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The role of membrane lipids in the formation and function of caveolae

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Abstract

Caveolae are plasma membrane invaginations with a distinct lipid composition. Membrane lipids cooperate with the structural components of caveolae to generate a metastable surface domain. Recent work has provided insights into the structure of essential caveolar components and how lipids are crucial for the formation, dynamics, and disassembly of caveolae. They also suggest new models for how caveolins, a major structural component of caveolae, insert into membranes and interact with lipids.

Introduction

Caveolae are an abundant feature of the plasma membrane of mammalian cells. These tiny invaginations of the cell surface, approximately 60-70nm in diameter, can occupy up to 50% of the surface area of certain cell types (Echarri and Del Pozo, 2015, Hansen and Nichols, 2010, Lamaze et al., 2017, Parton, 2018, Parton et al., 2020a)(Figure 1). Caveolae can provide a reservoir of membrane to prevent membrane damage (Sinha et al., 2011, Dewulf et al., 2019, Lo et al., 2015) and also have been linked to signal transduction, stress sensing, mechanotransduction, lipid regulation, and transendothelial transport (Echarri and Del Pozo, 2015, Hansen and Nichols, 2010, Lamaze et al., 2017, Parton, 2018, Parton et al., 2020a) (Figure 1).

It has long been known that caveolae are associated with specific membrane lipids. Early electron microscopic studies showed enrichment of sterols in caveolae (Montesano, 1979) and flattening of caveolae on cholesterol disruption (Rothberg et al., 1992). EM studies also showed that bacterial toxins that bind to surface gangliosides associated with caveolae (Montesano et al., 1982, Tran et al., 1987). These studies used cholera and tetanus toxins bound to gold particles and these multivalent particles were shown to drive clustering of the

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gangliosides in caveolae (Parton, 1994). Interestingly, GPI-anchored proteins, linked to the plasma membrane through a lipid anchor, showed similar characteristics with concentration in caveolae only upon clustering with antibodies (Mayor et al., 1994, Parton et al., 1994).

It is now clear that an understanding of the membrane lipids associated with caveolae is essential for understanding their formation, dynamics, and function. With the recent elucidation of the structure of caveolin, the major membrane protein of caveolae, we are now in a position to gain new insights into the role of membrane lipids in caveola formation but also to use caveolae as a general paradigm for understanding the role of membrane lipid-protein interactions in microdomain formation.

Caveola formation and dynamics

Before discussing recent insights into the membrane lipids associated with caveolae we will present a brief consensus overview of the formation, trafficking, and disassembly of caveolae and introduce the major structural proteins of caveolae, the caveolins and cavins. For more extensive reviews the reader is directed to recent articles (Busija et al., 2017) (Lamaze et al., 2017) (Matthaeus and Taraska, 2020) (Hubert et al., 2020a, Parton et al., 2020c, Parton et al., 2021).

Caveolae in vertebrate cells are formed by the cooperation of caveolins, small integral membrane proteins, and cavins, filamentous peripheral membrane proteins (Figure 1B, C). Both caveolins (1,2, and 3 in vertebrate cells) and cavins (Cavins1-3 in most cells and Cavin4 in striated muscle) form homooligomers and can also heter-oligomerise with other family members (Gambin et al., 2014, Hansen and Nichols, 2010, Kovtun et al., 2014, Ludwig et al., 2016).

Caveolins are embedded within the membrane with both N- and C-termini facing the cytoplasm. Like other integral membrane proteins, caveolins are synthesised cotranslationally in the endoplasmic reticulum and then traffic through the Golgi complex to the plasma membrane (Monier et al., 1995). This process is accompanied by the oligomerization of caveolin to the final mature state found in caveolae comprising multiple oligomeric complexes (8S comprising approximately 10-14 monomers) that come together to form a larger functional unit (Hayer et al., 2010). Formation of the higher order oligomers occurs via a cholesterol-dependent mechanism (Hayer et al., 2010) and is accelerated by cholesterol supplementation (Pol et al., 2005). Caveolins are also post-translationally modified by palmitoylation as they traverse the secretory pathway (Dietzen et al., 1995) (Parat and Fox, 2001).

At the plasma membrane, formation of caveolae is completed by the association of cavins forming characteristic surface invaginations of variable curvature (Matthaeus et al., 2022). Cavin1/PTRF is essential for caveola formation (Hayer et al., 2010, Hill et al., 2008). EHD2 (Moren et al., 2012, Stoeber et al., 2012) and pacsin2 (Hansen et al., 2011, Senju et al., 2011), as well as ROR1 in specific cell types (Yamaguchi et al., 2016) also associate with caveolae. Surface caveolae can bud from the plasma membrane, in a process negatively regulated by EHD2 (Moren et al., 2012, Stoeber et al., 2012), and fuse with early endosomes

before recycling back to the plasma membrane as a unit (Boucrot et al., 2011, Pelkmans et al., 2004). This cycle of budding, endosomal trafficking and recycling may not be crucial for endocytosis of many cargo proteins as membrane proteins are largely excluded from caveolae (Parton et al., 2020a, Shvets et al., 2015) but might be important in regulating surface levels of caveolae (Boucrot et al., 2011) and for lipid transport in specific cell types (Matthaeus et al., 2020).

Caveolae can also be disassembled in response to specific cellular conditions, such as increased membrane tension (Hetmanski et al., 2019, Sinha et al., 2011) (Del Pozo et al., 2021) and oxidative stress (Wu et al., 2023), suggesting that caveolae represent a metastable structure, built up by proteins and membrane lipids, ready to disassemble. Caveola disassembly can also influence plasma membrane lipid organization with increased clustering of phosphatidylserine (PS) and enhanced isoform-specific Ras signaling from lipid nanodomains upon loss of caveolae or experimental flattening of caveolae (Ariotti et al., 2014) or increased formin-dependent assembly of actin through increased availability of $PI(4,5)P_2$ (Teo et al., 2020).

