Caveolins and cavins in the trafficking, maturation, and degradation of caveolae: implications for cell physiology

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Abstract

Caveolins (Cavs) are ~20 kDa scaffolding proteins that assemble as oligomeric complexes in lipid raft domains to form caveolae, flask-shaped plasma membrane (PM) invaginations. Caveolae (“little caves”) require lipid-lipid, protein-lipid, and protein-protein interactions that can modulate the localization, conformational stability, ligand affinity, effector specificity, and other functions of proteins that are partners of Cavs. Cavs are assembled into small oligomers in the endoplasmic reticulum (ER), transported to the Golgi for assembly with cholesterol and other oligomers, and then exported to the PM as an intact coat complex. At the PM, cavins, ~50 kDa adapter proteins, oligomerize into an outer coat complex that remodels the membrane into caveolae. The structure of caveolae protects their contents (i.e., lipids and proteins) from degradation. Cellular changes, including signal transduction effects, can destabilize caveolae and

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produce cavin dissociation, restructuring of Cav oligomers, ubiquitination, internalization, and degradation. In this review, we provide a perspective of the life cycle (biogenesis, degradation), composition, and physiologic roles of Cavs and caveolae and identify unanswered questions regarding the roles of Cavs and cavins in caveolae and in regulating cell physiology.

Abbreviations:

8S-Cav – 8S20,w oligomer of Cavs
60S-Cavin – 60S20,w oligomer of cavins
70S-Cav – 70S20,w oligomer of Cavs
80S – 80S20,w complex of 70S20,w-Cav and 6020,w-Cavin
Ankrd13 – Ankyrin Repeat Domain-Containing Protein 13
β2AR – β2 adrenergic receptor
Cav – Caveolin
COPII – Coat protein complex II
CSD – Caveolin Scaffolding Domain
CSK – c-Src tyrosine kinase
CRAC – cholesterol recognition/interaction consensus sequence
DR1, DR2, DR3 – Disordered regions of Cavins
DRMs – detergent-resistant membranes (lipid raft membranes)
NOS – Endothelial nitric oxide synthase (NOS3)
ER – Endoplasmic reticulum
ERAD – ER-associated degradation
ERES – ER exit site
ESCRT – endosomal sorting complexes required for transport
FAPP-1, FAPP-2 – four phosphate adapter protein 1 and 2
Gα, Gβγ – Subunits of heterotrimeric G proteins
GPCR – G-protein coupled receptor
GPI – Glycosylphosphatidyl inositol
HR1, HR2 – Helical regions 1 and 2 of Cavins
MDCK – Madin-Darby Canine Kidney
MVB – Multivesicular bodies
NOS – Neuronal nitric oxide synthase (NOS1)
PM – Plasma membrane
PA – Phosphatidic acid
PC – Phosphatidylcholine
pCav1Y14 – Cav1 phosphorylated at tyrosine 14
PE – Phosphatidylethanolamine
PG – Phosphatidylglycerol
PI3K – Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI4P – Phosphatidylinositol 4-phosphate
Introduction:

Caveolins (Cavs) are ~20kDa oligomeric proteins required for the formation of caveolae, which are lipid raft plasma membrane (PM) domains enriched in proteins and lipids, including cholesterol and sphingolipids (154). Caveolae are flask-shaped membrane invaginations, formed...
by cis/trans-Golgi-PM transport of oligomeric Cavs embedded in cholesterol-rich membranes. Once at the cell surface, these complexes interact with cavins, adapter proteins that form oligomers and assist in membrane curvature (reviewed in (85, 144)). Based, at least in part, on the ability of Cavs to scaffold protein partners, caveolae play key roles in a variety of cellular responses, including: signal transduction (Table 1); transport of nutrients into and out of cells (e.g., insulin receptor activation stimulates translocation of the GLUT4 glucose transporter to caveolae in adipocytes (185)); and cellular entry of certain pathogens, toxins, and endocytic cargo (e.g., simian virus 40 (133), cholera toxin (136), albumin (120)). Caveolae and the functions they regulate have been recently reviewed: e.g., caveolae as membrane sensors and organizers (144, 164), in cytoskeletal interactions (70), endo/exocytosis (25), and signal transduction (186). Moreover, mutations in Cavs and Cavins are associated with a variety of diseases (Table 2).

There are three Cav isoforms: Cav1, Cav2, and Cav3. Cav1 and Cav3 are necessary and sufficient for caveolae formation in most tissues and striated muscle, respectively. Cav2 associates with Cav1 in hetero-oligomers and does not independently form caveolae (102, 201). Cavs have four domains (Figure 1): 1) an N-terminal domain, which includes a Cav1 phosphorylation site (Cav1Y14), ubiquitination sites in Cav1, and SUMOylation sites in Cav3; 2) an α-helical caveolin scaffolding domain (CSD) (implicated in protein-protein interactions, oligomerization, and inhibition of signaling proteins) with a cholesterol recognition/interaction consensus sequence (CRAC) and ubiquitination or SUMOylation sites; 3) a membrane domain that interacts with PM lipids through an atypical helix-turn-helix motif that exposes both the N-
and C-termini of Cav to the cytoplasm; and 4) a *C-terminal domain* with three palmitoylated cysteines involved in oligomerization, protein binding, and Cav stability.

Cavin proteins aid oligomerized Cavs to form flask-shaped caveolae. Four cavin isoforms have been identified: Cavin1 (also known as [aka] polymerase I and transcript release factor [PTRF]), Cavin2 (aka serum deprivation response protein [SDPR]), Cavin3 (aka serum deprivation response factor-related gene product that binds to C-kinase [SRBC]), and Cavin4 (aka muscle-restricted coiled-coil [MURC]). Cavins homo- and hetero-trimerize with two Cavin1 proteins and one Cavin1, Cavin2, or Cavin3 via an α-helical domain, bind to caveolae membranes by basic regions on the helical domains, and assist Cavs in the membrane curvature that forms caveolae (*Figure 2*).

Interactions of Cavs with binding partners during trafficking, at the PM, and as part of signal transduction events modulate the behavior of caveolae as structural and functional microdomains. This review follows the life cycle of Cavs from the ER to the lysosome and explores the roles of lipid and protein interactions within caveolae that contribute to aspects of cell physiology.

**Trafficking of Cavs and Cavins for the formation of caveolae**

Studies of caveolae formation have used primary cultures of epithelial cells (e.g., human lung microvascular endothelial cells) or fibroblasts (e.g., human skin fibroblasts, primary mouse embryonic fibroblasts), various epitheloid (e.g., Chinese hamster ovary, MDCK, Fischer rat
thyroid), fibroblast-like (e.g., CV-1 and COS-7 from African green monkey kidney, NIH mouse 3T3), adipocyte (e.g., 3T3-L1), cancer (e.g., MCF-7 and MDA-MB-231 breast cancer, PC3 prostate cancer, A431 epidermoid carcinoma) cells, and/or other commonly used systems (e.g., HeLa, HEK293, *E. coli* cells). Cav3, Cavin1, and Cavin4 studies have also been performed in primary skeletal or cardiac muscle isolates or in the immortalized cardiac myoblastic cell lines H9C2 (rat) or HL-1 (mouse). Some of these cells provide null (or near-null) backgrounds for Cav expression; for example, MCF-7 cells have very low Cav1 expression, do not express cavins and do not form caveolae and PC3 cells express Cav1 at high levels but do not express cavins (74, 93, 210). MDCK, A431, and MEF cells all express Cav1, Cavins 1-3, and form caveolae at the PM (13, 74, 193, 210). The diversity of cellular backgrounds in research on caveolae can contribute to differences in results; for example, in studies of polarized epithelial cells, apical and basolateral membranes perform distinct roles in endo/exocytosis, signal transduction, mechanosensation, adherence, junction formation, and/or migration compared to studies of caveolae in a sessile adipocyte, contractile primary skeletal muscle cell, or HEK293 cell (70). Cav1 and Cav3 may differ in their biogenesis of caveolae. However, most data on caveolae formation are based on studies of Cav1-expressing systems, in which the basic components and pathway of caveolae formation appear to be similar among different cell types and thus are the focus of this review.

