Re-examining structural features of caveolins: 25 years later

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Abstract

Caveolae are flask-shaped signaling platforms in the plasma membrane of cells. They broadly participate in different types of cellular processes including signaling, lipid homeostasis, caveolae-mediated endocytosis and pathogen invasion. The principal protein responsible for caveolae formation is the integral membrane protein, caveolin (caveolin-1, -2, -3). Caveolin oligomer assembly is believed to drive the membrane curvature that is a defining feature of caveolae. Capturing this phenomenon in biophysical studies has presented ongoing challenges for researchers over the past several decades. Much of what is reported about caveolin structure and oligomerization has resorted to isolating distinct regions of the protein for characterization. In this paper, major findings in the field of caveolae from the past several decades are summarized. Much of the discussion centers on caveolin-1, the most ubiquitous and minimal requirement for caveolae formation in non-muscle tissue. In the article, a comprehensive overview of what has been gleaned from cell biology and *in vitro* studies of caveolin is presented, summarizing both what we know and the challenges that remain. We attempt to reconcile structural features of caveolin and highlight unanswered questions, including but not limited to, the cytosolic disordered N-terminal region and its role in caveolin function. This work will help to illuminate new avenues for research in the field of caveolae, lipid rafts and disordered proteins. Important considerations about the challenges working with caveolin are conveyed in concert with a discussion about membrane proteins and the role of the membrane lipid bilayer on their structural conformation; followed by a critique of current approaches aimed at elucidating caveolin structure and oligomeric assembly.

Keywords: caveolin, caveolae, membrane proteins, structure, lipids, disordered proteins

Caveolae and caveolins

Caveolae are 50-100 nm diameter invaginations that are found at the surface of plasma membranes. While they can be ubiquitous, they are abundantly found in adipocytes, pneumocytes, fibroblasts, endothelial cells and striated and smooth muscle cells [1]. They are widely recognized as key players in signal transduction, purportedly enabling the congregation of signaling molecules to assemble and carry out their function. Noteworthy, the presence of many caveolae at the cell surface effectively increases the surface area of the plasma membrane allowing greater signaling capacity to take place. Caveolae are enriched in cholesterol and associated signaling proteins and believed to function as raft-like platforms at which multiple signaling events take place [2], [3]. Caveolin is the principle protein essential for caveolae formation (Cav1, 2, 3) [4]–[7]. Without caveolins, caveolae are unable to form [8], [9]. In fact, caveolae can be introduced synthetically to bacteria, which do not natively produce caveolae, by introducing the caveolin gene for expression [10]. Cav1 (α and β isoforms) is primarily responsible for caveolae formation in non-muscle tissues and is often found co-expressed with Cav2 while Cav3 is strictly found in muscle tissue-specific caveolae with the latter having the closest sequence similarity to Cav1 [11]. Both Cav1 and Cav3 are sufficient for caveolae formation [11]. Of the Cav1 sub-isoforms, Cav1 α forms caveolae more readily compared to Cav1 β with the latter rarely forming caveolae at all [12]. Cav2 is somewhat more elusive of the three major isoforms, but it does associate with other caveolins in the form of hetero-associations, and it is often found to co-localize with Cav1 [13], [14]. For years, the mechanism underlying the membrane curving ability of these proteins has stumped researchers. Early studies suggest that caveolins assumed a conformation whereby the cytosolic N- and C-terminal regions were separated by a single membrane spanning intramembrane domain (IMD) that was too long (approximately 34 residues) to be a single pass membrane protein, yet too short to be polytopic (two or more membrane passes). A single spanning transmembrane α-helix requires

approximately 20-25 residues to completely traverse both lipid bilayer leaflets of the plasma membrane, depending on the angle of insertion. Further, membrane proteins have the ability to influence the organization and enrichment of surrounding lipids in the membrane bilayer suggesting that the bilayered plasma membrane and the protein-lipid interactions that occur are critical to influencing, supporting and stabilizing their structure [15]. It has long been believed that caveolins associate in a homo- and hetero- manner to form high-order oligomeric complexes that give rise to the observed membrane curvature that are the hallmark of caveolae. The mechanism by which caveolins assemble and consequently bend the bilayer of the membrane remains unknown, although, there are a number of biophysical studies reporting a direct observation of Cav1 stoichiometry and assembly (oligomerization) *in vitro* in membrane mimetic systems, both detergent micelles and bicelles [16]–[18]. In these studies, all essential primary structural features of Cav1 were preserved including the hydrophobic IMD, which is shown to be necessary for caveolae formation [19].