Caveolar proteins and membrane lipids

Recent work points towards an integral role of membrane lipids in both the formation and dynamics of caveolae. The major structural proteins of caveolae, the caveolins and cavins, both show association with specific membrane lipids and many of these interactions have now been mapped in some detail. Caveolin tightly binds cholesterol (Murata et al., 1995, Thiele et al., 2000) but can also associate with cholesterol through indirect mechanisms as shown by *in vitro* studies with isolated caveolin domains (Epand et al., 2005, Wanaski et al., 2003) and by nanoscale mapping of caveolin-lipid association on plasma membrane sheets (Zhou et al., 2021). Caveolin can also be crosslinked to GM1 in the extracellular leaflet of the plasma membrane (Fra et al., 1995). Cavins, which have two basic helical regions (HR1 and HR2) flanked by acidic disordered regions (DR1, DR2 and DR3) (Kovtun et al., 2015), have a positively-charged binding site for $PI(4,5)P_2$ in the HR1 domain (Kovtun et al., 2014). This site is not essential for caveola formation but is ubiquitinated when cavins dissociate from the membrane leading to proteasomal degradation of the non-membrane bound cavin (Tillu et al., 2015). This ‘membrane-sensor’ domain can therefore ensure that cytosolic cavin levels are maintained at low levels.

The specific role of cavin1 in caveola formation has been linked to a conserved undecad repeat region in the HR2 region that binds PS *in vitro* (Tillu et al., 2018). This site regulates the stability of caveolae, with an increased number of repeats, as seen in particular cavin1 family members in evolution, being associated with increased caveolar stability (Tillu et al., 2018). This suggests that cavin1 has two distinct sites that bind membrane lipids. In addition, cavin1 can bind to the N-terminus of CAV1 (Tillu et al., 2021) and to ROR1 (Yamaguchi et al., 2016). These interactions are presumed to be of low affinity allowing disassembly of caveolae and release of cavins into the cytosol in response to changing cellular conditions.

Caveolae as a distinct lipid domain

These studies raise the question of the precise lipid composition of caveolae. As the majority of caveolae are attached to the plasma membrane at any point in time, the purification of caveolae is not trivial and current biochemical purification methods make isolation of caveolae with the neck region intact very challenging. However, lipidomic studies of purified caveolae have provided a quantitative picture of lipids in caveolae, showing enrichment of cholesterol, sphingomyelin, and GD3 (Ortegren et al., 2004). Two recent studies have provided more detailed insights into the association of specific lipids with caveolae and the role of those lipids in caveola formation and dynamics (Hubert et al., 2020b, Zhou et al., 2021). Interestingly, despite different microscopic techniques and the difficulty in localizing lipids at this resolution, the results are in good agreement. Firstly, using a combination of quantitative immunogold electron microscopy and FLIM/FRET a distinct set of lipids were shown to associate with expressed CAV1 and cavin1. CAV1 showed association with cholesterol, PS and $PI(3,4,5)P_3$ and cavin1 with $PI(4,5)P_2$, $PI(3,4,5)P_3$, and PA (Zhou et al., 2021). Interestingly this profile was quantitatively changed when CAV1 and cavin1 were co-expressed, representative of native caveolae, with $PI(3,4,5)P_3$ depleted but significant association with cholesterol, $PI(4,5)P_2$, PS, and PA. This profile was also dramatically altered by mutation of the $PI(4,5)P_2$ site in cavin1 with a significant decrease in association with all these lipids, not just $PI(4,5)P_2$. Similarly, mutation of the UC1 domain of cavin1 caused large-scale changes in lipid association with reduced association with $PI(4,5)P_2$, $PI(3,4,5)P_3$ and PA (Zhou et al., 2021). This indicates that both CAV1 and cavin1 contribute to the lipid environment in caveolae and that this is not a simple result of direct binding of clustered lipids but complex synergistic interactions between the proteins and membrane lipids. A second study used a complementary approach in which the association fluorescent lipids with caveolae as well as the lipid dynamics, were monitored by light microscopy after their introduction by liposome fusion into the plasma membrane (Hubert et al., 2020b). Cholesterol, sphingomyelin, and glycosphingolipids were all shown to be concentrated in caveolae but whereas cholesterol and sphingomyelin were actively sequestered in caveolae, glycosphingolipids diffused freely. The role of the other structural components of caveolae and how they contribute to the unique lipid code is currently unknown, though there are hints at additional roles in fine-tuning caveolar lipid composition (discussed below). Studies looking into the lipidomic composition of model caveolae generated by Caveolin-2 (CAV2) in a prokaryotic system demonstrated a distinct profile compared to caveolae generated by CAV1 (Walser et al., 2012). This hints at the possibility that incorporation of CAV2 into caveolin hetero-oligomers may represent an additional factor to alter caveolar lipid composition, though what these lipids represent in a mammalian cell membrane remains to be investigated. Together these studies are providing a glimpse of the complex nanoscale lipid environment of caveolae including the specific enrichment of particular membrane lipids.

Role of membrane lipids in formation and stability of caveolae

Recent studies point to the role of membrane lipids in regulating both the formation and dynamics of caveolae. Cholesterol and PS are required for caveola formation in mammalian cells (although in a model prokaryotic system they are not required for curvature generation

by CAV1 (Walser et al., 2012)). Cholesterol can regulate traffic of caveolins through the secretory pathway (Pol et al., 2005) and depletion of cholesterol from the plasma membrane causes flattening of the caveolar invagination and dissociation of cavins into the cytosol (Hill et al., 2008, Rothberg et al., 1992). Perturbation of cholesterol formation in cells accumulating desmosterol through inhibition of DHCR24 also caused structural heterogeneity and reduced curvature of caveolae (Jansen et al., 2008). Cells deficient in PS show reduced caveola formation which can be rescued by PS addition (Hirama et al., 2017, Zhou et al., 2021). Caveola formation, as judged by CAV1/cavin1 association, could be driven by fully saturated PS (DSPS, di18:0) and monounsaturated PS (DOPS, di18:1) but not mixed chain PS (POPS, 16:0/18:1) (Zhou et al., 2021) implicating specific PS acyl chains in facilitating formation of the caveolar domain.