Early steps in caveolae assembly: Membrane insertion, 8S-Cav oligomerization, and endoplasmic reticulum (ER) to Golgi transition

Caveolae are constructed in a complex, stepwise assembly process that involves: ER membrane insertion, oligomerization, and export of Cav; Golgi Cav-cholesterol association,
oligomerization, and export to the PM; palmitoylation near the PM; and addition of cavins at the PM to form invaginated structures (reviewed in (144, 173)) (Figure 3). The timeline from Cav translation to cavin addition at the PM in CV1 cells includes: ER exit site (ERES) localization and initial oligomer (8S-Cav) formation within 5 min; Golgi localization within 15 min; secondary oligomer (70S-Cav) formation, cholesterol addition, and PM translocation by ~60 min; and accumulation of cavin at PM 70S-Cav oligomers that occurs over >25 min (68). Cav1s are the primary protein components of caveolae, influencing membrane composition and protein content from translation to degradation. Thus, the translation and early oligomerization of Cav1s begin the caveolae biogenesis pathway.

Formation of caveolae is initiated by the co-translational ER membrane insertion of monomeric Cav1 in a signal recognition particle-dependent manner (68, 123). As quickly as 5 min after synthesis in the ER, Cav1 forms 8S20,ω oligomers (8S-Cav, 150-200 kDa with a minor species around 443-669 kDa) that is estimated to contain 7-14 Cav1s in a Cav1:Cav2 ratio of 2-4:1 (41, 68, 111, 123, 181, 184). The formation of 8S-Cav oligomers depends upon aa residues Cav166-70, Cav181-100 (i.e., the CSD), and Cav1134-178 (i.e., the C-terminus) (41, 113, 188). No molecular chaperone has been identified for the formation of 8S-Cav.

8S-Cav complexes translocate to the ERES, where recognition of a N-terminal diacidic sequence motif, Asp-X-Glu (Cav167DFE69), results in COPII vesicle-dependent export to the Golgi by ~15 min after synthesis (68, 124, 137). Disruption of this signal (e.g., Cav1D67G) delays ER export and leads to accumulation in tubular-reticular ER structures and lipid droplets (68, 137). Experimental overexpression of Cav1 exhibits similar trafficking errors, implying that this export
pathway may be a potentially saturable step in Cav maturation (64, 67). 8S oligomer formation is
a prerequisite for caveolae formation (124) but Cav1 monomers and small oligomers reach the
ER and may be subject to the same COP II-dependent export pathway as 8S complexes (68).

Intermediary steps in caveolae assembly: 70S-Cav oligomerization, cholesterol incorporation,
and PM insertion

Golgi-localized 8S-Cav oligomerizes by about 60 min after synthesis into 70S\textsubscript{20,w} complexes
(70S-Cav) of \(\sim 3.3\) MDa, predicted to comprise \(\sim 160\) Cav1 and Cav2 molecules in 15-25 8S-Cav
subunits (68, 150). Recent work suggests that the mature caveolae coat has a single 70S-Cav
unit; thus, 70S-Cav formation is the penultimate step of Cav maturation (5, 68, 111, 150, 219).
70S-Cav oligomerization is restricted to the Golgi: treatment with brefeldin A (which inhibits
protein transport from the ER to the Golgi) prevents 70S-Cav oligomerization (68).

During the oligomerization step, 70S-Cav-associated membranes attain a high content of
cholesterol, which is thought to involve a cholesterol recognition/interaction amino acid
consensus of Cavs (CRAC: -Leu/Val-X\textsubscript{1-5}-Tyr-X\textsubscript{1-5}-Arg/Lys- where X\textsubscript{1-5} represents one to five
residues of any aa). The CRAC of Cav1 \((^9\text{VTKYWFYR}^{101})\) induces cholesterol crystallization
in the membrane and results in deeper Cav1 peptide insertion (38). Cholesterol depletion reduces
70S-Cav complex formation, implying that oligomerization depends on membrane integration of
cholesterol (68). Cavs that lack the CRAC allow 8S-Cav oligomerization but can prevent Cav
association with lipid rafts in the Golgi and 70S-Cav formation, suggesting that 8S-Cav
oligomers are not sufficient to establish the cholesterol-rich environment of caveolae (113, 168).
The properties of the Golgi that allow 70S-Cav formation and cholesterol aggregation are not well-defined, nor are the chaperones or cofactors involved in assembly of 70S-Cav oligomers.

After oligomerization, 70S-Cav is exported to the PM from the cis/medial-Golgi network in carrier-dependent, dyanamin-2-independent secretory vesicles within 60 min of synthesis (68). Transport is directed by four phosphate adapter protein 1 and 2 (FAPP-1, -2) that interact with phosphatidylinositol-4-phosphate (PI4P), the primary phosphoinositide (PI) species in the Golgi in order to generate post-Golgi vesicles for transport (59, 68, 217). These small, uniform 70S-Cav-cholesterol vesicles can transport cargo, including glycosylphosphatidyl inositol (GPI)-linked proteins that associate with lipid rafts (68, 203). Vesicle fusion with the PM allows GPI-linked proteins to diffuse laterally while the Cav coat remains at the point of integration (68).

70S-Cav complexes encounter palmitoyl acyltransferases near the PM, which palmitoylate Cavs on 3 cysteines in the late membrane/early C-terminal domains (Cav1C133, C143, C156, Cav3C106, C116, C124) (Figure 1) (8, 32, 36, 141). Palmitoylation in many proteins is dynamic but Cav1 palmitoyl modifications are stable for at least 24 h in endothelial cells (141). Palmitoylation is not essential for membrane localization of Cavs. Although palmitoylation-deficient Cav1 mutants (Cav1Cys-) form high molecular weight oligomers in detergent-resistant membranes (DRMs, i.e., lipid raft domains) at the PM, they exhibit reduced stability (122, 142). The lipid affinities of Cav domains and palmitoylation sites can influence the oligomerization and stability of Cavs at the PM. The composition of the lipids in caveolae stabilizes proteins and influences protein function, as we will discuss subsequently.
Once 70S-Cav oligomers and their cholesterol-rich membranes reach the PM, ~60-80 cavin proteins accumulate in caveolae over >25 min and create a striated coat (detected by electron microscopy (68, 86, 112, 174)). Cavins can assemble into cytoplasmic complexes in the absence of Cav1 but cavins are gradually added to 70S-Cav at the PM (56, 68). Cavins are not primarily bound to Cavs at the membrane: Cavin3 is the only isoform known to directly interact with Cav1 (in the CSD and middle domains of Cav1 and Cavin3, respectively) (Figure 2) (121). However, cavins have binding sites for the anionic phospholipids phosphatidylserine (PS) and phosphatidylinositol (4,5)-bisphosphate (PI4,5P) that are locally concentrated in caveolae membranes (40, 56, 74, 111, 155, 220). All four cavin proteins have a C-terminal basic domain putatively involved in membrane association (66). Cavin1 also has an N-terminal leucine zipper domain (aa 53-75) that enables membrane association, while Cavin4 uses its N-terminal coiled-coil domain (aa 44-77) to localize to the PM (131, 224).