Caveolin expression has been strongly linked to signaling processes, and changes in its native expression affect important downstream signaling events. Physiological outcomes resulting from these altered expression profiles include the development of diseases such as cancers, complications related to skeletal muscle function (Cav3) such as limb girdle muscular dystrophy, and other disruptions in skeletal muscle signaling pathways [20], [21]. Its role in muscle-specific tissues has been comprehensively reviewed [22]. The vast body of literature describing reported physiological changes and onset of disease related to alterations in caveolin expression shows that it plays a key role in proper cell signaling and function [2], [23]–[29]. Importantly, a Cav1 breast cancer mutant, P132L, which is present in approximately 16% of human breast cancers was shown to form very strongly dimers, *in vitro*, suggesting a possible mechanism for the deleterious effects of this mutant [17], [23], [30], [31].

Caveolae are not static structures though they were once thought to be. Some proteins help to stabilize caveolae morphology and effectively increase their “half-life.” Cavin proteins (1-4

isoforms identified in mammals), are suggested to self-assemble in both hetero- and homo-oligomeric protein-protein and protein-lipid interactions to create a caveolar coat, increasing caveolae stability [32]–[36]. This additional caveolar coat disperses with specific cell stimuli leaving caveolae to adapt to changes in the plasma membrane. The exact mechanism of these interactions has not yet been elucidated biochemically and biophysically, and the nature of its caveolar interactions remain unresolved in terms of their necessity in facilitating caveolae generation. In the absence of Cavin, caveolae are still stable enough to be captured on most experimental timescales, which was reported earlier by Parton and co-workers [37]. Although some studies suggest Cavin-1 (PTRF) is required for caveolae formation, this is inconsistent with other reported studies [10], [33]. Caveolae structures, themselves, also participate in endocytosis, independent from clathrin-mediated endocytosis [38], [39]. They are implicated in lipid transport and homeostasis and some evidence suggests they may partake in facilitating host-pathogen invasion of cells [40], [41]. It is clear that caveolae are an integral, dynamic component of cell membranes and much has yet to be uncovered, on a molecular level, about the many interactions that occur at caveolar platforms, which are so crucial to proper cell function.

The unstructured N-terminal region and scaffolding domain

The structure of Cav1 is described as having distinct regions, a primarily unstructured (synonymously referred to as intrinsically disordered) N-terminal region (1-101), which includes a small region termed the scaffolding domain - due to its heavy interaction with signaling molecules. This relatively small region encompasses residues 61-101 [42]. The scaffolding domain was once thought to be the primary driver of oligomerization, arising from the observation that deletion of this region, 61-101, disrupted oligomerization *in vivo* [43]. Further, this region, by itself, is capable of oligomerizing *in vitro* [44]. The second half of the scaffolding domain, 82-101, is primarily thought to be involved in binding to various signaling molecules [45]–[50]. Caveolin has been linked to G-protein coupled receptors and their signaling partners both directly and indirectly through the organization of caveolar signaling platforms and the associated molecules Gα and

adenylyl cyclase [3], [51]–[53]. Aside from this role, the scaffolding domain has been implicated as the primary site of membrane attachment in earlier studies [42], [54]. While the scaffolding domain may help stabilize membrane attachment, the IMD interacts with the membrane most extensively due to its extreme hydrophobic properties both predicted by the primary amino acid sequence and based on experimental observations [55], [56]. Some studies suggest otherwise, however, in these studies the IMD construct used was fused to a soluble glutathione-S-transferase (GST) tag, which likely altered the solubility properties [42], [57].

