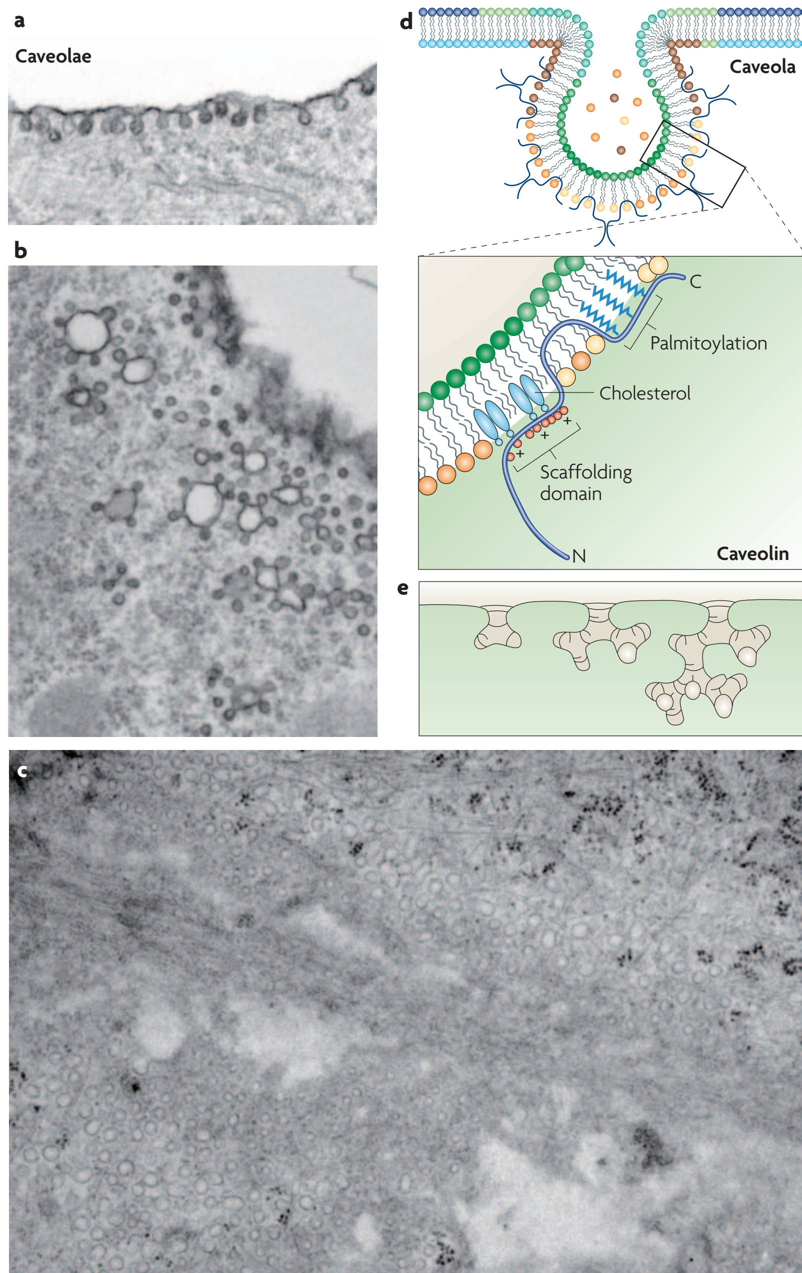

Figure 1 | **Caveolae and caveolins.** The diagrams show the main features of caveolae and caveolins. The electron micrographs in panels **a** and **b** show caveolae in adipocytes that have been surface-labelled with an electron-dense marker. Panel **c** shows a glancing section across the cell surface of a primary fibroblast that has been similarly labelled. Caveolae are evident as discrete flask-shaped pits, or circular profiles where the surface connection lies outside the plane of the section. Note the complex forms of surface-connected caveolae in the adipocytes (panels **a** and **b**), and the incredible abundance of caveolae in specific regions of the fibroblast surface (panel **c**). Panel **d** indicates how caveolin is inserted into the caveolar membrane, with the N and C termini facing the cytoplasm and a putative 'hairpin' intramembrane domain embedded within the membrane bilayer. The scaffolding domain, a highly conserved region of caveolin, might have a role in cholesterol interactions through conserved basic (+) and bulky hydrophobic residues (red circles). The C-terminal domain, which is close to the intramembrane domain, is modified by palmitoyl groups that insert into the lipid bilayer. The complex structures that are formed by interconnected caveolae can occupy a large area of the plasma membrane. The hypothetical formation of cubic membranes (panel **e**), which have adapted to allow the invagination of numerous caveolae, is depicted schematically[108]. These membrane invaginations can form with little energy input. Panel **e** is reproduced with permission from REF. 108 © (1995) Elsevier.

Synthesis and trafficking of caveolins. Caveolin is synthesized as an integral membrane protein in the endoplasmic reticulum (ER) in a signal recognition particle (SRP)-dependent manner[28]. The newly synthesized protein goes through a first stage of oligomerization which, at least *in vitro*, can occur in the ER[29]. Caveolin is then transported from the ER to the Golgi complex. A Golgi pool of newly synthesized caveolin is observed in many cell types, and this pool of the protein is not associated with detergent-resistant membrane (DRM)[30]. However, at some point in the biosynthetic pathway, caveolin associates with lipid rafts, becomes detergent-resistant and is organized into higher-order oligomers that are characteristic of the surface pool of the protein.