The basic oligomeric unit of cavin proteins is a trimer composed of two Cavin1 units with a monomer of Cavin1, Cavin2 or Cavin3 (Cavin4 has not been studied), interacting through helical region 1 (HR1); after HR1 trimerization, helical region 2 (HR2) undergoes conformational changes and forms an α-helical secondary structure, enabling further oligomerization (56, 111) (Figure 2). A 9 cavin complex forms from identical cavin trimers: Cavin1/Cavin2 and Cavin1/Cavin3 are mutually excluded from such trimeric-trimer-cavin complexes and segregate separately on the caveolae surface, implying that Cavin1/Cavin2 or Cavin1/Cavin3 oligomers may have distinct roles (56). Cavin-cavin interactions yield 60S_{20,w} complexes (60S-Cavin, predicted ~2.5 MDa) which, with addition of 70S-Cav, forms an 80S complex (68, 85).
Membrane invagination in a nascent caveola depends upon localized biophysical properties of the lipid bilayer and the intrinsic membrane-remodeling capacity of Cavs, cavins, and lipids (66, 86, 219) (Figure 3). Cav1 or Cavin1 expressed alone can independently influence membrane curvature and in mammalian cells can form tubular membrane structures (66, 216). Cavs interact with phospholipid headgroups of the membrane through a helix-turn-helix membrane domain that enters and exits from the cytosol (Figure 1) (178). Additionally, the three palmitoylated cysteines of Cavs stabilize Cav oligomers in the caveolae membrane (122, 142) and may participate in membrane curvature (178). Membrane lipids, such as PI4P in Golgi export vesicles, may also contribute to membrane curvature and caveolae invagination (50, 51).

A proposed mechanism of cavin-driven membrane remodeling is that alternation between basic HR1 and HR2 domains interspersed with acidic disordered regions 1, 2, and 3 (DR1-3) (Figure 2) allows DRs of one cavin trimer to recognize HRs of a trimer in the adjacent parallel striation and vice versa, thereby creating a flexible lattice of lateral cavin-cavin interactions around caveolae that exert curve-inducing forces on the membrane (85). Support for this idea includes a zigzag appearance of cavin striations around the caveolae coat, with cavin subunits alternating in proximity to adjacent striations (112). Details of membrane invagination are not well defined, for example, the roles of other binding partners of Cavs and cavins and of cytoskeletal components.

70S-Cav and 60S-Cavin together form mature, stable caveolae that have three layers: a cholesterol- and negatively-charged phospholipid-enriched membrane, 8S-Cav subunits organized into a palmitoylated 70S-Cav coat, and a 60S-Cavin complex that spirals around the
outside of the 70S-Cav coat. These mature caveolae (Figures 3 and 4) stabilize and protect CavS from degradation by preventing lateral movement and exchange of CavS and cavins (68, 208, 209). CavS and caveolae proteins are also protected from ubiquitination and depalmitoylation.

An example of this is Gα protein (GTP-binding proteins in αβγ heterotrimers): palmitoyl turnover of Gα proteins is increased by receptor activation and Gα dissociation from Gβγ and caveolae, implying that caveolae may exclude acyl thioesterases (81, 103, 126, 223). Cavins are also protected from degradation within caveolae: a PI binding site on HR1 of Cavin1 is a major ubiquitination site that is obscured by membrane association (210).

Cav1 has a reported half-life (t1/2) of >24 h (69, 106, 141), although some studies report other values (27, 44, 76). Cavin1 knockdown destabilizes caveolae and reduces the t1/2 of Cav1 to 7 h (74). Similarly, truncation constructs of Cav1 that cannot oligomerize have a decreased half-life and do not form stable caveolae (113). Cav3 appears to have a shorter t1/2 than Cav1 (5.5-7 h) and mutant forms of Cav3 can have an even shorter t1/2 (45-60 min) as a result of ubiquitination and proteosomal degradation (54, 55, 204). Cavin1 has a t1/2 that ranges between 5-8 hr; however, in the absence of Cav1, the t1/2 decreases by almost half (210).

Thus, in the process of caveolae biogenesis, unstructured 18-21 kDa Cav monomers assemble in a tightly controlled fashion to form 8S-Cav, 70S-Cav-cholesterol, and finally, 80S-Cav-Cholesterol-Cavin complexes that are protected from degradation.

Processes of caveolae disassembly and degradation
Stability of caveolae at the PM requires that Cav and cavin oligomers remain tightly associated. Disruption of that structural stability by mechanical stress, endocytic activity, or signal transduction pathways dissociates cavin from caveolae and leads to endocytosis of Cavs (Figure 4). In contrast to the maturation and stability of 70S-Cav, which requires constitutive, stable palmitoylation, caveolae disassembly and 70S-Cav dissociation involve dynamic PTMs of Cavs that include phosphorylation, S-nitrosylation, and ubiquitination.

Cavs are subject to multiple endocytic pathways, two of which are depicted in Figure 4: Caveolae, including the 70S-Cav complex, can be endocytosed as a result of Src-dependent phosphorylation (3, 21, 128, 206) or the PM can undergo mechanical stresses that flatten caveolae and dissociate 60S-Cavin complexes, after which Cav is ubiquitinated (UbCav1) and endocytosed (9, 84). The pathways are likely more complex than described below and in Figure 4. Key to both pathways are Cavin dissociation, endocytosis, Cav ubiquitination, and endosomal processing of UbCav1, in which mono- and poly-UbCav1 is directed to intraluminal vesicles for lysosomal degradation (68, 84) and poly-UbCav1 undergoes proteosomal processing (9).

Cavin dissociation occurs in both pathways portrayed in Figure 4. If caveolae lose structural integrity in response to mechanical stress or vesiculation, 60S-Cavin dissociates into the cytosol and separates into trimeric-trimer-cavin units (56, 85, 108, 196). Because cavin associates with the lipids of caveolae, its departure does not directly affect 70S-Cav localization to the PM (40, 69, 74, 155). For example, although Cav3, Cavin1, and Cavin4 co-localize at the PM of myocytes, during hypo-osmotic stress Cav3, but not Cavin1 or Cavin4, is present in membrane blebs, suggesting that membrane stress lead to dissociation of the Cavins but not Cav3 (108).
Cholesterol depletion with U18666A (which inhibits cholesterol trafficking in cells) also results in 60S-Cavin dissociation from the PM (69).

Cytosolic Cavin1 is susceptible to ubiquitination on Lys residues within PI binding sites (i.e., groupings of surface-exposed basic residues) in the HR1 region that are occluded by membrane contact (Figure 5) (210). Mutation of the five Lys and Arg residues in this region reduces Cavin1 ubiquitination and turnover by the proteasome (210) without substantially influencing Cav1 co-localization or caveolae formation (86). Such data imply that the co-localization of the PI binding/ubiquitination sites selectively protects Cavin1 from ubiquitination while bound to caveolae and leads to the ubiquitination of cytosolic Cavin1 (210).

