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Minireview

A new paradigm for membrane-organizing and -shaping scaffolds

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Abstract The clathrin, COPI and COPII scaffolds are paradigm vesicle coats in membrane trafficking. Recent advances in our understanding of the caveolar coat have generated a new paradigm. It represents those membrane coats, where a considerable part of the protein component is lipid modified, and integrated into the cytosolic leaflet of the vesicle membrane by a hairpin-like hydrophobic structure. Such coat proteins are permanently associated with membranes, and form oligomers early after synthesis. These oligomers assemble into a coat that has high affinity for particular lipids, creating lipid microdomains within the membrane. The combined protein–lipid structure should be considered as the scaffold that entraps ligands, either through affinity with the protein or with the lipid component, and that has the ability to shape membranes. Besides scaffolds assembled by *caveolins*, scaffolds assembled by *reticulons* and PHB domain-containing proteins such as the *reggielflotillin* proteins fit this paradigm.

Keywords: Caveolin; Reticulon; Flotillin

1. Introduction

The past 30 years have seen a tremendous increase in our understanding of basic membrane trafficking principles and how these are regulated, and this is summarized and extensively discussed in many reviews [1–5]. The prime paradigm in our molecular understanding of membrane trafficking is the 'vesicle coat'. Vesicle coats concentrate ligands in budding structures at donor membranes, generate membrane curvature, aid in pinching off vesicles, and recruit specific SNAREs for correct targeting to downstream 'target membranes'. The classical vesicle coats (COPI, COPII, Clathrin) disassemble into cytosolic subunits and dissociate from the vesicle once these processes have taken place. What is left is a 'naked vesicle' that contains sorted cargo destined for a downstream compartment, the correct SNAREs that ensure the vesicle will indeed fuse with membranes of the right destination compartment, and a whole set of regulatory molecules, orchestrated by a specific RabGTPase. Upon fusion, soluble cargo is released into the lumen of that compartment, and the membrane lipids and trans-membrane proteins that were present in and made up the vesicle membrane diffuse into the target membrane.

Multiple layers of complexity exist to control the basic principle outlined above. The many individual molecular events must be correctly timed and coordinated, rates and kinetics of vesicle trafficking modulated, and downstream targeting controlled. In addition, various physiological processes impinge on these machineries at multiple levels to modulate membrane-dependent signal transduction and to adapt synthesis and membrane trafficking of particular cargo to the physiological needs of the cell. Membrane trafficking is therefore involved in both processing incoming signals, and in translating them into the correct physiological output.

More recent concepts in membrane biology have however not yet been fully integrated into our general basic understanding of membrane trafficking. These concepts emerge from the discovery that lipids organize laterally within the lipid bilayer to form, transiently or more stably, microdomains in which certain lipid species are concentrated while others are depleted [6,7]. The lipids themselves are believed to generate part of the driving force behind lipid microdomain formation, since certain lipid species tend to be less miscible with other lipid species within the bilayer. In addition, certain protein anchors (e.g. GPI) or certain protein trans-membrane domains, might favour a particular lipid microenvironment over another, leading to preferential partitioning into one or another microdomain. The concept of the so-called 'lipid rafts', membrane microdomains especially enriched in glycosphingolipids and cholesterol, is under extensive scientific scrutiny and debate [8,9]. While no consensus has yet been reached on whether lipid raft formation in mammalian cells can be lipid-driven (it certainly can be in model membranes), it is clear that this process can be protein-driven. Such polarized views are likely to be inappropriate for cellular membranes, where microdomain formation must be a complex interplay of lipid–lipid, lipid–protein, and protein–protein interactions. The proteins involved are either multivalent cytosolic or extracellular proteins that bind headgroups of specific lipids, or membrane-integrated proteins with affinity for particular lipid species that form multimeric scaffolds. In this review, we will consider particular members of this latter group as a new paradigm of membrane-shaping and -organizing proteins that operate according to new, poorly understood principles.

2. Biosynthesis and assembly of membrane-integrated coats: the caveolar coat

The biosynthesis of scaffolds built from membrane-integrated proteins starts in the endoplasmic reticulum (ER),

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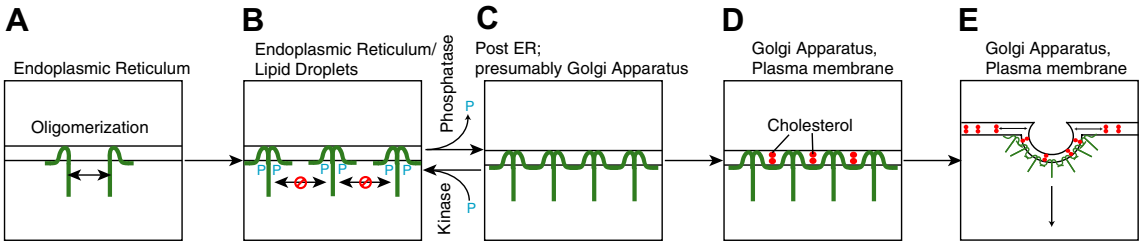