The fact that the Golgi and plasma membrane pools of the protein differ in their characteristics indicates that exit from the Golgi complex is linked to this change in the properties of caveolin. Exit from the Golgi complex is accelerated by the addition of cholesterol[30] and inhibited after glycosphingolipid depletion[31]. It is also associated with masking of specific caveolin epitopes (as shown using antibodies that recognize the Golgi pool, but not the plasma membrane pool, unless cholesterol is removed from the plasma membrane)[30]. Light microscopy using CAV1-GFP (green fluorescent protein) revealed that defined quanta of caveolin form in the Golgi complex and are transported directly to the plasma membrane[32]. The quantal size is similar to that of surface caveolae, which indicates that exit from the Golgi is associated with caveolin assembly (oligomerization and association with cholesterol and glycosphingolipid-rich lipid-raft domains) to form a mature caveola-like exocytic structure (we propose the term 'exocytic caveolar carrier'), which is destined for the plasma membrane (FIG. 2). Whether this carrier is made up of only a mature caveola or has other non-caveolar membrane associated with it is as yet unknown.

How the assembly of a caveolin-enriched domain is linked to the budding of a mature caveolar carrier at this specific stage of the secretory pathway is not yet clear. However, the domain-induced budding model for lipid-raft-associated vesicle formation implies that glycosphingolipids and cholesterol might well be crucial factors in this process[23]. Budding in this model is driven by the energy derived from the increase in the line tension that emanates from the increasing size of the lipid-raft domain that forms the caveolar carrier. A growing (phase-separated) domain will eventually reach a critical size, beyond which budding becomes energetically favourable[33]. In line with this model, caveolar carriers would be formed late in the secretory pathway, presumably in the late Golgi compartments in which glycosphingolipids and cholesterol are present at high levels, rather than immediately after the synthesis of caveolin in the ER. However, whether induction of the budding of the carriers involves further regulation, such as phosphorylation, is still unclear[34].

Post-Golgi trafficking of caveolins. Some of the other components that are associated with the formation of caveolar carriers during Golgi exit (and/or caveolin post-Golgi

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Box 1 | **Defining caveolae**

One problem in the field has been laxity concerning the definition of caveolae. We would like to restrict the term caveolae to invaginations of the plasma membrane with a diameter of 60–80 nm. These invaginations are formed by the polymerization of caveolins and contain a subset of lipid-raft components, including cholesterol and sphingolipids. Caveolae might exist as single pits or can form a cluster of caveolae with non-caveolar membrane between the pits (FIG. 2). Clathrin-coated pits can even be observed in membrane continuity with caveolae within these clusters[59]. Caveolae can flatten out into the plasma membrane, thereby losing their caveolar identity. The term flat caveolae is a misnomer that only caused confusion in the field.

Signal recognition particle
(SRP). A complex of polypeptides and RNA involved in synthesis of proteins on membrane-bound ribosomes of the ER. SRP interaction with a specific signal on the nascent polypeptide dictates co-translational insertion of the protein into the ER.

Detergent-resistant membrane
(DRM). DRM fractions remain insoluble after cold Triton X-100 extraction. This is a crude biochemical measure for lipid-raft association.

Exocytic caveolar carrier
A carrier produced in the Golgi that resembles a fully formed caveola in caveolin density. We suggest the terms endocytic caveolar carrier and recycling caveolar carrier for budded caveolae or caveolae recycling back to the cell surface.

transport) are also starting to be elucidated. The SNARE protein syntaxin-6 is involved in the delivery of CAV1, glycosylphosphatidylinositol (GPI)-anchored proteins and the ganglioside GM1 to the plasma membrane[35]. However, CAV1 is not required for efficient plasma membrane delivery of GPI-anchored proteins[36]. By contrast, dysferlin[37], the angiotensin receptor[38], the insulin receptor[39] and the stretch-activated channel short transient receptor potential channel-1 (TRPC1)[40,41] seem to depend on caveolin for efficient surface delivery. These proteins have been shown to be inefficiently transported to the plasma membrane in cells, which either lack caveolins, or which express mutant Golgi-accumulated forms of caveolins. When examined in detail, at least some of these proteins did not localize to caveolae at the cell surface[38]. Whether these proteins are transported to the plasma membrane in caveolar carriers or through other exocytic pathways but in a caveolin-dependent manner (FIG. 2) awaits further detailed characterization. In epithelial cells, CAV1 and CAV2 are targeted to the basolateral surface and form caveolae, whereas caveolae are not normally observed on the apical surface where only CAV1 is found[42,43]. CAV1 presumably travels as a cargo protein from the Golgi to the apical surface in distinct, non-caveolar-carriers, which are not yet defined. A more general question is whether caveolae or caveolar carriers only incorporate cargo that is bound to the outer or inner leaflet, or whether they can also accommodate transmembrane proteins without perturbing the caveolin polymeric network in the lipid-raft cluster.