The remainder of the soluble, cytosolic N-terminal region is unstructured, although some report α-helical and β-sheet character. It is not required for caveolin trafficking and subsequent caveolae formation [19]. Here, we postulate that the unstructured characteristic of the N-terminal region is precisely what allows it to interact with the extensive number of signaling partners reported and not necessarily the helical scaffolding domain, exclusively [53]. Few have directly investigated putative interactions at the unstructured end of the N-terminal domain (1-60), but we highlight the capacity to interact with many signaling partners as a direct consequence of its disorder.

Disordered proteins or regions (IDP/Rs) are structurally dynamic, lack appreciable secondary and tertiary structure and, as a consequence, can assume multiple conformations. This has been suggested as the basis for many important regulatory interactions such as transcription factor binding at sites of DNA [58]–[60]. They also play a role in the assembly of intracellular condensates giving rise to distinct liquid-liquid phase transitions that are a hallmark feature of membrane-less organelles both in the cytoplasm and nucleus of mammalian and other higher-order organisms [61]. For the purposes of this discussion, we will focus on IDP/Rs and how their structural flexibility enables more complex functions as it relates to the N-terminal region of Cav1. IDP/Rs are present in all organisms, however, it has been suggested that their presence increases with organism complexity allowing for more versatile functions to play out [62]. Mechanistically, IDP regions function in a variety ways, giving rise to disordered-ordered transitions upon binding depending on their requisite binding partners [61], [63]. Under these

159 circumstances, IDP/Rs can assume a folded protein structure in an induced-fit like manner as a
160 result of interacting with one of many species. Alternatively, IDP/Rs can remain primarily as a
161 flexible, polypeptide chain. They can also undergo post-translational modification (PTM) in a
162 regulatory manner as well [64], [65]. They vary in length from 10 to 30 residues (long) or more,
163 and this is but one of many factors that affects their behavior, propensity to fold into different
164 conformations, and even their ability to interact with one another [66]. The disordered N-terminal
165 region of Cav1 is approximately 60 residues. Compared to Cav2 and 3, it is longer by 16 and 27
166 residues, respectively. Figure 1 illustrates the primary sequence of the three caveolin isoforms
167 with regions of structural interest for characterization highlighted. Notably, all three caveolin
168 sequences contain a consensus "caveolin signature sequence" encompassing residues,
169 "FEDVIAEP," located within the N-terminal region, including the scaffolding domain, of all three
170 proteins. Its role is not clear, but it appears to be strictly conserved across all isoforms [14]. In
171 Cav1 this sequence lies within the scaffolding domain where heavy interactions with other
172 signaling molecules occurs, so it may be critical in supporting these interactions.
173 In addition to the associated physiological effects of caveolae/caveolin interactions, some
174 studies report direct interactions with signaling partners at the N-terminus, independent of the
175 scaffolding domain. A sterol carrier protein, SCP-2, interacts with residues 33-59 in an
176 electrostatically driven binding interaction [50]. This may help to shed light on the mechanism of
177 cholesterol enrichment in caveolae if not its role in lipid homeostasis [67]. A direct interaction with
178 caveolin and cholesterol has been suggested through binding at the heavily implicated CRAC
179 (cholesterol recognition amino acid consensus) motif, which is present in the scaffolding domain
180 (residues 94-101) of Cav1 [68]. This motif has been shown to recruit cholesterol in DPPC bilayers.
181 Cholesterol enrichment, whether a consequence of direct caveolin interactions or due to indirect
182 preferential phase behavior (in lipid membranes), may help to explain how caveolae can assume
183 a meta-stable state in the absence of facilitating proteins like Cavin. Interestingly, mutations at
184 the N-terminus (including the scaffolding domain) have led researchers to point to a direct cause