Src-dependent phosphorylation of Cav1 is implicated as a control point of clathrin-independent membrane protein internalization and macromolecular cargo endocytosis (80, 120, 145, 158). Phosphorylation of Cav1 by Src kinases on an N-terminal tyrosine (p-Cav1Y14) is an interaction that requires C-terminal palmitoylation of Cav1C156 and the myristoyl plus basic motif of Src (95, 104, 120, 149, 195, 205, 211, 221). p-Cav1Y14 also adds a binding site on Cav1 to the SH2 domain of Src, thereby scaffolding this kinase more closely to Cav1 after Src activation (61).

Cav1 phosphorylation and sustained Src signaling initiates endocytosis by phosphorylating dynamin-2 to close off and detach caveolae from the membrane, recruiting actin and the actin regulator cortactin, and cross-linking p-Cav1Y14 to filamin A (3, 20, 88, 117, 128, 177, 206). p-Cav1Y14 regulates the duration of Src activity by binding kinases and phosphatases: p-Cav1Y14 recruits c-Src tyrosine kinase (Csk) to inhibit Src via SrcY527 phosphorylation, while recruitment
of Src homology 2 domain-containing protein tyrosine phosphatase 2 (SHP-2) interferes with Csk-p-Cav1\textsuperscript{Y14} complex formation and preserves Src activity (Figure 6) (79, 169). The balance between Csk and SHP-2 action on p-Cav\textsuperscript{Y14} thus may control Src-mediated caveola endocytosis.

Cav1\textsuperscript{Y14} phosphorylation also destabilizes 70S-Cav oligomers by altering the relationships between oligomerized Cav1: pCav1\textsuperscript{Y14} or phosphomimetic Cav1\textsuperscript{Y14D} increases the intermolecular distance between Cav1s in caveola vesicles, resulting in dissociation of higher-order Cav1 oligomers (232). Phosphorylation-induced loss of 70S-Cav integrity may be a mechanism by which the previously stable structure is made accessible to ubiquitination enzymes, acyl-protein thioesterases, and other degradative influences. Addition of a cell-permeable CSD peptide, which directly blocks Src-dependent Cav1\textsuperscript{Y14} phosphorylation, stabilizes large oligomers (232).

Mechanical effects on the PM that flatten caveolae initiate another type of caveolar disassembly. Flattening can result from cholesterol depletion (87, 214), hypo-osmotic stress (108), and substrate stretch (196, 210). The flattening of caveolae after mechanical stress may be a protective mechanism that provides a reservoir of PM to prevent membrane tension and injury by increasing PM surface area (108). Caveolae in skeletal muscle cluster in PM surface-connected rosettes; under hypo-osmotic stress, caveolae density decreases and reduces the ratio of rosettes to independent caveolae (108). Treatment of mouse lung endothelial cells with methyl-\textbeta-cyclodextrin, which depletes the PM of cholesterol, increases membrane tension during hypotonic stress (196).
The flattening of caveolae is ATP- and actin-independent. During recovery from nonlethal hypotonic stress, caveolae re-form by integrating the PM-localized pool of Cav1 with Cavin1 within 10 min to form an equal number of caveolae; this requires ATP but not actin remodeling (196). Chronic and repetitive shear stress, such as at the luminal PM of endothelial cells exposed to laminar flow, can increase PM caveolae number without increasing mRNA, suggesting that signaling induced by shear stress increases Golgi processing and export of Cav1 (16, 143). The number of caveolae increases only at the shear-exposed membrane, where they may play roles in translocating proteins to caveolae and activation of signaling cascades (16, 143, 227). Caveolae flattening thus protects the PM from rupture by reducing PM tension during transient stress.

Absence of cavins from caveolae through mechanical detachment, cholesterol depletion, or knock-down disrupts 70S-Cav oligomers into their 8S-Cav subunits and induces Cav1 ubiquitination, caveolae-independent packaging, and endocytosis of UbCav1 to the early endosome (69). The cholesterol-rich membranes surrounding 8S-Cav units are transported to the endosome and lysosome, indicating that Cavs can maintain their membrane localization even with loss of the 70S-Cav structure (127).

Cav1 separated from mature caveolae can be monoubiquitinated on six N-terminal lysines (Cav1^K5, K26, K30, K39, K47, K57) (84), polyubiquitinated on Cav1^K86 (9), and may have other states of ubiquitination. The most prevalent ubiquitination is monoUbCav1. PolyUbCav1 is divided into two groups: 64% Lys-63-linked and 28% Lys-48-linked (18, 69, 170). p-Cav1^Y14 undergoes Cav1^K86 polyubiquitination and proteosomal degradation (9). Additionally, Cav1 and DRMs are implicated in the inhibition of lysosomal function, which may influence Cav1 degradation (193).
Cav3 ubiquitination of specific lysines has not been shown; however, a small ubiquitin-like modifier (SUMO) can be added by the E3 ligase PIASγ to lysines on Cav3 that are homologous to the ubiquitin sites on Cav1, suggesting that such sites may be loci for ubiquitination (47).

After ubiquitination and translocation of Cav to endosomes, it is degraded by proteasomal and lysosomal pathways (9, 69). Mono- and polyUbCav1 are packaged within multivesicular bodies (MVB) by endosomal sorting complexes required for transport (ESCRT) and an AAA+-type ATPase, valosin-containing protein (VCP; aka p97, Cdc48) (18, 69, 84, 170). VCP uses conformational changes from ATP hydrolysis to extract polypeptides from larger assemblies of oligomers or membranes, regulates endosome size and sorting, and with the cofactor UBXD1 enables MVB formation (17, 29, 33, 162, 170, 175, 228). Two mutually exclusive VCP cofactors, UBXD1 and Ankrd13 regulate VCP interaction with monoUbCav1 and mono/polyUbCav1, respectively and can drive Cav segregation to intraluminal vesicles (17, 18, 84, 170). VCP preferentially interacts with higher-order Cav oligomers (8S-Cav, ~150-200 kDa major species and 443-669 kDa minor species (68)) but it is unknown whether VCP also interacts with 70S-Cav (18, 170, 181). 8S-Cav and VCP interaction is disrupted by the depletion of membrane cholesterol, suggesting that VCP-dependent regulation of Cav oligomers occurs after cholesterol membrane integration in the Golgi and that cholesterol remains in the local membrane environment of Cavs during endocytosis and endosomal sorting (170).

PolyUb chains on residue Cav1K86 direct it to proteasomes for degradation. The proteasomal inhibitor MG132 reduces Cav1 degradation but also prevents acidification of Cav1-containing endosomes; thus, Cav1 may be packaged in MVB before proteasomal and endosomal processing.
(Figure 4) (69). Because VCP is typically involved in membrane protein extraction and proteasomal transport (17) and UbCav1 interacts with VCP, VCP complexes may also be involved in the proteasomal pathway of UbCav1 degradation; however, the process by which polyUbCav1 is trafficked to the proteasome is not known.

Importantly, tagged, overexpressed, and/or mutant Cav constructs are subject to defective 8S-Cav and 70S-Cav oligomerization, lipid raft exclusion, intracellular accumulation/aggregation, and increased turnover (18, 64, 67, 69, 84, 170). However, many studies use tagged Cavs to assess turnover, intracellular Cavs, or interaction of Cavs with partners. Cav1 oligomerization is also influenced by experimental tagging: overexpression of GFP-, mCherry- or C-myc-tagged Cav1 yields aberrant Cav1 oligomers that do not interact with endogenous Cav1 or Cav2 (64). Tags and overexpression can also affect localization to lipid rafts: tagged mutant Cav1P132L (a mutation in multiple diseases) is more sensitive to oligomer and lipid raft disruption than is Cav1WT (64). Differences in the reported t1/2 of Cav1 can at least partially be attributed to the use of tagged proteins: in one study, endogenous Cav1 and Cav1-HA had t1/2’s of >24 h and 13.6 h, respectively (69). Because of the role of VCP in ER-associated degradation (ERAD, in which misfolded or incomplete protein assemblies are dislocated from the ER and degraded by the ubiquitin-proteasome system (228, 229)), tagged/overexpressed Cav1 in the biogenesis pathway may be a target of VCP and yield non-physiologic results. Careful attention thus must be paid in Cav trafficking and signaling studies to identify possible artifacts created by expression constructs with abnormal trafficking characteristics.