These studies show that while caveolins and cavins can directly interact, these interactions are presumably of low affinity as they are highly sensitive to the lipid environment. For example, depletion of PS or cholesterol leads to caveolar instability, as indicated by reduced caveolin-cavin interaction and disruption of caveolar morphology (Hill et al., 2008, Hirama et al., 2017, Rothberg et al., 1992, Zhou et al., 2021). This indicates that the caveolar domain is built up from multiple low affinity interactions - protein-protein, protein-lipid, and presumably lipid-lipid - to generate a metastable domain. Each of these interactions alone is not sufficient to make a stable caveolar domain meaning that perturbation of any of the components can cause caveola disassembly.

Role of membrane lipids in formation and budding of caveolae

Increasing evidence suggests a role for cholesterol and other membrane lipids in not only the formation of caveolae but also as a key regulator of their budding from the plasma membrane. In an elegant approach, fusion of liposomes with cultured cells allowed delivery of specific lipids into the plasma membrane (Hubert et al., 2020a, Hubert et al., 2020b). Cholesterol was shown to cause increased caveola curvature and caveolar budding. This process is negatively regulated by EHD2 (Hubert et al., 2020b) and by dynamin (Larsson et al., 2023) and therefore may represent a physiological process triggered by specific lipids. The stimulation of endocytic trafficking by cholesterol is consistent with earlier studies showing budding of caveolae in fibroblasts (Sharma et al., 2004) and cultured adipocytes in response to cholesterol addition (Le Lay et al., 2006). Similar results were obtained with the glycosphingolipid, LactosylCeramide (LacCer), again consistent with earlier studies using different approaches (Sharma et al., 2005, Sharma et al., 2004). These studies provide compelling evidence that caveola dynamics are regulated by the lipid composition. In this way caveolae can act as sensors of specific plasma membrane lipids, with lipids regulating their surface levels. There are further exciting implications of these studies. The fact that curvature and budding can be driven by changes in specific lipid species and that caveolin oligomerization alone appears to be sufficient for caveola formation and fission in a model system raises the possibility that the budding is driven by lipids through their effect on caveolin-induced membrane curvature. The role of accessory proteins such as EHD2 (Moren et al., 2012, Stoeber et al., 2012) and dynamin (Larsson et al., 2023) might be to regulate this lipid-driven process.

Lipid interactions with the caveolin scaffolding domain

The conserved caveolin scaffolding domain (CSD) of CAV1 (amino acids 81-101 in mammalian CAV1) has been proposed to regulate signaling through interaction with proteins containing a caveolin binding domain (CBD) (Couet et al., 1997, Sargiacomo et al., 1993). However, this direct interaction model has been challenged in a number of studies (Byrne et al., 2012, Collins et al., 2012, Huang et al., 1997, Jung et al., 2018). This model also appears incompatible with the recent cryoEM structure of the caveolin oligomer that suggests that the CSD region is involved in stabilizing contacts beween Cav1 protomers ((Porta et al., 2022), see below). Yet the profound effects of peptides corresponding to the CSD of CAV1, either linked to GFP or fused to a cellular penetrating peptide, are striking and highly specific. This includes specific inhibition of eNOS in cells and *in vivo* (Bucci et al., 2000) and inhibition of clathrin-independent endocytosis (Chaudhary et al., 2014). An interesting possibility is that these effects are actually mediated through lipid modulation. The CSD region contains a putative cholesterol-binding CRAC domain (Yang et al., 2014, Hoop et al., 2012, Epand et al., 2005). When expressed in cells a CSD-GFP fusion protein associates with plasma membrane domains enriched in $PI(4,5)P_2$, $PI(3,4,5)P_3$ and cholesterol (Zhou et al., 2021). Moreover, expression of the CSD fusion protein increased the nanoscale clustering of PIP2 and cholesterol as shown by quantitative immunoelectron microscopy (Zhou et al., 2021). *In vitro* binding experiments also show that CSD-peptides bind to liposomes and promote the formation of cholesterol-rich domains that lead to cholesterol crystallization (Epand et al., 2005). This was not proposed to be through a direct interaction but through generation of a specific lipid domain. Similarly specific clustering of NBD-PS and NBD-cholesterol was observed upon addition of CSD peptides to liposomes (Wanaski et al., 2003), although it should also be noted that the insolubility of CSD peptides suggested that at least experiments using residues 83-101 should be treated with caution owing to the formation of micelles (Arbuzova et al., 2000) (as also observed by (Huang et al., 1997)).

In summary, while a number of studies suggest that peptides corresponding to the CSD of caveolin have very specific effects on cells and even *in vivo*, for example on regulation of eNOS or on clathrin-independent endocytosis, these effects might at least partially be driven by lipid interactions. Whether this reflects the properties of this region of caveolin when incorporated into the caveolin oligomer or is unrelated to the physiological function of caveolin remains unclear.

Disassembly of caveolae; possible role for lipid peroxidation

The metastable caveolar domain of caveolins, cavins and membrane lipids can disassemble in response to cellular stress. For example, UV treatment or oxidative stress causes loss of caveolae from the plasma membrane and dissociation of cavins into the cytosol. Recent work suggests that lipid peroxidation can trigger caveolar disassembly and cavin release in response to oxidative stress allowing cavins to regulate the oxidative stress response (Wu et al., 2023). As lipid peroxidation can change the physical properties of the target lipids, polyunsaturated omega 6 acyl chains such as arachidonate/C20:4 at the sn2 position of membrane glycerophospholipids, this raises the possibility that these lipids in the non-

oxidized state, are essential for caveola stability. Unsaturated acyl chains are required in highly curved membranes and for a range of cellular processes, including vesicle formation and ion channel opening (Barelli and Antonny, 2016). It is therefore interesting to speculate that such lipids, through their specific biophysical properties, are required for caveola formation and as sites for regulation through peroxidation. Incorporation of C22:6 into cells poor in polyunsaturated phospholipids, for example, dramatically increases endocytosis, correlating with a decrease in plasma membrane rigidity. The high membrane curvature in caveolae, most notably in the neck region (Parton et al., 2020b) may require the high flexibility of polyunsaturated acyl chains, particularly in the z direction (normal to the bilayer) (for review see (Barelli and Antonny, 2016)) making the caveolar domain particularly susceptible to lipid peroxidation.