and effect of the scaffolding domain on proper neuronal nitric oxide synthase (nNOS) activity [69]. In this study, again, a direct interaction, *in vitro*, was also investigated and confirmed. Further, endothelial nitric oxide synthase, eNOS, is inactivated upon binding to the Cav1 scaffolding domain in a regulatory manner, maintaining it in a structurally inactive conformation [70]. The scaffolding domain, by itself, was also able to inhibit eNOS activity when delivered systemically to mice as a potential therapeutic treatment [71]. In addition to NOS regulation, the scaffolding domain also reportedly interacts with critical proteins in pathogenic species, such as gp41, a prominent protein of the HIV viral envelope [72]. This was also demonstrated in studies where synthetic peptides that captured the binding domain of gp41 (CBD1) to Cav1 were generated and the resulting complexes were capable of eliciting an immunogenic response [73]–[75]. These studies support a purported role for caveolin in caveolae-mediated pathogenesis and infection. Lastly, Cav1 reportedly binds to dynamin-2 through interactions at the scaffolding domain. [38], [76]. Dynamin promotes "pinching off" during the endocytosis process in clathrin-mediated endocytosis and its binding to Cav1 may support internalization through a similar mechanism [40]. Much of the reported direct interactions involve the caveolin scaffolding domain leaving the function of the remaining N-terminal region less well-defined.

While the N-terminal region may not be as heavily implicated in binding to signaling molecules as the scaffolding domain, compelling evidence suggests it may play more of a regulatory role in caveolae assembly through phosphorylation by v-Src, a non-receptor tyrosine kinase, at N-terminal tyrosine residue 14, Y14. Phosphorylation of tyrosine 14 resulted in flattening, aggregation, fusion and overall destabilization of caveolae [77]–[79]. It is reasonably hypothesized that phosphorylation at the N-terminal Y14 leads to charge-charge repulsion, which disrupts caveolin oligomers and promotes disassembly and possibly, triggering endocytosis. Cav1 also serves as a substrate for other non-receptor tyrosine kinases in a cell-type dependent manner [80]. Of the two Cav1 isoforms identified in humans, the α isoform contains 3 tyrosine residues,