Box 2 | **Caveolae in disease**

Although the exact physiological roles of caveolae continue to be a matter of some debate, there is strong evidence for an association of caveolar dysfunction with human disease[7]. Caveolin-1 (*CAV1*)-null cells show increased proliferation, and loss of CAV1 accelerates tumourigenesis[122,123]. In some breast cancers, CAV1 is downregulated, and a number of sporadic mutations in CAV1 have been detected in samples of human breast cancer[124–126], correlating specifically with oestrogen-receptor-α-positive status[127]. In some tumours, CAV1 can also promote tumour survival and growth[128], and CAV1 has been associated with the progression of prostate carcinoma[129,130].

CAV3, the muscle-specific caveolin isoform, is also strongly linked to disease. Many mutations in CAV3 have now been described, and the mutant proteins are associated with a number of human muscle disorders including limb girdle muscular dystrophy and rippling muscle disease[131–134]. The mutant CAV3 proteins often show mislocalization to the Golgi complex[135] and cause a reduction in the surface levels of CAV3 and other muscle plasma membrane proteins, such as the membrane-repair protein dysferlin[37,136].

Endocytosis of caveolae

Caveolae at the cell surface form a stable functional unit that is generated by oligomerized caveolin and associated proteins and lipids. Fluorescence recovery after photobleaching (FRAP) studies have shown that caveolins in caveolae are relatively immobile, but mobility can significantly increase upon cholesterol depletion[44,45]. This finding has been confirmed by fusing cells with differently coloured caveolins; the caveolae remain red or green and do not form mixed structures[32].

The caveolar unit is also maintained as a stable structure upon endocytosis[44]. Budded caveolae, here known as endocytic caveolar carriers, can fuse with the caveosome in a RAB5-independent manner (BOX 3; FIG. 3), or with the early endosome in a RAB5-dependent manner. The caveolar unit can then be recycled back to the plasma membrane for reuse while maintaining the tight association of caveolin oligomers in a stable unit[44]. Caveolar carriers can also fuse back to the plasma membrane without the involvement of an intermediate station, a process that is also subject to tight regulation[21]. In view of the apparent stability of the caveolar unit, an interesting question is how fusion with the plasma membrane or with caveosomes is achieved, and whether this requires partial disassembly of the caveolar coat. Furthermore, it is unclear whether caveolae always bud as single units, as shown in recent ultrastructural studies[46], or whether they can also detach from the membrane as part of larger domains with one or more caveolae connected to non-caveolar membrane.

Caveolae remain for long periods at the plasma membrane[46], but their internalization can be stimulated by various agents. These include the SV40 virus, which uses caveolae for entry into cells and stimulates caveolar budding[8,47], as well as sterols and glycosphingolipids[48]. Despite the different agents used to stimulate caveola internalization, there are common mechanisms involved in these pathways with a crucial role for dynamin[49–51], Src kinases, protein kinase C (PKC) and actin recruitment[8,47,48]. Also other clathrin- and caveolin-independent, lipid-raft endocytosis pathways have been identified that differ from the caveolar pathway in their characteristics[31,46,52,53]. This complicates the interpretation of studies of non-clathrin-mediated endocytic routes. Downregulation of CAV1 and the resulting loss of caveolae does not block the endocytosis of many lipid-raft markers — including cholera toxin, which binds to GM1 (REFS 53,54) — highlighting the multiple endocytic mechanisms that are involved in the internalization of these markers.

Caveolae and cell adhesion. A large-scale RNA interference screen examining the role of specific kinases in caveolar endocytosis has provided key insights into this process and its regulation[55]. The results not only present a new global view of the interplay between different endocytic pathways but also provide fascinating insights into the possible function of caveolar endocytosis. For example, several kinases that regulate SV40 endocytosis are associated with the regulation of cell adhesions, focal contacts and extracellular matrix (ECM) interactions[55].

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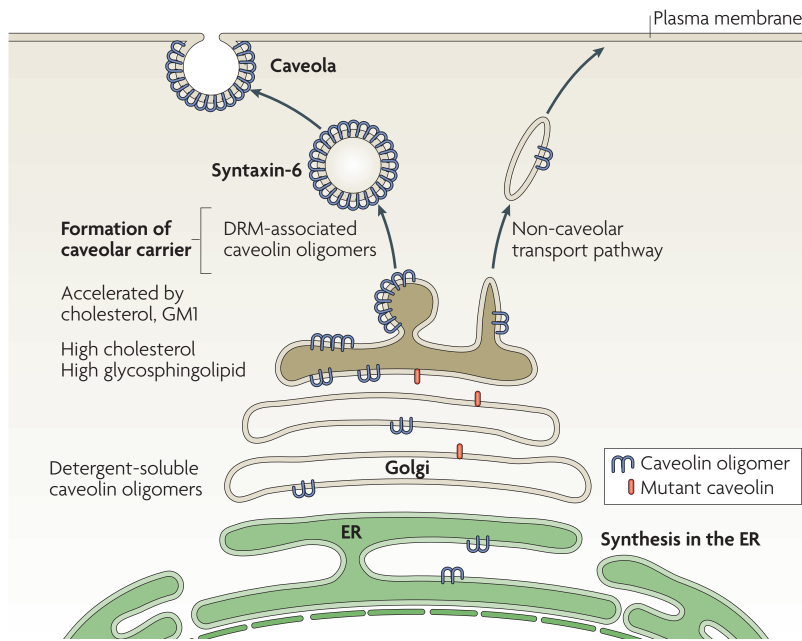