In this model omega6 acyl chains may be important for caveola formation. In contrast omega3 fatty acids (such as docosahexanoic acid C22:6) appear to disrupt caveolae (Chen et al., 2007, Li et al., 2007, Ma et al., 2004), possibly replacing the omega6 acyl chains required for caveola formation. Intriguingly, the Mfsd2a protein acts as a flippase for omega3 lipids such as DHA in the endothelial cells of the blood brain barrier and its knockout increases caveola formation (Andreone et al., 2017) consistent with an inhibitory effect of omega3 fatty acids on caveola formation.

The cryoEM structure of CAV1 suggests potential mechanisms of lipid sorting

Recent work has now uncovered a critical piece of the puzzle of how caveolins and caveolae sort lipids: a 3.5 Å resolution cryoEM structure of the human CAV1 8S complex (Porta et al., 2022). The CAV1 8S complex has a wheel-like appearance, consisting of an outer rim, inner spoke region, and central hub, with an overall diameter of ~14 nm (Figure 2). It is composed of 11 tightly packed CAV1 protomers symmetrically organized in a spiral pattern around a central beta barrel formed by the C-terminal most residues of each protomer (Figure 2). Consistent with CAV1's monotopic topology, both the N- and C-terminal-most residues face the same side of the complex. The opposite side of the complex, corresponding to its membrane facing surface, is strikingly flat (Figure 2). This has important implications for how the complex likely associates with membranes, as discussed in more detail below.

The atomic model of the complex revealed residues 49-177 of the protein are ordered, allowing the locations of all of the previously described regions of the protein to be identified in the context of the multimeric complex (Figure 2). The signature motif, scaffolding domain, and oligomerization domain are all located in the outer rim of the complex. These regions contribute extensively to protomer-protomer interactions, explaining their role in oligomerization and the high degree of conservation of the signature motif. The scaffolding domain is helical and contains both a putative CRAC and putative CARC motif (Figure 3). However, it seems unlikely that they bind cholesterol in context of the intact complex, for reasons discussed further below.

The scaffolding domain is immediately followed by a region called the intramembrane domain. This domain is primarily helical as previously suggested (Lee and Glover, 2012).

In contrast to previous models, however, it does not adopt a hairpin structure. Instead, this region of Cav1, together with the newly-described helical spokes, contributes to the formation of a flat and expansive membrane facing surface of the complex. The intramembrane domain also has a second structural role: its helices lie underneath the oligomerization domain of the adjacent protomer, thus contributing to protomer-protomer interactions that exist across the entire length of each protomer. Indeed, with the exception of the central cavity created by the beta barrel, the protomers are so closely packed that there is essentially no free space within the complex (Figure 2). This strongly suggests that the outer rim and spoke region of the CAV1 complex exclude other proteins and lipids.

The raised hydrophobic outer rim, large flat hydrophobic membrane-facing surface, and the tightly packed nature of the protomers, strongly suggest the CAV1 complex associates with membranes in a manner that differs substantially from previous models (Figure 4) and from almost all previously characterized membrane proteins. In particular, the structure suggests CAV1 sits deeply in the bilayer, displacing a large patch of lipids from the cytosolic leaflet of the cytoplasmic leaflet of the plasma membrane. It thus predicts that CAV1 generates a unique, localized membrane nanodomain, consisting of a protein disc on the cytoplasmic leaflet of the membrane and a lipid monolayer on the other (Figure 4).

This new model raises several potential mechanisms for how CAV1 might sort lipids (Figure 5). The patch of membrane that sits directly above the CAV1 complex is one obvious site where lipid sorting may occur. For example, to prevent hydrophobic mismatch, lipids with a particular chain length could be recruited to extracellular leaflet directly above the CAV1 complex. The choice of lipids likely depends both on the composition of the surrounding membrane and how deeply the CAV1 complex sits in the bilayer. For example, if the CAV1 complex is slightly thinner than the average width of the cytoplasmic leaflet, lipids with longer acyl chains may be selectively recruited to the extracellular leaflet to fully solvate the hydrophobic membrane facing surface of the complex. Furthermore, the model makes a strong prediction that the covalently attached palmitate chains would interdigitate across the center of the bilayer, inserting into the extracellular leaflet of the plasma membrane (Figures 4, 5). Each Cav1 protomer contains 3 palmitoylation sites, adding up to a total of 33 for the entire 8S complex. The presence of these saturated fatty acids could in turn recruit raft-preferring lipids such as cholesterol and glycosphingolipids, as well as GPI-anchored proteins, which are also typically composed of saturated acyl chains (Benting et al., 1999, Maeda et al., 2007).

In addition to sorting lipids on the extracellular leaflet above the complex, CAV1 could also sort lipids via direct protein-lipid interactions on the outer rim of the membrane. One attractive possibility is that specific lipids could preferentially associate with the outer rim of the complex. It seems unlikely that CAV1's putative CRAC motif is functional in this capacity, as cholesterol bound to this site on the 8S complex would be predicted to sit almost perpendicular to the plane of the membrane (Figure 3). The CARC motif is also not likely to bind cholesterol, due to a lack of accessibility for binding (Figure 3). It is nevertheless possible that either the CRAC motif or CARC motif that is located on the same stretch of the scaffolding domain (Figure 3) could interact with cholesterol when peptides comprising this region of the protein are studied in isolation (Yang et al., 2014, Hoop et al., 2012,

Epand et al., 2005). This could potentially explain the specific lipid sorting properties of peptides containing the CSD, including Cavtratin, discussed above. The hydrophobic cavity within the central beta barrel of the CAV1 8S complex is yet another potential binding site. It is open to the cytoplasm and is capped with a ring of charged residues that could potentially interact with lipid headgroups. The narrowest accessible diameter of the cavity is ~15 angstrom, raising the possibility that it could accommodate several lipids.