Composition and roles of the caveolae lipid microenvironment
The protein complex of caveolae comprises 80S-Cav multimers: ~160 membrane-bound, palmitoylated Cavs bound in close proximity to cholesterol-rich membranes and complexed with ~50 cavin oligomers. Interactions between Cavs, cavins, lipids, and other proteins create an unique protein, lipid, and lipoprotein microenvironment. Cavs shape this environment not only through cholesterol binding and palmitoylation but also by interaction with the inner leaflet of membrane lipids by a hairpin helix-turn-helix membrane domain. This domain includes Cav1T91, K96, Y97, R101, Y118, which preferentially interact with phospholipid headgroups of the cytosolic PM leaflet, and Cav1G108 which interacts with the exofacial leaflet (5, 96, 178). Cavins are dependent upon the lipid composition of caveolae because they have affinities for negatively charged phospholipids (PS, PI(4,5)P) and cholesterol in caveolae membranes and their lipid binding may occlude ubiquitination sites, thereby protecting them from degradation (40, 56, 74, 111, 155).

Other proteins localize to caveolae by association with Cavs, the caveolae membrane, and/or scaffolding proteins that associate with caveolae. Major factors that influence these interactions are caveolae lipid composition, PTMs, and activation state changes that exchange proteins between caveolae and cytosolic or PM domains.

The lipidome of caveolae appears to depend on the cell type studied, the method used to isolate caveolae, and physiological “state” (e.g., mechanical stress or signal transduction events). Cavs are inserted and exit through the cytoplasmic leaflet of the PM, which is enriched in PS, phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC), while the outer leaflet is enriched in sphingolipids (including glycolipids) and phosphatidylcholine (PC) (116, 212). Caveolae isolated without detergents have more than twice the molar
concentration of lipid per mg of protein than do PM membranes (6, 156). These isolates are highly enriched in phospholipids, cholesterol, and sphingomyelin (SM) when compared to non-caveolae membranes: caveolae contain 3-5 times more PS, 2-5 times more cholesterol and SM, 2-4 times more phosphatidic acid (PA), and ~2-fold more PE (which composes around 40% of all membrane phospholipids) (Figure 5) (6, 156). However, studies disagree about PC and PI concentrations: a concanavalin-A affinity chromatography method of caveolae isolation in murine L-cell fibroblasts identified a 15-fold increase in PC and 3-fold increase in PI within caveolae, whereas an Optiprep density gradient centrifugation method in KB human epidermal cancer cells expressing exogenous Cav1 found a minimal increase in PC or PI species (6, 156). Caveolae membranes contain higher concentrations of unsaturated, saturated, and mono-unsaturated fatty acid species (6). Caveolae isolated with 1% Triton X-100 are enriched in cholesterol and SM without the glycerophospholipid enrichment seen in non-detergent-isolated lipid rafts (156). Thus, detergent treatment of PMs may preferentially extract exoplasmic leaflet lipids but not anionic inner leaflet glycerophospholipids (153). However, when Cav1 is not present in cells, non-detergent lipid rafts contain similar proportions of lipids as do those isolated from Cav1-expressing cells except that cholesterol is reduced (156). Thus, caveolae are distinct from other PM lipid rafts primarily due to caveolin and its interaction with cholesterol.

Cholesterol has a large role in the formation and structure of caveolae. Because the integration of cholesterol is necessary for 70S-Cav assembly from 8S-Cav subunits, cholesterol is essential for caveolae formation; however, it is also necessary for the stability, structure, and function of caveolae at the PM. Cholesterol increases the ordering and packing of lipid membranes and has numerous effects, for example, increasing resistance to disruption by mechanical stress (42, 194),
membrane thickness (172), and depth of Cav CSD peptide insertion into membranes (38). Cholesterol depletion distorts caveolae, implying that PMs do not maintain an invaginated morphology without cholesterol (151, 174). Substitution of cholesterol with its precursor, desmosterol, reduces Cav affinity for sterol and results in enlarged caveolae with increased Cav1Y14 phosphorylation (78).

Proteins associate with PM lipids through transmembrane domains, membrane-interacting sites, and/or fatty acid PTMs; however, conformational changes, binding site obstruction, and de-lipidation can alter lipid affinities, which may influence movement of proteins into and out of caveolae. Protein-protein, protein-lipid, and lipid-lipid associations can reciprocally stabilize and concentrate binding partners (83). For instance, membrane cholesterol and other lipids within caveolae influence conformational plasticity of the β2AR, receptor ligand affinity, activation/inhibition, and G-protein affinity and activation; importantly, lipids that stabilize the β2AR concentrate in its local membrane (34, 91, 114, 157). Certain lipids can also be allosteric modifiers of β2AR activity (34). Membrane lipid composition thus influences the stability and behavior of the receptor, which can influence the lipid composition in its local environment. In conjunction with evidence that agonist-stimulated β2AR dissociates laterally from lipid rafts to undergo β-arrestin-mediated clathrin endocytosis and degradation (34, 60, 233), localization within or outside caveolae may influence receptor behavior independent of protein scaffolds, activators, and downstream effectors. Therefore, by aggregating certain lipid species within caveolae, Cavs may stabilize proteins in conformations that can regulate ligand affinity, protein-protein interactions and signal transduction; in addition, conformational changes and/or PTMs
that change lipid affinities may alter protein localization in caveolae (91, 103, 114, 157, 199, 223).

Stable membrane-bound homeostasis and stimulus-dependent dynamic behavior or modification occur with other caveolae-resident proteins. For example, nitric oxide (NO) production in endothelial cells is a key regulator of vascular smooth muscle relaxation. Within caveolae, eNOS is constitutively bound and inhibited by Cav1 (58, 118). Src activity enabled by Cav1 binding can lead to eNOS dissociation from Cav1 and eNOS activation: Src phosphorylates Cav1Y14, which causes dissociation of eNOS; Src phosphorylates PI3K, which phosphorylates Akt, which phosphorylates uninhibited cytosolic eNOS at the activating Ser1177 site; and p-eNOSS1177 produces more NO to stimulate smooth muscle relaxation (Figure 7) (11). Each component of this pathway has membrane and/or protein affinities that change upon activation or modification.

Proteins can cycle out of caveolae and be activated; others may cycle into them upon stimulation.

Fatty acylations (e.g., palmitoylation, myristoylation, GPI-link formation) influence caveolae localization and protein association in caveolae. Palmitoylation does not influence Cav1 stabilization in cholesterol-enriched membranes but interacts with other lipid components of the membrane and perturbs conformational dynamics (46). Cavs are palmitoylated near the PM after oligomerization, implying that palmitoylation has a limited role in lipid species selection or Golgi-localized oligomerization (32, 36, 141). However, the caveolae lipid environment can aggregate proteins (e.g., GPCRs, Ga’s, Ras, c-Src), with fatty modifications (53, 199, 213). In some cases, acylation is required for protein-protein associations: c-Src is localized to caveolae as a consequence of its myristoylation and binds to Cav1 through CavC156 palmitoylation (95,
203, 213). CavC\textsuperscript{156} can be S-nitrosylated secondary to eNOS activation, acting as potent negative regulator of Cav palmitoylation and thus palmitoylation-dependent binding activity.