Fig. 1. Hypothetical model of coat assembly. (A) After co-translational insertion into the ER membrane, six or seven caveolin monomers form SDS-resistant oligomers. (B) To prevent unwanted polymer assembly at this stage, oligomers are phosphorylated by an unknown kinase at serine80 (S80). (C) Phosphorylated oligomers are transported to the Golgi complex where they are dephosphorylated by an unknown phosphatase. This triggers polymerization and increased cholesterol affinity. (D) Cooperative polymer assembly and high-affinity binding to cholesterol leads to formation of a caveolar coat, leading to (E) membrane curvature and budding of caveolar vesicles.

where they are integrated into the lipid bilayer by the translocon (Fig. 1A, B). Caveolin (Cav1, Cav2, and Cav3) synthesis occurs at the ER membrane, where single molecules are co-translationally integrated into the bilayer [10,11]. They do not span the membrane, but form a hairpin loop within the bilayer, causing both the N- and the C-terminus of the protein to be cytosolic. Caveolins form SDS-resistant oligomers (hexa- or heptamers) soon after synthesis and still in the ER, probably by coiled–coil interactions of cytosolic N-terminal domains [10,12]. These oligomers leave the ER and are transported by the secretory pathway to the Golgi complex [13] (Fig. 1C). If onward traffic is prevented and caveolin accumulates in the ER, the protein is increasingly found on lipid droplets [14–16].

Somewhere before caveolin oligomers reach the plasma membrane but after they have left the ER, they are assembled into a polymeric caveolar coat (Fig. 1). Assembly, which involves a fixed number (between 100 and 200) of Cav1 molecules [17], shields a particular antigenic domain of the protein, while it creates new antigenic domains [18]. This allows the use of certain antibodies to distinguish between unassembled and assembled caveolar coats. Apparently, newly synthesized caveolin on the ER, caveolin on lipid droplets and on part of the ER-Golgi intermediate compartment (ER-GIC) or the perinuclear Golgi complex is recognized by one type of antibodies, while caveolin on more peripheral Golgi-derived membranes, on intracellular caveolar vesicles, caveosomes and endosomes, and on caveolae connected to the surface is recognized by another type of antibodies [19]. Antigenic properties of newly synthesized Cav1 also reappear when pre-assembled caveolar coats are disassembled on the cell surface by active cholesterol extraction [18], indicating that cholesterol has a structural/stabilizing role in coat assembly. In addition, Cav1 organized into a caveolar coat appears to have higher affinity for cholesterol than oligomeric or monomeric caveolin [20], suggesting a cooperative model of coat assembly involving proteins and lipids.

It can be assumed that other structural proteins participate in forming caveolar coats, but these are currently not known. Also structural insights are completely lacking. Early scanning electron microscopy images gave the impression of spiral-like coats on the cytosolic surface of caveolae [21], but there is currently no evidence that this is indeed the defining appearance of a caveolar coat. The protein composition of these spiral-like assemblies has not been determined, nor do we know any physical characteristics like flexibility and tendency to self-assemble. Unfortunately, the limited information has not yet provided any intuitive models, like that in the case of clathrin coats.

3. Common properties of caveolins, reggie/flotillin proteins and reticulons

A number of membrane-integrated proteins have been described that share topological principles of caveolins (Fig. 2). One class carries an evolutionarily conserved protein domain called the prohibitin homology (PHB) domain, of which the reggie/flotillin proteins are well-known members [22,23]. The PHB domain could have a hairpin-like topology, similar to