Finally, we envision additional lipid sorting mechanisms that depend on synergistic interactions between caveolin complexes, or between CAV1 and other proteins. For example, interactions between 8S complexes that occur during 70S complex formation- a process known to be cholesterol dependent - could generate regions of specialized lipid composition between adjacent complexes. Direct or indirect interactions between CAV1 and cavin-1 within caveolae could lead to futher remodeling of these inter-complex membrane patches. For example, the ability of cavin1 to bind PS could potentially recruit PS to membrane patches between Cav1 8S complexes. This in turn could modulate local cholesterol levels as a consequence of the close interplay between the two lipids (Maekawa and Fairn, 2015). Clearly, much work remains to be done to test these and other possibilities.

Summary and Future Perspectives

These new advances allow us to propose a new working model for the formation of caveolae. In Figure 6 we provide a scheme of the major protein and lipid interactions involved in assembling caveolae, from synthesis of caveolin in the endoplasmic reticulum, caveolin oligomer assembly in the Golgi complex, delivery to the plasma membrane, and association with cavin proteins through protein-protein and protein-lipid interactions (Figure 6). This complex pathway enables the formation of caveolae specifically at the plasma membrane where the metastable domains can rapidly disassemble in response to stress and modulate intracellular signalling.

Despite the rapid advances in our understanding of caveolin, caveolae, and lipid sorting over the past few years, many critical questions still remain unanswered. For example, although it is widely recognized that caveolae sequester cholesterol and cholesterol is critical for the integrity of caveolae, how and why this is the case is unknown. How different caveolin family members each contribute to lipid sorting, and how disease mutations of caveolins impact this process is also not yet clear. Relatedly, a frameshift mutation of CAV1 associated with pulmonary arterial hypertension and congenital generalized lipodystrophy, was found to exhibit reduced colocalization with cavin-1 in MEFs (Han et al., 2016). Whether this reflects decreased protein-protein interactions between CAV1 and cavin-1, decreased PS levels in mutant caveolae, or both has yet to be determined.

Another important question is how lipids influence the structure and assembly of the caveolin complex itself. The finding that the human Cav1 can assemble into 8S complexes in *E. coli* membrane emphasizes that its native lipid environment is not essential for 8S complex formation and rules out a requirement for cholesterol in this process. However, recent evidence suggests that assembly of 8S complexes may occur at the level of the Golgi complex rather than immediately following biosynthesis of the protein (Morales-Paytuvi et

al., 2022). This raises the possibility that in mammalian cells 8S complex assembly may depend on lipid composition, membrane thickness, and/or membrane mechanical properties in ways that are not yet understood.

Finally, very recent evidence now suggests that in cells lacking cavin-1, CAV1 can generate a newly described class of membrane invaginations, termed dolines (Lolo et al., 2022). These structures are significantly less regular in size and shape than caveolae; however, like caveolae, dolines flatten in response to increased membrane tension, albeit at lower tension than is necessary for caveolar flattening (Lolo et al., 2022). These structures could be relevant not only in mammalian cells where CAV1 is expressed without cavin-1, as sometimes is seen in cancer cells (Gould et al., 2010), but also in the many non-vertebrate organisms that express caveolins but naturally lack cavin-1 (Hansen and Nichols, 2010). For example, large invaginations observed in the Ascidian *Ciona intestinalis* that are dependent on caveolin but of distinct morphology to mammalian caveolae may represent doline-like structures (Bhattachan et al., 2020). In view of our increasing understanding of caveolin-lipid interactions as summarised here, it will be interesting to test if dolines form in a lipid-dependent manner, show lipid sorting abilities, and if aspects of their function depend on specific membrane lipids.

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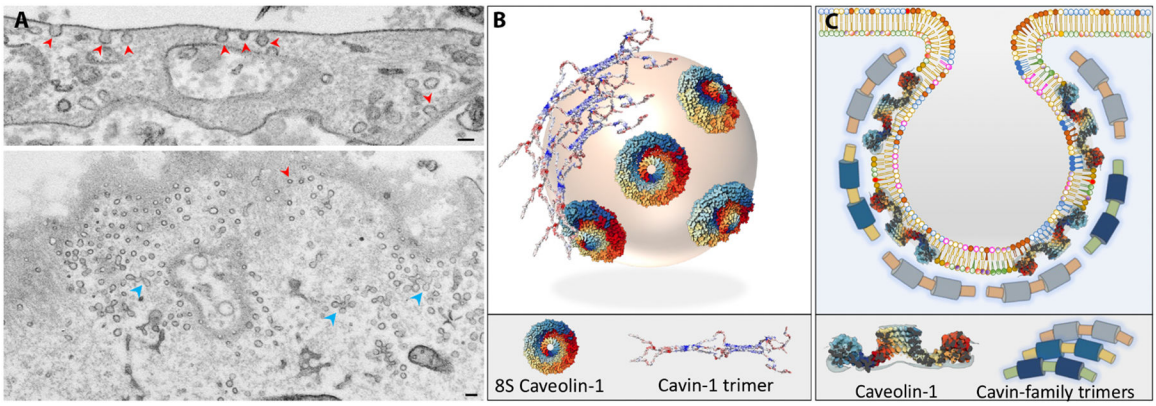

Figure 1.

A) Electron micrographs showing single caveolae (red arrowheads) and interconnected groups of caveolae (blue arrowheads) in cultured adipocytes. The top panel shows the characteristic flask-shaped caveolae connected to the plasma membrane and the bottom panel shows a glancing section across the surface of the cell in which caveolae appear as vesicular profiles. Bars, 100nm. B) Schematic of the cavolar structure showing the potential organisation of Cavin1 trimers and 8S caveolin-1 oligomers. C) Cross-sectional depiction of caveolin, cavin and lipids in the plasma membrane.