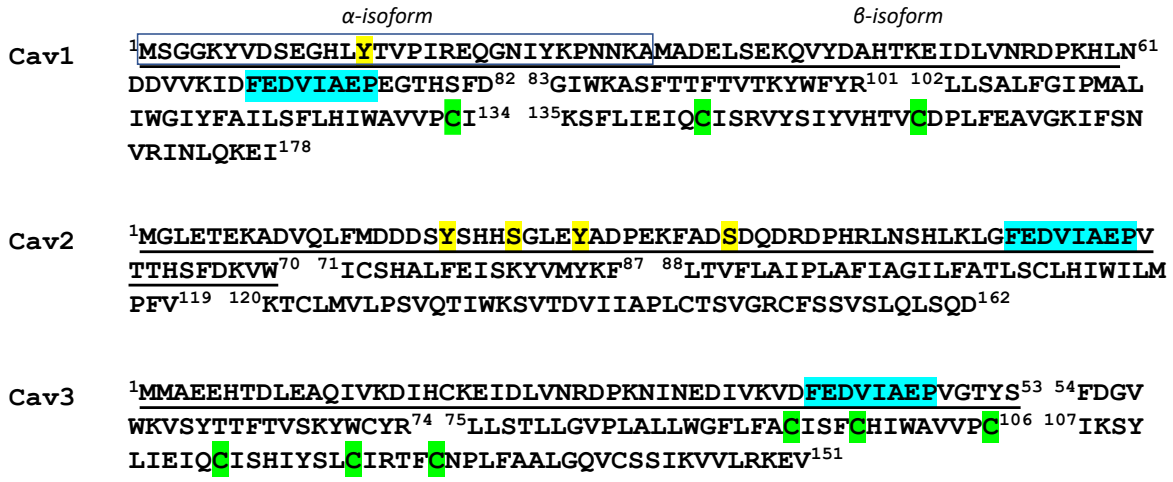

Figure 1. Primary sequence features of human caveolin-1, -2, -3 (Cav1, 2, 3). The N-terminal regions are underlined (not including the scaffolding domain). Sites of phosphorylation at the N-terminus are highlighted in yellow and the "caveolin signature sequence" is highlighted in blue. Three sites of palmitoylation in Cav1 are noted in green. Each distinct region of structural characterization is numbered accordingly in all three isoforms. There are no reports of Cav2 palmitoylation at the C-terminal region, although it also contains three signature cysteine residues corresponding to those in Cav1 and Cav3. A recent report shows Cav3 has six potential sites of palmitoylation in its membrane interacting region including the C-terminal domain. These sites have been highlighted in green. There are no reports of phosphorylation at the N-terminus in Cav3.

while studies show residue Y14 is preferentially phosphorylated by tyrosine kinases [48], [77]. Through tyrosine phosphorylation, the N-terminal region of caveolin serves as more of an indirect regulator of signaling through the intracellular dissociation of caveolae signaling platforms. Studies suggest tyrosine phosphorylation is responsible for caveolae disassembly, yet the molecular basis of caveolae- induced endocytosis initiation is still unclear. Perhaps other interactions through additional binding partners at the N-terminus are responsible for triggering endocytosis. Interestingly, caveolae still maintain the capacity to form even in the absence of the N-terminal region when Cav1 is expressed [19], [32]. One study concludes, based on site-directed mutagenesis of six N-terminal lysine residues, that Cav1 is capable of being ubiquitinated and by way of a protein degradation complex, VCP-UBXD1, is tagged for trafficking to the lysosome during late endocytosis [81]. In the absence of these lysine residues, caveolae turnover is reduced and they accumulate in requisite cells. In Cav2, serine residues 23 and 36 along with tyrosine residues 19 and 27 have been reported as sites of phosphorylation [82], [83]. Few studies are yet to report corresponding sites of phosphorylation

at the N-terminal region of Cav3, although, mutagenesis studies of other key residues at the N-terminus show disruptions that lead to complications in growth factor signaling [84].

The membrane and MP structure

Early reports that attempted to dissect caveolin structure suggested that the scaffolding domain was largely responsible for Cav1 membrane association and oligomerization, but this is still somewhat controversial [42], [53], [54]. Since these early studies, substantial efforts have been directed at characterizing the IMD and understanding its role in membrane insertion and shaping the membrane morphology of caveolae. Taken together it is likely that the scaffolding domain, while serving as a highly dynamic active site for signaling molecules, also facilitates membrane attachment, but in a more obligatory role. It fails to drive homo-oligomeric interactions of Cav1 *in vitro* in both detergent micelles and in bicellar, systems [16], [17]. Ironically, while caveolin oligomerization is attributed to shaping the bilayer membrane, the membrane itself also strongly influences membrane protein (MP) structure. This delicate interplay is what serves to stabilize MPs and induce changes in the physical properties of the bilayer such as membrane thickness and lipid order and disorder to name a few [85]. The native lipid bilayer surrounding many membrane proteins also heavily influences protein function. Diacylglycerol kinase (DAGK), for example, exhibits remarkably different levels of enzyme activity depending on whether it is reconstituted in detergent micelles or a lipid bilayer [86]. Many considerations of the membrane and its impact on MPs and their structure and function has been reviewed extensively by Cournia and co-workers [87]. Caveolin, an integral membrane protein embedded in the lipid bilayer, requires the same contextual consideration in order to understand the driving forces behind its membrane curving capability. Current studies of Cav1 structure continue to aim at elucidating a physiologically relevant structure in order to derive a better understanding of the molecular mechanics underlying its ability to shape the membrane. The consensus in the field is that oligomerization is a key factor in driving caveolae assembly. While this may true, the protein itself, does not appear to have properties sufficient enough to drive self-association or oligomerization