Much is unknown regarding the facilitation by fatty acylation of membrane, Cav1 binding, and caveolae localization. The complexity of these interactions is illustrated by the behavior of G\textalpha isoforms. G\textalpha isoforms are CSD-associated and subject to dynamic palmitoylation but G\textalpha\textsubscript{i/o/z} possess an additional myristoylation site that localizes them to caveolae (53, 125, 213). Palmitoylation of G\textalpha and all three Cav1 cysteines is required for binding (53). Palmitoylation-deficient, but not myristoylation-deficient, G\textalpha\textsubscript{i} is trafficked to caveolae but binding to Cav1 is abrogated, implying that caveolae localization requires myristoylation but Cav1 binding requires palmitoylation and thus suggesting a two-step mechanism for G\textalpha\textsubscript{i} localization and association with Cav1 (53). By contrast, G\textalpha\textsubscript{s} has no myristoylation site and thus its PM localization and subsequent palmitoylation depend on its association with isoprenylated G\textbeta\gamma subunits (125). Localization of G\textalpha is also dependent upon its activation state: GPCR-mediated activation of G\textalpha subunits induces G\textbeta\gamma dissociation, separation from Cav1, and accelerates G\textalpha depalmitoylation, thus delaying re-association of G\textalpha and Cav1 (103, 223). Cav1 expression also modifies the palmitoylation state of certain proteins (12), and palmitoylation of Cav1\textsuperscript{C143+C156} is required for the efficient transport of GPI-anchored proteins to the cell surface (203). Taken together, these data demonstrate that fatty acylations may induce differential localization and Cav binding behavior between protein isoforms, thus altering the stoichiometry or function of signal transduction proteins.
In summary, the microenvironment of caveolae represents a multifaceted, interrelated, and dynamic collaboration between Cavs, cholesterol, cavins, membrane lipids, and other membrane-interacting proteins. Forces within and outside caveolae can influence signal transduction paradigms in a variety of ways. Prior research has only begun to address this complexity.

Discussion and unanswered questions

This review focuses on the biogenesis and degradation of caveolae microdomains and the roles of the lipid and protein components in those microdomains and in aspects of cell physiology. Much is known regarding caveolae biogenesis and degradation but many questions remain.

Caveolae biogenesis is sensitive to changes in protein structure, oligomerization, cholesterol, and PTMs. For example, numerous mutations in Cav1, Cav3, Cavin1, and Cavin4 have been associated with human diseases (Table 2). Most of these mutations cause defects in Cav localization and loss of morphological caveolae and result in diseases in adipose tissue (Cav1, Cavin1) (19, 161), pulmonary endothelium and smooth muscle (Cav1) (7), skeletal muscle (Cav3) (89, 119), and cardiac muscle (Cav3, Cavin1, Cavin4) (92, 161, 171). Some Cav1 mutations occur in certain cancers (65, 105, 146). Cav biogenesis can be disrupted by epitope tags, Cav constructs, and Cav overexpression that recapitulate some disease-related defects (4, 64, 67, 113, 187, 188, 202, 225). The disease-related mutations in these proteins reinforce the idea that the caveolae biogenesis system is physiologically relevant in numerous tissues. Normal Cav function may be tightly controlled by factors and/or chaperones that require unaltered Cav subunits to properly oligomerize, traffic to and function in the PM, and be degraded.
8S-Cav must be formed before COP II-dependent ER export to generate 70S-Cav in the Golgi. As Cav proteins localize to ERES within 5 min of synthesis and reach the Golgi by 15 min, 8S-Cav oligomerization is a relatively rapid process (68). The lack of substantial secondary or tertiary structural stability in Cav may make this initial oligomerization step slower if Cav is modified or mutated. Indeed, if given more time, conditions that facilitate protein folding (e.g., incubation at 30°C or 10% glycerol supplementation), or inhibition of proteasomal degradation (e.g., with MG-132), some trafficking-deficient Cav3 is competent to reach the PM, accrue in lipid rafts, and be protected from premature degradation (54). Even so, chaperones and information regarding the requirements for 8S-Cav oligomerization remain unknown.

Tagged, mutant, and/or overexpressed Cav can form aggregates larger than 8S-Cav (64, 67, 204) and raise the question: how do 8S-Cav oligomers assemble into immobile and protected 70S-Cav structures with cholesterol in the Golgi? A mixture of incomplete Cav oligomers and monomers can be transported to the Golgi, but 8S-Cav does not form outside of the ER (68). Might unknown factors facilitate 70S-Cav assembly from 8S-Cav subunits (38, 64)? Perhaps long-lived caveolae structures require strict steric precision to prevent degradation, such that sequence changes lead to premature loss of oligomers, e.g., when Src-dependent Cav1Y14 phosphorylation wedges apart 8S-Cav clusters at the PM (232). Without 70S-Cav-cholesterol and 60S-Cavin complexed at the PM, “exposed” ubiquitination, palmitoylation, and/or phosphorylation sites may direct Cav to degradation (54).
Another poorly understood aspect is the recognition pathway for 70S-Cav export from the Golgi. 8S-Cav is partially competent to reach the PM, but aggregates substantially larger than 8S-Cav are unable to reach the PM and may accumulate insufficient cholesterol to become buoyant lipid rafts (168). Are 70S-Cav-driven changes in Golgi lipid membrane composition a prerequisite for efficient export? Although FAPP-1 and -2 are components of the secretory pathway, their binding target PI4P is the most prevalent Golgi phospholipid and not unique to 70S-Cav (59, 217). Is there a cofactor that recognizes cholesterol, another enriched lipid, or Cav oligomers and drives the 70S-Cav Golgi export pathway?

Alternating acidic/basic domains in neighboring striations of the outer cavin coat have been proposed to exert forces for membrane curvature, but more information is needed regarding cavin recruitment, the role of cavins in caveolae curvature, determinants of cavin stability and detachment of the cavin complex (68, 108, 196). Tomographic reconstruction of caveolae complexes has yielded promising data on the ultrastructure of the complete caveolae coat (111, 112) and the crystal structure of cavin (86) has helped advance understanding of cavins. By contrast, the lack of a crystal structure for Cavs has hampered the accrual of more precise information of aspects of their structure and function.

The degradation pathways of caveolae and their resident proteins are less well-defined than those of biogenesis. In view of the trafficking and aggregation problems of tagged Cav constructs, their use may reveal information that identifies the sequelae of structural instability. Such “artifacts” can be useful, e.g., HA-Cav1 has a shorter $t_{1/2}$ than wild-type Cav1, thus facilitating cell culture experiments (18). However, most studies of Cav degradation cited in this review used tagged
Cav1 to draw conclusions about the endocytic, endosomal, lysosomal, and/or proteasomal degradation pathway of Cavs (18, 69, 84, 170). The lack of data on the degradation of endogenous Cavs and cavins (85) is thus an important gap in terms of normal cell physiology.

In summary, sixty years after their discovery (140), caveolae remain enigmatic dark caves. Our understanding of Cavs, cavins, their maturation and degradation processes, and the dynamic complexity of their lipid and protein components remains a work in progress.