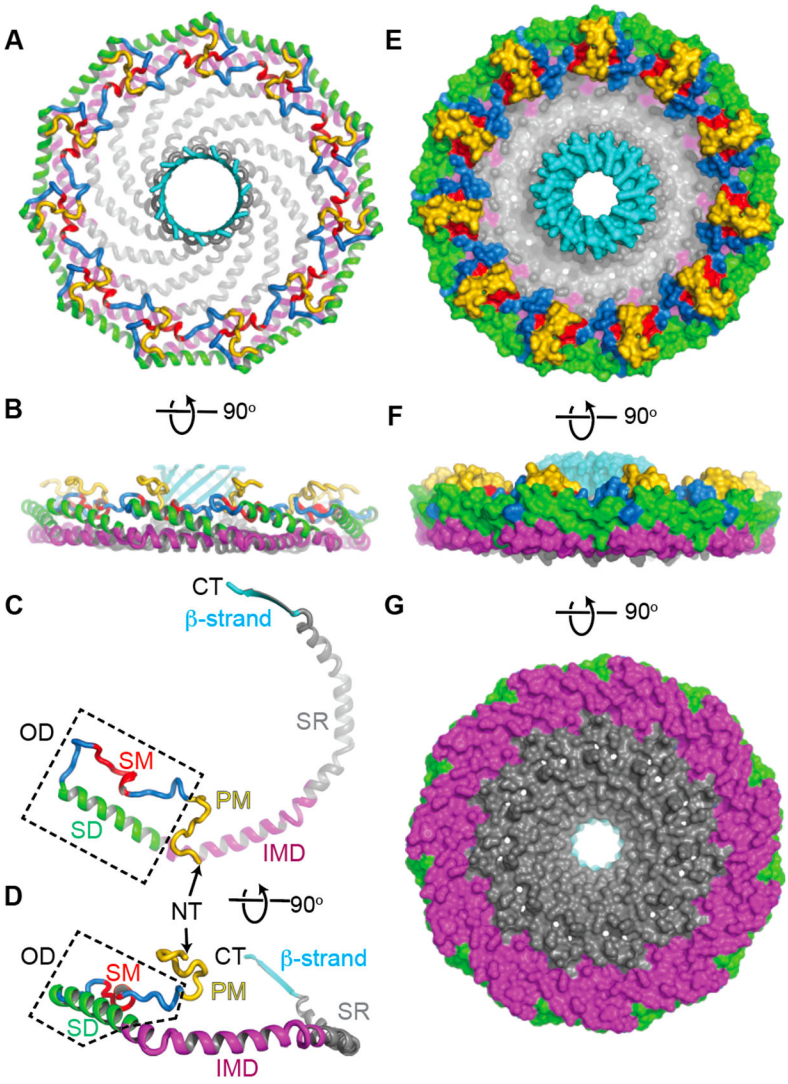

Figure 2. Organization of the Cav1 complex as revealed by the cryoEM structure of human CAV1.

(**A-B**) Atomic model of residues 49-177 of the CAV1 8S complex. (**C-D**) Structure of a single CAV1 protomer. Orientations match those in panels A and B. (**E-G**) Space filling model of the 8S complex. A, and H, cytoplasmic face; B, and F, side view; G and J, membrane facing surface. Color coding in A-G is as follows: red, signature motif; green, scaffolding domain; purple, intramembrane domain; gray, spoke region; cyan, beta strand. The oligomerization domain, which contains the scaffolding domain and signature motif, is highlighted with a dashed box. Adapted from Porta, JC, B Han, A Gulsevin, JM Chung, Y Peskova, S Connolly, HS McHaourab, J Meiler, E Karakas, AK Kenworthy and MD Ohi (2022). Molecular architecture of the human caveolin-1 complex. Sci Adv 8(19): eabn7232.

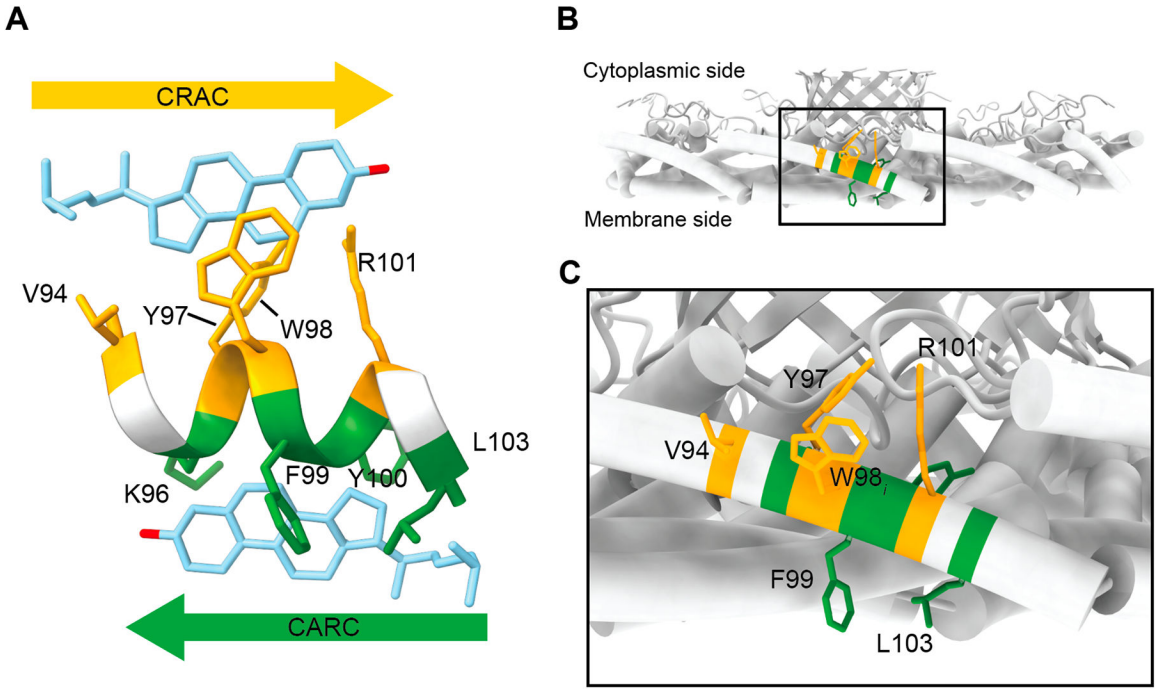

Figure 3. Position of the CRAC motif in the structure of the Cav1 complex.