Figure 2 | **Caveolin biosynthesis and trafficking to the plasma membrane.** Caveolin is synthesized in the endoplasmic reticulum (ER) and is then transported to the Golgi complex as detergent-soluble oligomers. When caveolin exits the Golgi complex, the oligomers associate with glycosphingolipid- and cholesterol-enriched lipid-raft domains, as judged by detergent-resistant membrane (DRM) association. Caveolar carriers that contain quanta of caveolin are produced and fuse directly with the plasma membrane in a process that requires the SNARE protein syntaxin-6. In addition, we propose that caveolin can be transported to the plasma membrane by other carriers. Many caveolin mutants that have been associated with disease conditions accumulate in the Golgi complex and are not transported to the plasma membrane efficiently[135] (as indicated by mutant caveolin (red) in the figure). Expression of these mutants seems to cause a more complete block in Golgi exit, and be more harmful, than total loss of caveolin[37,139,140]. One explanation for this is that proteins that exit the Golgi through the caveolar pathway are facilitated by caveolin, but can also follow other pathways, presumably less efficiently, if caveolin is absent. However, caveolin mutants (which cannot form caveolar carriers) perturb the exit pathway and trap their cargo in the Golgi complex so cargo are unable to 'escape' through other routes.

SNAREs
(Soluble *N*-ethylmaleimide-sensitive factor attachment-protein receptors). A protein family that consists of a cognate group of integral and peripheral membrane proteins that are required for bilayer recognition and fusion during membrane trafficking.

Caveosome
A neutral pH endosomal compartment that lacks classic endosomal markers but contains markers that are internalized through caveolae.

Other studies strengthen the link between caveolar endocytosis and cell adhesion. Glycosphingolipids, which stimulate caveolar endocytosis, cause clustering and internalization of integrins through a pathway that involves caveolae; this pathway is inhibited by cholesterol perturbation, CAV1 knockdown or dominant-negative mutants of dynamin and is dependent on Src kinase and PKC[56]. Fibronectin internalization and degradation is also inhibited by knockdown of CAV1 (REF. 57).

Caveolar endocytosis also seems to be important under conditions in which cells lose adhesion to their substratum[58]. The detachment of fibroblasts triggers internalization of the ganglioside GM1 from the plasma membrane in a caveolin-dependent manner. The internalization of GM1 causes loss of Rac1 from the plasma membrane and thereby suppression of Rac1 activation. During the detachment process, Tyr14-phosphorylated CAV1 is localized in caveolae. This phosphorylation event is required for endocytosis — as shown using *Cav1*$^{-/-}$ cells in which wild-type CAV1 or a non-phosphorylated CAV1 mutant had been reintroduced. The striking, rapid removal of GM1 and other lipid-raft components from the cell surface due to internalization of caveolae, as proposed, is surprising in view of the limited enrichment of GM1 in caveolae under control conditions, as shown using both morphological and biochemical techniques[22,59,60]. It has been proposed that CAV1 is required for the coordination of the detachment stimulus, which induces a massive lipid-raft clustering process (possibly macropinocytosis[61]), and leads to domain-induced inward budding of lipid-raft components and proteins, followed by internalization. Taken together these studies show strong links between caveolar endocytosis and the regulation of cell adhesion. But how these findings relate to the apparently normal development of *Cav1*-null mice, which completely lack caveolae, is still unclear.

Other roles for caveolar endocytosis. Endocytosis through caveolae has been extensively studied in endothelial cells but still remains a matter of much debate. Caveolae are particularly abundant in vascular endothelial cells *in vivo*. Endocytosis and transcytosis through caveolae have been claimed to be involved in the transcellular transport of albumin[62,63]. However, the levels of tissue albumin seem normal in *Cav1*$^{-/-}$ mice[11]. In fact, loss of caveolae *in vivo* through small interfering (si)RNA-mediated downregulation of CAV1 resulted in an increase in vascular permeability to albumin[64]. Ultrastructural studies indicate that the increased vascular permeability to albumin might be a result of the dilation of interendothelial junctions[64], which is consistent with another study showing higher transvascular transport of solutes in *Cav1*-null mice[65]. This study shows similar passive transport across the endothelial monolayer in the *Cav1*-null and wild-type mice, arguing against a significant role for caveolar transcytosis in transport across the endothelium *in vivo*[65]. A clear-cut role for caveolae in this process is not proven at present, and might be complicated by the effects of CAV1 loss on the activity of endothelial nitric-oxide synthase (eNOS) and the resulting changes in vascular permeability[66]. In addition, as with other ligands and in other cell types, the dependence of albumin endocytosis on caveolae is not absolute and it cannot be excluded that other pathways take over in *Cav1*-null cells.

Other diverse roles for caveolar internalization have been described, for example, the endocytosis of epidermal growth factor receptor (EGFR) and transforming growth factor receptor (TGFR)[67,68]. Again, these and other studies remain to be confirmed in view of the evidence for multiple clathrin-independent endocytosis pathways[31].