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**TABLE 1: Examples of caveolae-associated signal transduction proteins. Adapted from (147).**

<table>
<thead>
<tr>
<th>Signal transduction protein</th>
<th>Mechanism(s) of association</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G Protein-Coupled Receptors (GPCRs, e.g., adrenergic, adenosine, angiotensin-1, opioid, serotonin)</td>
<td>Transmembrane (TM), fatty acylation (FA), Cav association (Cav)</td>
<td>(47, 49, 71, 139, 226)</td>
</tr>
<tr>
<td>Steroid hormone acceptors (e.g., estrogen receptor α)</td>
<td>TM, FA, Cav</td>
<td>(2, 23, 99)</td>
</tr>
<tr>
<td>Transforming growth factor-β receptors (e.g., Bone Morphogenic Receptor II)</td>
<td>TM, Cav</td>
<td>(163)</td>
</tr>
<tr>
<td>Tyrosine kinase (e.g., insulin, EGFR, NGF, IGF, PDGF)</td>
<td>TM, FA, Cav</td>
<td>(28, 75, 152)</td>
</tr>
<tr>
<td>inositol 1,4,5-triphosphate receptor (IP3R)</td>
<td>TM, FA, Cav</td>
<td>(48, 189)</td>
</tr>
<tr>
<td><strong>Ion channels, reporters, and exchangers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$-ATPase</td>
<td>TM, Cav</td>
<td>(189)</td>
</tr>
<tr>
<td>Ca$^{2+}$ pumps (e.g. Na(+)–Ca(2+) exchanger type 1)</td>
<td>TM, Cav</td>
<td>(31)</td>
</tr>
<tr>
<td>L-type Ca$^{2+}$</td>
<td>TM, Cav</td>
<td>(31, 110)</td>
</tr>
<tr>
<td>Large-conductance Ca$^{2+}$-activated K$^+$</td>
<td>TM, Cav</td>
<td>(222)</td>
</tr>
<tr>
<td>Transient Receptor Potential Cation (TRPC)</td>
<td>TM, Cav</td>
<td>(90, 109, 129, 192)</td>
</tr>
<tr>
<td>Na/K-ATPase</td>
<td>TM, Cav</td>
<td>(63, 107)</td>
</tr>
<tr>
<td>Voltage-gated K$^+$</td>
<td>TM, Cav</td>
<td>(1, 26, 115)</td>
</tr>
<tr>
<td>K$_{ATP}$</td>
<td>TM, Cav</td>
<td>(180)</td>
</tr>
<tr>
<td><strong>Kinases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Src-family</td>
<td>FA, Cav</td>
<td>(35, 94, 95, 101, 104, 148, 197, 200, 207, 218)</td>
</tr>
</tbody>
</table>
Protein Kinase A | Cav | (73, 97, 166, 167, 180)
Protein Kinase Ca, ζ | Cav | (45, 159, 179)
p42/p44 Mitogen Activated Protein Kinase | Cav | (10, 37, 52)
p38 Mitogen Activated Protein Kinase | Cav | (10)
Phosphatidylinositol-4,5-bisphosphate 3-kinase | PI(4,5)P₂ binding | (191, 198)
Protein kinase B | PI(3,4,5)P₃ binding | (62, 82, 100, 135, 190, 191, 198, 230, 231)

**Other post-receptor components**

Heterotrimeric GTP binding (G) protein α subunits | FA, Cav | (15, 26, 49, 71, 77, 101, 103, 130, 134)
Heterotrimeric GTP binding (G) protein βγ subunits | FA | (77)
Ras | FA, Cav | (160, 176, 199)
Adenylyl Cyclases (AC, e.g., AC3, 5, 6) | TM, Cav | (39, 71, 72, 138, 139)
Cyclic nucleotide phosphodiesterase (PDE3B) | Cav | (132)
Endothelial nitric oxide synthase (eNOS/NOS3) | Cav | (22, 24, 43, 57, 58, 73, 98, 165, 183, 215)
Neuronal nitric oxide synthase (nNOS/NOS1) | Cav | (14, 22, 30, 58, 182, 215)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Clinical Features</th>
<th>Mutated protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bernardinelli-Seip Congenital</td>
<td>Lack of adipose tissue, hypertriglyceridemia, insulin resistance, diabetes mellitus,</td>
<td>Cav1, Cavin1</td>
<td>(19, 161)</td>
</tr>
<tr>
<td>Condition</td>
<td>Description</td>
<td>Gene(s)</td>
<td>Notes</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Generalized Lipodystrophy types 3 (Cav1) and 4 (Cavin1)</td>
<td>Hypertrophic cardiomyopathy, hepatic steatosis</td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>Pulmonary arterial hypertension</td>
<td>Pulmonary vascular remodeling and proliferation, high pulmonary arterial blood pressure, right ventricular failure</td>
<td>Cav1</td>
<td>(161)</td>
</tr>
<tr>
<td>Limb-Girdle Muscular Dystrophy type 1C</td>
<td>Symmetric, progressive, proximal weakness of the limb girdle muscles, myoglobinuria, myotonia, elevated serum creatine kinase</td>
<td>Cav3</td>
<td>(89)</td>
</tr>
<tr>
<td>Rippling Muscle Disease</td>
<td>Mechanically-triggered contractions of skeletal muscle; subsequent electrically silent muscle contraction cascades</td>
<td>Cav3</td>
<td>(92)</td>
</tr>
<tr>
<td>Long QT Syndrome</td>
<td>Extended Q-T interval on electrocardiogram, arrhythmias, ventricular fibrillation</td>
<td>Cav3, Cavin1</td>
<td>(161)</td>
</tr>
<tr>
<td>Sudden Infant Death Syndrome</td>
<td>Sudden death of an infant unexplained by medical history or autopsy</td>
<td>Cav3</td>
<td>(92)</td>
</tr>
<tr>
<td>Hypertrophic or Dilated Cardiomyopathy</td>
<td>Thickened myocardium, non-ischemic cardiomyopathy, reduced cardiac function</td>
<td>Cav3, Cavin1, Cavin4</td>
<td>(161, 171) (92)</td>
</tr>
<tr>
<td>Cancer (implicated)</td>
<td>Breast, prostate, ovarian, and pancreatic</td>
<td>Cav1</td>
<td>(65, 105, 146)</td>
</tr>
</tbody>
</table>


Lee J, and Glover KJ. The transmembrane domain of caveolin-1 exhibits a helix-break-helix structure. *Biochim Biophys Acta* 2012.


177. **Rozelle AL, Machesky LM, Yamamoto M, Driessens MH, Insall RH, Roth MG, Luby-Phelps K, Marriott G, Hall A, and Yin HL.** Phosphatidylinositol 4,5-bisphosphate...


Figure Legends

Figure 1: Structure and post-translational modifications of Caveolin-1 (Cav1) and Caveolin-3 (Cav3).

Cav1 and Cav3 have four primary domains: N-terminal domains (orange) with multiple ubiquitination/SUMOylation sites on both Cavs, as well as a splice site and phosphorylation site on Cav1; scaffolding domains (blue) that form α-helices and are inserted into the membrane, with a cholesterol recognition/interaction amino acid consensus (CRAC) composed of the eight residues proximal to the membrane domain; helix-turn-helix membrane domains (fuchsia) that exit the membrane at a palmitoylation site; and C-terminal domains (green) with two more palmitoylated cysteines and two SUMOylation sites on Cav3.