(**A**) The scaffolding domain of Cav1 contains a putative cholesterol-binding CRAC motif (yellow) and CARC motif (green). (**B**) Position of the putative CRAC and CARC motifs on the cryoEM structure of the 8S Cav1 complex. For simplicity, the α-helix they are located on is shown as a cylinder. (**C**) Zoomed in view of the CRAC and CARC motifs.

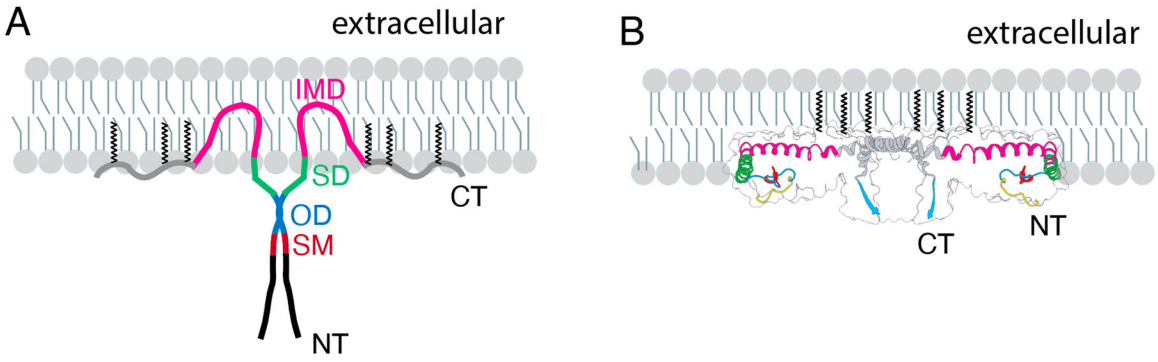

Figure 4. The cryoEM structure of Cav1 suggests a new model for how Cav1 sits in the plasma membrane and potentially sorts lipids.

(A) Previous models typically suggested the intramembrane domain of CAV1 forms a wedge shape in the bilayer and the C-terminal domain forms a helix that sits at the membrane interface, as is characteristic of many amphipathic alpha helices. This model predicts that the lipids in the vicinity of Cav1 form a traditional bilayer structure and the bulk of the N-terminal domain of the protein resides in the cytoplasm. It also implies that palmitates bound to Cav1 insert into the cytoplasmic leaflet of the membrane. (**B**) The cryoEM structure paints a very different picture for how Cav1 associates with the plasma membrane than suggested by previous models. In particular, the raised, hydrophobic outer rim and flat hydrophobic membrane facing surface, combined with the tightly packed nature of the complex suggest it sits deeply in the bilayer, excluding a large patch of lipids from the cytosolic leaflet of the plasma membrane as a result. This model implies that bulk of the protein, including the scaffolding domain, is nearly completely embedded in the membrane bilayer and that the palmitates interdigitate into the extracellular leaflet. In both A and B, the signature motif (SM) is shown in red, the scaffolding domain (SD) in green, and the intramembrane domain (IMD) in magenta. In panel B, the spoke region (SR) is shown in gray and the C-terminal beta strand is cyan. NT, N-terminus; CT, C-terminus. Adapted from Porta, JC, B Han, A Gulsevin, JM Chung, Y Peskova, S Connolly, HS McHaourab, J Meiler, E Karakas, AK Kenworthy and MD Ohi (2022). Molecular architecture of the human caveolin-1 complex. Sci Adv 8(19): eabn7232.

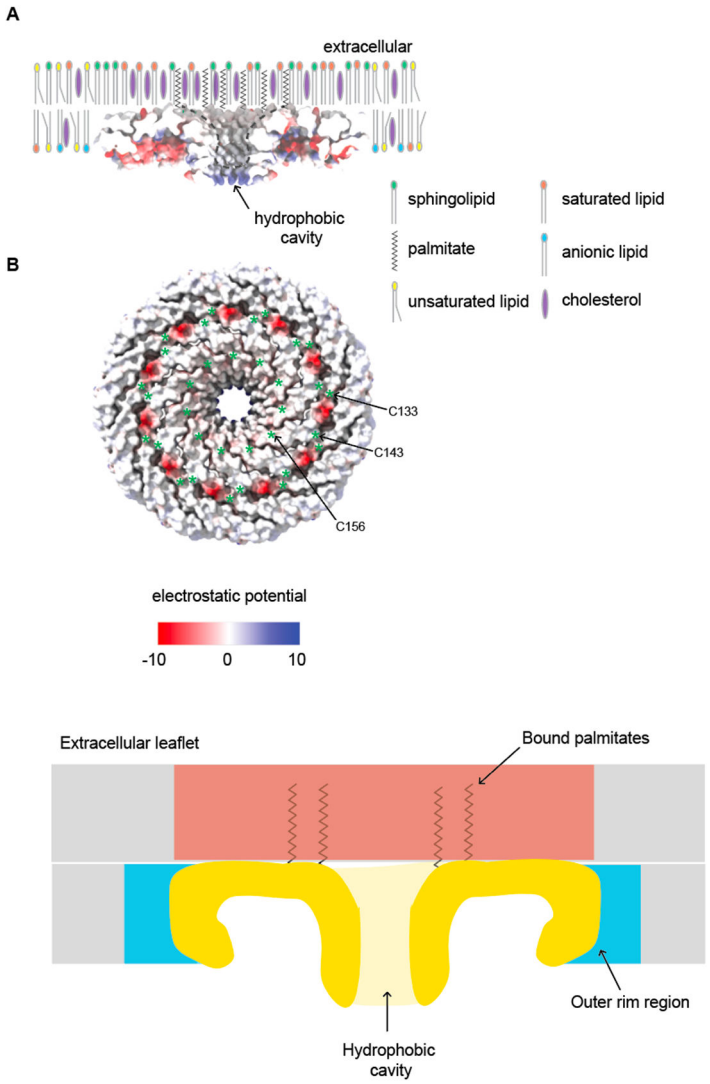

Figure 5. Potential mechanisms of lipid sorting by caveolin.