Caveolae and large-scale membrane changes

Small viruses such as SV40 and polyoma virus (of approximately 45-nm diameter) enter cells through caveolae[8,69,70], and it has been suggested that a number of larger pathogens — which are far too large for endocytosis by a single caveola — also make use of caveolae as an endocytic vehicle. By exploiting this entry pathway, pathogens avoid degradation in lysosomes. Such pathogens include FimH-expressing *Escherichia coli*[9] and *E. coli* K1 (REF. 71), *Pseudomonas aeruginosa*[72] and *Porphyromonas gingivalis*[73]. By exploiting this entry

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Box 3 | **Features and properties of the caveosome**

The caveosome was identified by Helenius and Pelkmans as a distinct endosomal compartment that fuses with SV40-containing caveolae[8,44,47] (FIG. 3). This compartment has specific properties:

- The caveosome is negative for markers of endosomes on the clathrin-mediated endocytic pathway, such as early endosomal protein-1 (EEA1) and internalized transferrin.
- The caveosome has a neutral lumenal pH, which is in contrast to early endosomes, late endosomes and lysosomes, which have an acidic pH.
- The caveosome has some of the properties of a sorting compartment. For example, cholera toxin within the caveosome is sorted to carriers that are destined for the Golgi complex, whereas caveolin-1 (CAV1) returns to the plasma membrane. SV40 exits from the caveosome and moves along microtubule-dependent carriers to the endoplasmic reticulum.

It should be noted that not all CAV1-labelled vacuolar structures within the cell are caveosomes, as has been assumed in some electron-microscopy studies. Many cells have caveola-covered structures that seem to be intracellular vacuoles, but are actually connected to the plasma membrane out of the plane of section (see, for example, FIG. 1). An extreme example of this unusual plasma membrane organization is seen in adipocytes[137].

pathway, pathogens avoid degradation in lysosomes. The bacteria do not use caveolae as such, but they seem to be internalized in caveola-rich areas of the cell[74] through a non-clathrin cholesterol-dependent pathway, and often locate to caveolin-containing vacuoles within the cell. Although these bacteria are not internalized within caveolae, and the apical surface of epithelia where invasion often occurs lacks caveolae[42,43], several studies have shown an effect of caveolin downregulation on the entry process (see, for example, REF. 73).

How does loss of CAV1 affect the invasion process? The gross membrane rearrangements that are required to accommodate the invading pathogens might require coordination of membrane and cytoskeletal changes that are consistent with a general role for caveolin in these processes, rather than as an endocytic carrier. For example, group A streptococci bind to the ECM protein fibronectin, which in turn binds to integrins[74]. These interactions then trigger the uptake of streptococci by large, encapsulating invaginations. This process resembles the dramatic internalization of ganglioside-rich domains that occurs after breaking focal adhesions[58]. Both processes involve caveolins and could be shaped by massive lipid-raft clustering.

Another large-scale invagination process in which caveolae have been implicated is the transcellular migration of lymphocytes across the endothelium[75]. CAV1 downregulation partially inhibits this process. Clustering of the endothelial adhesion molecule intercellular adhesion molecule-1 (ICAM1) was proposed to trigger transcytosis through caveolae; however, the role for caveolae in ICAM1 endocytosis remains controversial[76]. Caveolins have also been implicated in phagocytosis[77] and cell motility[78]. The latter could involve regulation of the focal-adhesion machinery and/or regulation of endocytic events. As will be discussed further below, caveolae might be well-equipped to sense changes in the membrane that are required for these large-scale surface rearrangements.

Caveolae and signalling

The number of papers linking caveolae to specific signalling pathways has grown exponentially. Are caveolae the universal signalling regulators originally proposed? If so, how do cells regulate signalling in the absence of caveolae, both in those cells that naturally lack caveolae (lymphocytes, for example) or in genetically modified systems (such as *Cav1*$^{-/-}$ mice)? Are the scores of proteins that immunoprecipitate with CAV1 — including receptors and downstream signalling components, channels, structural proteins and enzymes — actually concentrated in caveolae? Purification of caveolae by immuno-isolation techniques, and analysis by immuno-electron microscopy have rarely supported these claims (see, for example, REF. 79) and in the absence of functional data these data might have to be re-examined (BOX 4). There are so many conflicting claims that scepticism should be maintained until further supporting data become available.

Universal signalling regulators? A key feature of the signalling model is the interaction between the conserved scaffolding domain of caveolin (amino acids 82–101 of CAV1) and signalling proteins[80]. However, this domain also has membrane-binding activity[81,82], and might even insert into the caveolar membrane[83], possibly as an in-plane amphipathic helix[27]. Antibodies against this region do not label caveolin in caveolae at the cell surface, but do label Golgi-based caveolin[30], possibly reflecting masking of the scaffolding domain during caveola formation. The caveolin-signalling hypothesis would predict that, in cells lacking caveolae, the distribution of signalling proteins would be affected. However, this is not the case; siRNA-mediated knockdown of CAV1 in aortic endothelial cells did not affect lipid-raft association or targeting of any of the signalling proteins that were tested in recent studies[84].

How can interactions between the scaffolding domain of caveolin and signalling proteins be explained? These interactions have been well-characterized in the case of eNOS and particularly through the use of peptides from the CAV1-scaffolding domain[85]. One possibility is that the non-caveolar pool of CAV1, at the cell surface[86] or in the secretory pathway[38], is responsible for some of these interactions. In support of this, transgenic overexpression of CAV1 inhibits eNOS activation without increasing caveola formation[87]. This hypothesis could explain other reported interactions between expressed caveolins and signalling molecules, in situations where their presence in caveolae is not observed. Caveolae could provide a reservoir of caveolin molecules that are only released under specific cellular conditions. This hypothesis must be examined carefully in future studies.