Figure 2: Structure of Cavins

A. Cavins are cytosolic proteins with three disordered regions (DR1, DR2, DR3) alternating with two helical regions (HR1 and HR2). Two Cavin1’s homo- and hetero-trimerize with a Cavin1, Cavin2, or Cavin3 monomer via an extended coiled-coil structure in HR1, which drives HR2 to generate an α-helical structure. Cavin1 has membrane binding sites, including a phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2)-binding site composed of four basic regions in close proximity on HR1 and a phosphatidylserine (PS)-binding basic site on HR2.

B. Cavins detected on cellular membranes with electron microscopy form rod-like structures that are 5.5 nm wide and 23.4 nm long and associate laterally in 7 nM intervals. One idea, as described in the text and shown here, proposes that electrostatic interactions between alternating
acidic DRs (orange) and basic HRs (purple) may facilitate these lateral associations while permitting flexibility in the cavin coat complex.

Figure 3: Maturation of Caveolae

Cav1 and Cav2 monomers are co-translationally inserted into the endoplasmic reticulum (ER) membrane and swiftly oligomerized into 8S-Cav oligomers containing 7-14 Cavs. These oligomers are transported to ER exit sites (ERES) within 5 min of synthesis for COPII-dependent transport to the Golgi apparatus 15 min post-synthesis. In the Golgi, cholesterol crystalizes in the membrane and assists in the formation of 70S-Cav complexes composed of 18-25 8S-Cav subunits by ~60 min after synthesis, whereupon 70S-Cav is transported to the PM by a four phosphate-adapter protein (FAPP-1, -2)-dependent secretory vesicles. Near or on the PM, palmitoyl acyltransferases palmitoylate 70S-Cav oligomers. Also on the PM, cavin proteins that trimerize in the cytosol gradually aggregate on the 70S-Cav membrane over the course of more than 25 min and assist in membrane curvature. Mature caveolae consist of three layers: a cholesterol and anionic lipid-rich membrane embedded with a palmitoylated 70S-Cav coat, which is surrounded by a striated oligomerized 60S-Cavin coat.

Figure 4: Disassembly and Degradation of Caveolae

Caveolae disassembly begins at the plasma membrane. Examples shown here are Src-dependent phosphorylation of Cav1 and membrane stretch. Cav1 phosphorylation initiates dynamin-dependent vesiculation that distorts caveolae, dislodging cavin trimers, and leads to ubiquitination of Cavs (UbCav) during vesicular transport to the early endosome. Mechanical stress displaces cavin from the membrane, followed by ubiquitination and subsequent
endocytosis of Cav1 to the early endosome. Endosomal UbCav and cholesterol associate with
valosin-containing protein (VCP) and are packaged in multivesicular bodies by endosomal
sorting complexes required for transport (ESCRT) complex proteins for transport to and
degradation in the late endosome/lysosome. Some polyUbCav is degraded by the proteasome.

**Figure 5: Caveolae membrane composition and caveolae targeting signals**

Caveolae membranes are enriched in phospholipids, sphingolipids, and cholesterol. This diagram
depicts the distribution of membrane lipid species within a caveolae bilayer. Cholesterol is
concentrated within caveolae, representing a third of all lipids, and is present in both leaflets of
the bilayer. Sphingomyelin is slightly more prevalent than PC in the exofacial leaflet of caveolae,
while PE composes more than half of the cytoplasmic leaflet. Also in the cytoplasmic leaflet, the
anionic phospholipids PI, PS, and PA compose the minor fraction of lipid content with PI
roughly twice as prevalent as PS and PA at half the concentration of PS. Proteins can be targeted
to caveolae through transmembrane domains (e.g., GPCRs, CavS), fatty acylations (e.g., Src
myristoylation) and membrane binding domains (e.g., Cavin1 PI and PS binding domains).

**Figure 6: Cav1 phosphorylation influences Src signaling**

A. Cav1Y14 phosphorylation creates a Src Homology 2 (SH2) binding site that regulates Src
activity through binding of Src and recruitment of two cytosolic Src-regulating enzymes: C-
termin al Src Kinase (CSK) and SH2 domain-containing non-transmembrane protein tyrosine
phosphatase (SHP-2). B. When recruited to caveolae by pCav1Y14, CSK deactivates Src by
phosphorylating SrcY530; however, pCav1Y14 binding to SHP-2 blocks CSK recruitment and
prevents SrcY530 phosphorylation, increasing the duration of Src activity. Thus, Cav1
phosphorylation permits a rheostat-like control of Src signaling.

**Figure 7: Caveolae-cytosol exchange of eNOS increases NO production**

A. Within caveolae, Src phosphorylates Cav1, leading to the release of eNOS from Cav1 inhibition (blue arrows). Src also phosphorylates and activates PI3K, which phosphorylates and activates Akt. B. In the cytosol, free eNOS is phosphorylated by Akt, which increases NO production.
A

Cavin-1/Cavin-1 Homotrimer

Disordered Region 1
Helical Region 1
Disordered Region 2
Helical Region 2
Disordered Region 3

PI(4,5)P2 Binding Sites
PS Binding Site

Acidic
Basic
Acidic
Basic
Acidic

Cavin1/Cavin2 Heterotrimer
Cavin1/Cavin3 Heterotrimer

CAVIN-3: MEMBRANE BINDING
CAVIN-3: CAV1 BINDING

B

~23.4 nm

~5.5 nM
~7 nM
ER Exit Site
CIS/Medial Golgi
8S-Cav Oligomerization
Co-translational Insertion
Cholesterol Crystallization
70S-Cav Oligomerization
CopIi Transport
Co-translational Insertion
Cholesterol Crystallization
70S-Cav Oligomerization
Pi4P-Dependent Golgi Export

Cav Palmitoylation, Cavin Assembly
Cavin-Induced Membrane Curvature

70S-Cav Pre-invagination

Golgi Apparatus

8S-Cav + Cholesterol

Mature Caveola

Plasma Membrane
Mature Caveola -> Early Endosome

CAVIN DISSOCIATION

VCP- AND ESCRT-DEPENDENT MULTIVESICULAR BODY FORMATION

LYSOSOMAL DEGRADATION

PROTEASOMAL DEGRADATION

Late Endosome

Mature Caveola

CAV1 UBIQUITINATION

CAVIN DISSOCIATION

CAV1 PHOSPHORYLATION

VESICULATION

Mature Caveola

CAV1 PHOSPHORYLATION

VESICULATION

MECHANICAL STRESS

CAVEOLAR FLATTENING

Plasma Membrane

UbCAV1 ENDOCYTOSIS

UbCAV1

ENDOCYTOSIS

CAV1

Later Endosome

Lysosome

MonoUbCav1

PolyUbCav1

8S-Cav + Cholesterol

8S-Cav + Cholesterol

8S-Cav + Cholesterol

8S-Cav + Cholesterol

8S-Cav + Cholesterol

8S-Cav + Cholesterol
Caveolar Membrane Composition

Unbiased Lipids
- Cholesterol
- Exoplasmic Leaflet Lipids
  - Sphingomyelin
  - Phosphatidylcholine

Cytoplasmic Leaflet Lipids
- Phosphatidylethanolamine
- Phosphatidylinositol
- Phosphatidylserine
- Phosphatidic Acid

Fatty Acylation
Transmembrane Domains
Membrane Binding Domains

GPCR

Src

PS-binding
PI-binding
Cavin1 Homotrimer
A

Cav1

Y14 PO4

SHP-2

CSK

Active Src

B

Cav1

Y14 PO4

CSK

SHP-2

Inactive Src

Y530 PO4