(**A**) Space filling model of the membrane facing surface of Cav1, color coded to depict electrostatic potential and highlight the position of palmitoylation sites (green *). (**B**) A cross section through the 8S complex embedded in a membrane bilayer showing the position of the palmitates and lipid species potentially enriched within the caveolin 8S domain. (**C**) The 8S complex (yellow) embedded in a membrane bilayer. The complex could potentially sort lipids in contact with its outer rim (blue), membrane-facing surface (pink), or bound palmitates (black). It could also potentially sequester lipids in the hydrophobic cavity of the beta barrel (light yellow). Panel A is adapted from Porta, JC, B Han, A Gulsevin, JM Chung, Y Peskova, S Connolly, HS McHaourab, J Meiler, E Karakas, AK Kenworthy and MD Ohi (2022). Molecular architecture of the human caveolin-1 complex. Sci Adv 8(19): eabn7232. © The Authors, some rights reserved; exclusive licensee AAAS. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC) http://creativecommons.org/licenses/by-nc/4.0/”

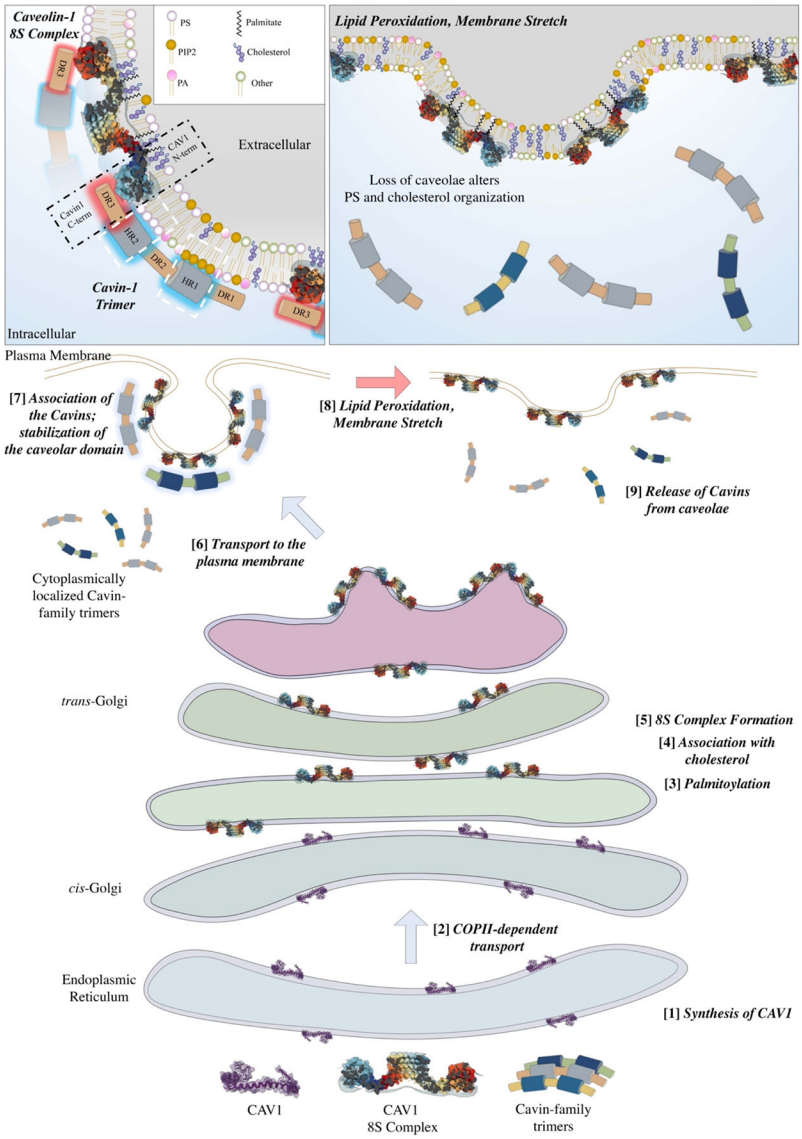

Figure 6. Intracellular Trafficking of Caveolin; from synthesis to caveola formation and cellular stress response.

[1] Cav1 is synthesized in the rough endoplasmic reticulum (RER). [2] From the RER, Cav1 is trafficked to the Golgi complex via COPII-dependent vesicular transport as monomers or low molecular weight oligomers. [3-5] As Cav1 matures through the Golgi it is palmitoylated, associates with cholesterol and forms the 8S complex. [6-7] 8S complexes are transported to the PM where they recruit soluble Cavin1, Cavin1:Cavin2 and Cavin1:Cavin3 trimers. The coalescence of both these protein families with specific lipids at the PM generates the metastable caveolar domain. (Inset: Top Left) Schematic of Cav1 8S complex, Cavin1 trimer and lipids in caveolae. The interaction between Cavin-1 and Cav1 requires amino acids 330-345 in Disordered Region 3 (DR3) on Cavin1 that has been postulated to associate with the disordered N-terminal region of Cav1. Cav1 associates with cholesterol, PS and $PI(4,5)P_2$ whereas Cavin1 binds $PI(4,5)P_2$ via the Helical Region 1 (HR1) domain, PS through the HR2 domain and sequesters PA within caveolae. These

proteins function together to recruit the distinct lipid profile that is observed in caveolae. [8] Caveolae flatten in response to stress stimuli such as lipid peroxidation or mechanical stretch. (Inset: Top Right) Stretch or lipid peroxidation results in the alteration in the lipid profile of caveolae, dissociation of the cavin-coat proteins and loss of the quintessential caveolar morphology. [9] Release of the cavin proteins from the plasma membrane mediates intracellular signaling.