These questions aside, there is evidence of a role for caveolins in specific signalling pathways that is strongly supported by functional studies. In addition to a role in eNOS regulation[88,89], several other pathways show a crucial dependence on caveolins[84,90,91]. Caveolae have also been implicated in the regulation of channels and in calcium signalling[92,93], although the precise role of caveolae or non-caveolar caveolins is not yet clear.

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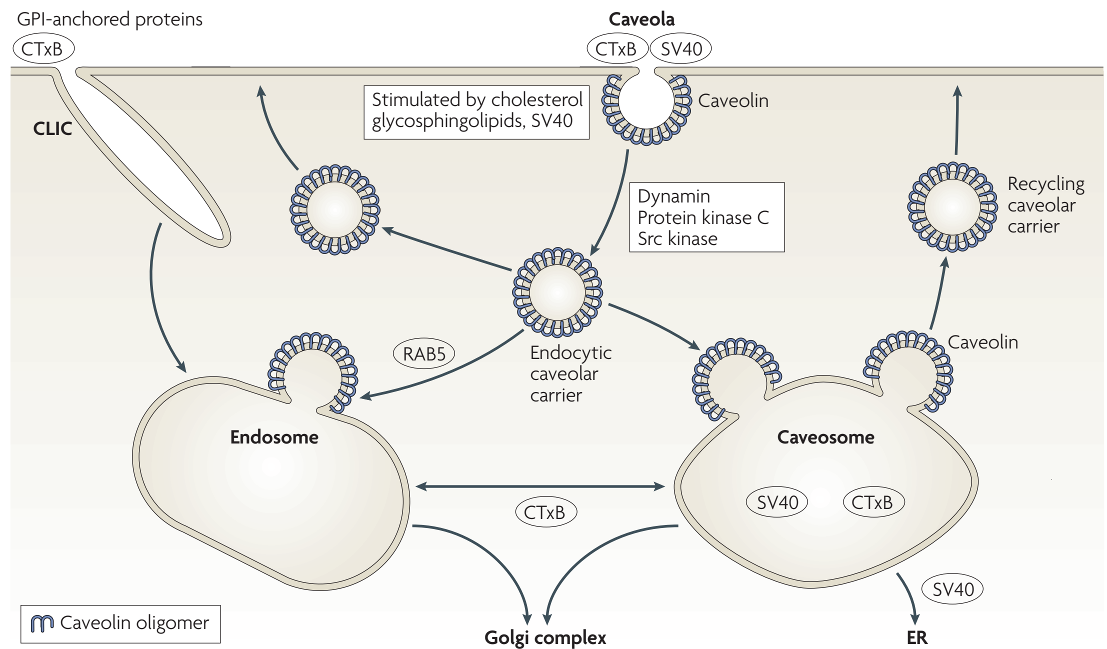

Figure 3 | **Caveola endocytosis.** Caveolae at the cell surface can bud into the cell carrying cholera-toxin-binding subunit (CTxB) and SV40 in a process that is regulated by dynamin, protein kinase C and tyrosine kinases, such as Src kinases. Caveolae bud off to form endocytic caveolar carriers that fuse with the caveosome or with the early endosome, or can fuse back to the plasma membrane without the involvement of an endosomal intermediate[8,44,141]. From the caveosome, SV40 is transported to the endoplasmic reticulum (ER) and recycling endocytic caveolar carriers carry caveolin back to the plasma membrane. CTxB is transported to the Golgi complex, possibly through early endosomes. Note that in cells with or without caveolae there is at least one other main endocytic pathway that involves clathrin- (and caveolin)-independent carriers (CLICs), which can endocytose the same surface markers into endosomes[46]. Glycosylphosphatidylinositol (GPI)-anchored proteins are predominantly internalized through this pathway.

Caveolins and lipid regulation

Caveolae are extremely abundant in adipocytes, and increasing evidence has linked caveolae to lipid regulation in this specialized lipid-storage cell as well as in other cell types. CAV1 interacts with cholesterol, binds to fatty acids[94] and associates with lipid droplets (the site of cellular lipid storage[95,96]) under specific cellular conditions in cultured cells and *in vivo*[97–99].

To examine the role of caveolins in lipid regulation, researchers have studied the effect of *Cav1* expression in cells with low endogenous caveolin levels, as well as looking at the effect of caveolin mutants. Expression of *Cav1* facilitates the uptake of fatty acids into cells[100], and increases the levels of free cholesterol and cholesterol export[101,102]. Studies of fibroblasts and peritoneal macrophages from *Cav1*-null mice showed a slight decrease in cholesterol synthesis and increased esterification in both cell types[103]. A caveolin truncation mutant constitutively associated with lipid droplets and perturbed cellular lipid regulation, which decreased surface levels of free cholesterol and increased neutral lipid storage in lipid droplets[99,104]. Taken together, these studies all point to a role for CAV1 in sterol and fatty-acid regulation, both at the plasma membrane and possibly in lipid droplets; however, the detailed mechanisms are still unclear. The importance of this process *in vivo* is emphasized by the requirement for CAV1 for efficient liver regeneration and mouse survival after partial hepatectomy[105]. The lack of CAV1 caused a dramatic reduction in lipid-droplet formation during the liver-regeneration process. Survival could be dramatically increased by providing glucose as an alternative energy source[105].