outside the framework of a cell [17], [16]. This could be explained by a number of factors. The membrane mimetic system used in these studies was somewhat minimalist in that it contained a homogeneous lipid composition in both bicelles (planar bilayer region) and in the detergent system. While both systems lacked an appreciable amount of cholesterol and other enriching lipids commonly found in the plasma membrane of mammalian cells, the mimetic systems did preserve the dynamic nature of the membrane allowing for both lipids and protein (reconstituted Cav1) to freely associate in solution on the timescale of the experiments described. Nonetheless, the protein, itself, did not retain the properties required to drive self-association suggesting a more integral role of the surrounding membrane lipids. Conversely, the pervasive breast cancer mutant, P132L, does have properties sufficient to drive self-association in the absence of these factors. This further suggests that the lipid mimetic system used in this analysis was sufficient to support protein-protein interactions if they were, in fact, relevant to the assembly of oligomers.

We surmise that the historical challenges of working with caveolins *in vitro* may be due to inherent structural instability during recombinant expression and purification compounded by its inherent conformational flexibility, particularly within the IMD. There are few appreciable intramolecular forces that would be significant enough to drive preferential association between the two hydrophobic intramembrane helices in a stabilizing manner within the lipid bilayer. From the chemical properties of the primary sequence of the residues in the IMD we can infer that stabilization of the position of the two helices, with respect to one another, must depend on or at the very least is heavily influenced by the surrounding membrane itself and/or the flanking N-terminal scaffolding domain and C-terminal regions (135-178). A combination of experimental analyses and simulations by Rui and co-workers support the case for interactions between the intramembrane helices that are subsequently stabilized by the surrounding lipid bilayer [88]. The combination of these interactions ultimately gives rise to a U-shaped conformation of the IMD within the bilayer.

The C-terminal region has been implicated in earlier studies in membrane attachment and oligomerization similar to the scaffolding domain [54]. A closer look at the C-terminal region reveals three sites of palmitoylation at cysteine residues 133, 143, 156, which are suggested to facilitate anchoring to the membrane in a manner similar to the scaffolding domain, although, the exact role is less understood and not as well studied [54], [89], [90]. In addition to the possibility of membrane anchoring, palmitoylation is also thought to play a role in facilitating cholesterol recruitment to caveolae and thereby stabilizing membrane curvature, which is supported by simulation experiments, but this notion would benefit from further analyses. Palmitoylation is not required for proper caveolae formation or trafficking of Cav1 to caveolae [91]. To date, only a bioinformatics-based prediction of the C-terminal domain structure suggests it assumes a long α-helix [89]. A recent report shows Cav3 is also palmitoylated at its C-terminal region as well [92].

Structural flexibility enables caveolin to adopt multiple conformations

Aside from the soluble, disordered nature of the N-terminal region of caveolins, its unusual topology continues to complicate its three-dimensional structural analysis. With few verifiable functional assays to confirm the physiological significance of any reported three-dimensional structure or assembly of oligomers, *in vitro*, it continues to present challenges and intrigue. A comprehensive overview of the literature and progress over the last several decades has undoubtedly provided a wealth of knowledge and insight into caveolin oligomeric structure and function. From these studies, it is apparent that caveolin (Cav1) appears to have the remarkable ability to adopt multiple conformations, which have been captured using a variety of experimental and computational approaches (Figure 2) [18], [19], [88], [93], [94]. However, what can be concluded about the nature of its structure and assembly from these analyses? Studies that attempt to characterize caveolin structure in the absence of a lipid bilayer overlook a key component that undoubtedly influences structure, oligomeric assembly and stabilization. The significance of the bilayer in contributing to MP structure may vary as well, depending on the protein. For a protein such as caveolin, which arguably has more conformational freedom than