Lipid droplet
A lipid-storage organelle that comprises a core of triacylglycerol and/or cholesterol esters surrounded by a phospholipid monolayer.

Lipid sensing and storage. Adipocytes have attracted considerable interest in view of the density of caveolae in these cells and their vital role in lipid regulation. *Cav1*$^{-/-}$ mice show decreased adiposity and are resistant to diet-induced obesity[106]. Caveolae at the cell surface of adipocytes have been implicated in triacylglycerol synthesis, but there is also evidence for the redistribution of CAV1 from the cell surface to lipid droplets upon treatment with cholesterol[107] and upon stimulation of lipolysis[98]. The cholesterol-stimulated association of CAV1 with lipid droplets is inhibited by agents that perturb caveola internalization whereas caveola budding is stimulated by cholesterol[107]. These data indicate a novel, as-yet-uncharacterized, pathway by which CAV1 reaches the lipid droplet, and also link the endocytic role of caveolae with lipid sensing and storage. The intermediate stations on this pathway are unknown, although it is clear that caveolae cannot directly fuse with the lipid droplet, which is enclosed by a phospholipid monolayer. Rather, caveolin might flip from the cytoplasmic leaflet of one membrane into the monolayer. The functional importance of this pathway is unclear; however, the finding that adipocytes from *Cav1*$^{-/-}$ mice show decreased levels of free cholesterol in the lipid droplet[107] indicates an important role in regulating cholesterol trafficking to, or association with, the lipid droplet.

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Box 4 | **Approaches to functionally characterize caveolae**

Several functions have been assigned to caveolae and caveolins but many of these functions must be critically reassessed. In many cases, numerous strategies were used for studying these structures and all of them contributed to the conclusion of caveolar involvement in a specific process and/or the association of proteins with caveolae. These approaches include the following:

- cholesterol depletion
- fractionation using detergents or detergent-free methods
- immunofluorescence
- immunoprecipitation.

There are problems associated with each approach and even their combined use can provide misleading results. First, cholesterol depletion is not specific to caveolae; even in cells that lack caveolae, cholesterol depletion affects many cellular processes. Second, detergent-insoluble preparation and detergent-free methods of purification do not yield caveolar preparations[138]. Studies of caveolae have also been confounded by the small size of caveolae and the resolution of light microscopy. Numerous studies have provided evidence for the colocalization of particular proteins with caveolin proteins using light microscopy, but inspection of the images shows only partial overlap, consistent simply with the observation that two surface proteins are present on the plasma membrane.

The immunoprecipitaton of caveolins with hundreds of different proteins is also puzzling. In some cases, solubilization conditions are unlikely to have caused the dissociation of detergent-resistant membranes (for example, immunoprecipitation from detergent-resistant membranes without further solubilization or with mild detergents). The vast array of proteins that have been shown to immunoprecipitate with caveolin must be considered with a measure of scepticism if the results are not confirmed by independent techniques. In several cases, the association of proteins with caveolae have not been confirmed by immuno-electron microsocpy or immuno-isolation; however, an interesting possibility is that non-caveolar caveolin might explain some of these results. As outlined in the main text, the study of caveolin-null animals and the small interfering (si)RNA-based knockdown of caveolins has started to provide exciting new insights into the involvement of caveolae and caveolins in specific cellular processes.

Caveolae as lipid-raft stores. One astounding property of caveolae is the stability of their lipid–protein assembly. Caveolae seem to be largely static surface structures at the plasma membrane, and so far we only know of viruses, exogeneous gangliosides or cholesterol that can trigger their internalization. In some cells, such as endothelial cells, caveolae represent more than one-third of the plasma membrane surface area. Some of these caveolae fold to form ‘bunches of grapes’ of connected caveolae (FIG. 1), which prompted Tomas Landh to postulate that these structures could represent cubic membrane infoldings that can fold and unfold with little energy input[108].

Why do some cell types have so many caveolae attached to their plasma membranes? Could caveolae function as stores for sphingolipids and cholesterol? These stores could ensure a supply of lipid-raft components to the cell when needed. Caveolae could also be used as a device to regulate plasma membrane surface area. Some cell types have such a high density of caveolae that flattening of the caveolae could contribute significantly to an increase in the accessible surface area of the cell.

We therefore postulate that mammalian cells could use two different organelles to store lipids: lipid droplets and caveolae. If this is the case, regulatory circuits that coordinate their functions in different cell types must be in place.

Mechanosensation
The sensing of mechanical stimuli, for example stretch or flow, by cells.

Caveolae as mechanosensors

One of the most exciting insights into caveola function that has been derived from the use of caveolin-null mice and knockdown systems is the demonstration of a role for caveolae in mechanosensation (for a review see REF. 109). The ability to sense membrane forces might underlie many of the functions of caveolae in diverse cell types, including endothelial and smooth-muscle cells.

Flow sensors in endothelial cells. The surface of endothelial cells is sensitive to changes in hydrostatic pressure and shear stresses. Chronic exposure to shear stress resulted in increased plasma membrane levels of CAV1 due to redistribution of CAV1 from the Golgi complex to the plasma membrane, which caused an increased surface density of caveolae[110,111]. These changes are accompanied by increased mechanosensitivity and activation of specific signalling pathways (including eNOS and mitogen-activated protein kinase (MAPK)) and increased phosphorylation of CAV1. Subsequent studies confirmed and extended these findings and linked integrins to CAV1 phosphorylation[112].