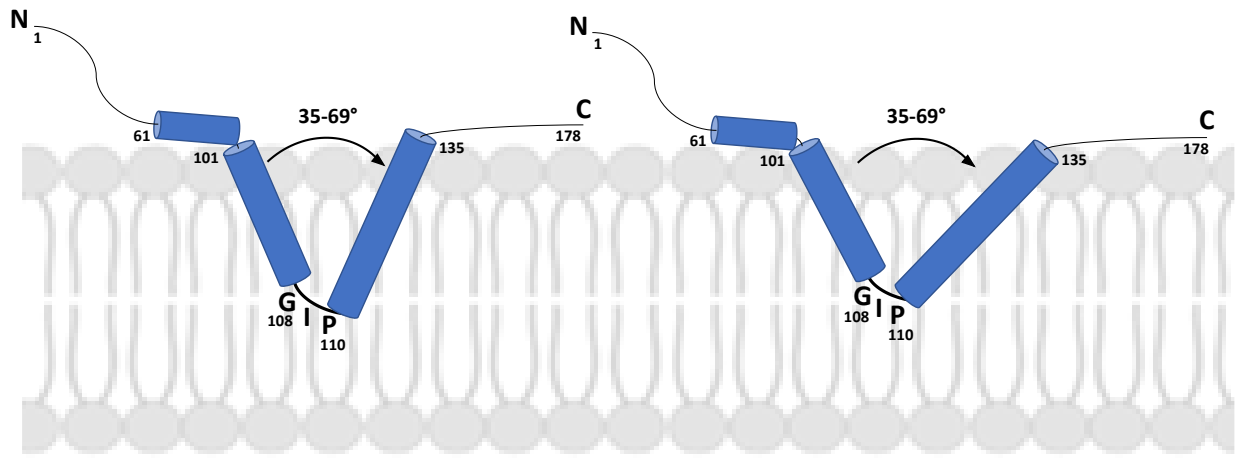

Figure 2. Topological depiction of caveolin-1 illustrates the intramembrane domain (IMD) and the range of conformational angles the two intramembrane helices assume with respect to one another. These findings are reported in Rui *et al.* 2014.

other membrane proteins, we would expect the lipid bilayer to heavily influence its three-dimensional structure and oligomeric assembly [95]. This idea is loosely supported by Rui and co-workers [88], who in their landmark study, addressed the question of Cav1 IMD 3D conformational orientation in the context of a lipid bilayer.

The effects of the membrane lipid nano-environment on MP structure has also been recently reviewed by Lyman and Levental [96]. Other membrane mimetic systems such as detergent micelles are dynamic compared to their lipid bilayer forming counterparts and can destabilize membrane protein structure over time [97], [98]. Further, cholesterol appears to contribute in some way to stabilize caveolae (caveolin oligomers) in native membranes and are enriched at caveolae structures (see above). Its affect on Cav1 oligomerization is also unclear, but would be worth exploring in a biophysical analysis. Finally, the emerging class of caveolae associating proteins, Cavins, adds another layer of complexity to the caveolae/caveolin assembly problem. Conflicting reports suggest Cavins are required for caveolae formation, yet it is still not clear how they interact with caveolae and the surrounding membrane lipids to accomplish this. The role of Cavins in assuming a stabilizing coat assembly similar to clathrin-coated pits seems more plausible, especially if caveolae are highly dynamic in nature, which has also been suggested.

Conclusion and Future Perspectives

We re-examined structural features of caveolins looking back at decades of comprehensive work that has been done to characterize individual regions of these proteins. We re-evaluated distinct structural components, emphasizing the disordered N-terminal region and the role it plays in signaling and caveolae assembly. We highlight the “caveolin signature sequence,” which is conserved in the N-terminal region of all three caveolin isoforms and how the exact role of this sequence is still unknown. This article also discusses exciting new advances in our understanding of caveolin oligomeric assembly while considering the role the lipid bilayer serves in the native conformation of the protein. We chose to revisit this body of work to address the many outstanding questions that remain as we move into a new era of experimental capabilities for molecular visualization. These new capabilities will and already have begun to advance our critical understanding of molecular structure and higher-order assembly. What once proved substantially difficult for structural biologists to tackle, *in vitro*, is now becoming more accessible through the advancement of new tools such as cryo-EM and more sophisticated simulations capabilities. We look forward to the next decade of structural biology in anticipation of what more we will learn.

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