The role of caveolae in mechanosensing is strengthened by recent *in vivo* studies using *Cav1*$^{-/-}$ mice[113]. *Cav1*-knockout mice were crossed with mice expressing CAV1 under an endothelium-specific promoter to assess the precise role of endothelial caveolae *in vivo*. These studies showed a role for CAV1 and caveolae in mechanotransduction and remodelling of blood vessels. *Cav1*-null mice showed defects in chronic flow-dependent remodelling, and in acute flow-dependent dilation; both effects were rescued by CAV1 re-expression in the endothelium, which indicates that caveolin/caveolae might represent a flow sensor.

The endothelial phenotype that has been observed after chronic shear stress is similar to that observed in eNOS-deficient mice in response to flow[114]. In fact, a decrease in eNOS activation was observed in the *Cav1*-null mice[113]. This observation elegantly demonstrates that coupling of the flow stimulus to eNOS activation is lost in the absence of CAV1 and caveolae. This model might seem paradoxical in view of the postulated role of caveolae in the negative regulation of eNOS, and the increased nitric oxide production that has been reported in *Cav1*$^{-/-}$ mice. Based on the above findings, Yu and colleagues proposed that the coupling of flow to eNOS activation might require caveolae, whereas activation of eNOS by agonists is a different process that might be hyperstimulated in the absence of caveolae owing to the loss of the inhibitory influence of CAV1 on eNOS function[113].

Stretch-induced cell-cycle progression. Ongoing studies of caveolae in smooth-muscle cells also support a role for caveolae in mechanosensing. Smooth-muscle cells that have been subjected to cyclic stretch show rapid redistribution of CAV1 to focal contacts[115]. In control cells, stretch triggers cell-cycle progression through the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB) pathway, the MAPK ERK, c-Src, and integrins. This response is inhibited upon transient downregulation of CAV1 or in *Cav1*$^{-/-}$ smooth-muscle

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cells, which indicates an essential role for CAV1 in this process. This requirement was recapitulated in an *in vivo* system[115].

Molecular mechanisms of mechanosensing. Caveolae have been shown to harbour G_q proteins that could be activated by unknown mechanotransducers[116], as well as a sphingomyelinase that could produce ceramide from caveolar sphingomyelin stores in response to specific stimuli[117]. Integrin-mediated connectivity between the ECM and the cytoskeleton has also been implicated in mechanotransduction[118]. Caveolin and caveolae have been tightly connected to integrins and the focal-adhesion machinery. Caveolae can also be removed from the surface by endocytosis in response to specific stimuli, which provides a mechanism to regulate surface mechanosensing activity.

However, one question still remains. How might caveolar functions be regulated by mechanical strain? Could the specialized lipid composition of caveolae be involved? One possibility is that mechanical stress could cause changes in membrane elasticity[119]. This is determined by the lipid composition of the bilayer. Membrane elasticity regulates the hydrophobic coupling between a membrane-spanning protein and the surrounding bilayer. Changes in cholesterol concentration have been shown to have a specific role in modifying the elastic properties of this coupling[120]. Therefore, it is not unreasonable to speculate that mechanosensitive cholesterol–protein couples could be part of the transducing machinery. Mechanical stress and the addition of lipids to cells cause a similar caveolar response. Only further work will clarify whether such mechanisms really are operating. Sens and Turner[121] have recently analysed the issue of budded microdomains as tension regulators from the biophysical viewpoint. They came to the conclusion that caveolae could perform such a role by flattening out into the plasma membrane as a response to increases in surface tension.

Conclusions and perspectives

Caveolae are emerging as important surface-associated organelles that have vital functions in diverse cellular processes. Caveolae are formed by the polymerization of caveolins, which leads to the clustering and invagination of a subset of sphingolipid–cholesterol lipid rafts. In some cell types, considerable reservoirs of lipid-raft components are stored in these surface invaginations. These structures are stable; the bulk of the caveolar caveolins can only be released by dissociation of the caveola structure. But how this disassembly process is regulated is not known.

Nevertheless, there is evidence for a second pool of non-caveolar caveolins in focal adhesions. Detachment of cells from the substratum by the breaking of their focal adhesions leads to massive caveolin-dependent endocytosis. Bacteria might use similar mechanisms, which are possibly also triggered by integrin activation. Other stimuli for caveolar endocytosis include viruses, exogenous glycosphingolipids or addition of cholesterol. How internalization is regulated physiologically and for what purpose is not known; however, caveolar endocytosis seems to be linked to the regulation of the cellular lipid balance.

The most intriguing property of the caveolin/caveolae system is its involvement in mechanosensing. Many of the apparently unrelated functions of caveolae might be related to the ability of caveolae to sense changes in membrane tension or to sense other changes in the physical properties of the plasma membrane (for example, those caused by lipid loading). In this model, caveolae would coordinate physical membrane changes with intracellular signal transduction pathways. How caveolae are structured to carry out these different functions remains to be explored. However, the tools are now there to resolve how caveolae function at the molecular level in cell physiology and pathology.

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