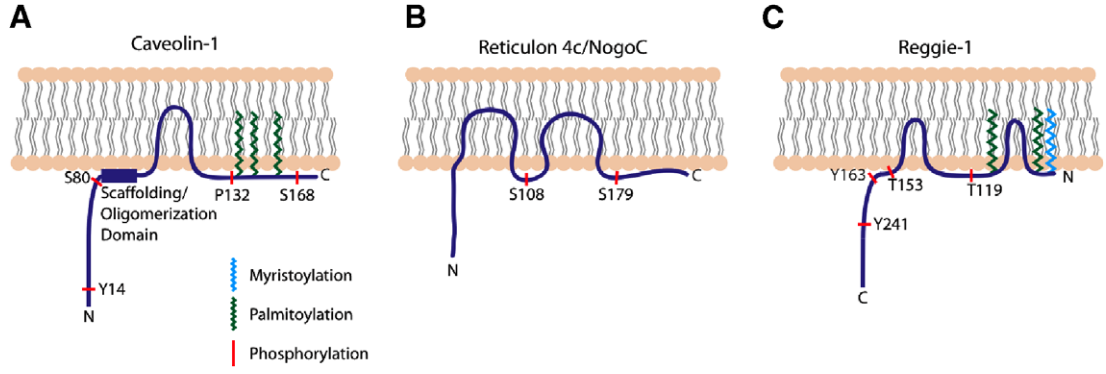

Fig. 2. Putative membrane topologies of membrane-integrated coat subunits. Membrane topologies of Caveolin-1 (A), Reticulon 4c/NogoC (B) and Reggie-1 (C) are depicted. They have an overall similar appearance, contain at least one membrane-integrated, but not -spanning, hairpin-loop domain, and have several phosphorylation sites on both sites of the hairpin loop near the lipid bilayer.

the membrane domain of caveolins. The reticulons, in particular Rtn4c/NogoC and DP1, have two hydrophobic segments that adopt this unusual hairpin-like topology [11]. There are additional shared properties between these protein families. They all partition in detergent-resistant membranes, they form oligomers [22] (Rtn3 oligomerizes via its cytosolic N-terminal domain like caveolins [24]), and they contain several putative phosphorylation sites. Furthermore, caveolins and reticulons are found on membranes with high positive curvature (caveolae and peripheral ER tubules, respectively). Most likely this curvature is induced by a local concentration of several hairpin loops inserted into the cytosolic leaflet, which creates a larger surface on the cytosolic than on the luminal side of the membrane. In agreement with this, overexpression of caveolins creates more caveolae-like structures, while overexpression of reticulons creates more ER tubules. Membrane insertion has not been studied to this extent for PHB domains, and it is therefore not known to which extent reggie/flotillin proteins create positive curvature on membranes. That they are present on a particular type of endocytic vesicles may suggest that they participate in shaping those membrane carriers [25].

Caveolins are involved in generating spherical shapes and their grape-like assemblies, but also in forming tubules (e.g. T-tubules in muscle cells by Cav3 [26]), while reticulons have until now only been reported to be involved in generating tubules of the peripheral ER [11]. Perhaps a particular stoichiometry and ordering of caveolar coat subunits allows inducing curvature in one or in two dimensions, while reticulon complexes can only induce curvature in one dimension. Structural information of these polymers is required to address these questions. The types of shapes that reggie/flotillin proteins can form are not known.

4. Cyclic, regulated coat assembly and disassembly

If a cyclic assembly and disassembly reaction exists for this type of coats, it must be principally different from classical coats that are built from cytosolic subunits. For reticulons and reggie/flotillin proteins information on cyclic assembly and disassembly is currently very limited. For caveolar coats, and in particular Cav1, there is evidence for such cycling (Fig. 3). An important difference with classical coats is that a cycle of Cav1 polymer assembly and disassembly is not necessarily connected to a single membrane trafficking step between two compartments. Pre-existing caveolar vesicles can dock on, fuse with and leave from target membranes in a kiss-and-run manner without fully disassembling their coat [27]. When specific ligands in these vesicles are either captured or released during the kiss-and-run cycle, for instance upon receiving a specific cue in the respective compartment, directional transport and sorting can still be achieved.

Nevertheless, there is some evidence that suggests that the caveolar coat may undergo triggered assembly and disassembly [18,28] during biosynthesis of the caveolar coat, and when caveolin is targeted to lipid droplets. In addition, as mentioned before, some experiments suggest that caveolin polymers have higher affinity for cholesterol than caveolin monomers or smaller oligomers [20]. Combined, this limited evidence suggests an intriguing, although highly speculative model of coat cycling, which is possibly linked to cholesterol sensing and/or transport. The model does not incorporate the possibility that caveolins can also be cytosolic, based on one report that a dominant-negative mutant of dynamin, which is involved in vesicular transport, may perturb this cycle [20]. However, other models that take non-vesicular modes of caveolin transport into the equation may certainly be valid, and extensive experimentation is required to test other models.

A phospho-mimetic mutant of Cav1 at serine80 (S80E) is not able to reach the cell surface [29], does not partition into detergent-resistant membranes (DRMs, a crude method to study potential partitioning into lipid rafts), and has lower affinity for cholesterol than wildtype Cav1 [30]. A phosphodeletion mutant of serine80 (S80A) however has a high affinity for cholesterol, comparable to wildtype Cav1. This suggests the existence of a phosphatase that dephosphorylates Cav1 at serine80 to allow coat assembly, leading to an increased binding of cholesterol, and partitioning into DRMs. Full coats are however not formed in the ER, but somewhere in the ERGIC or Golgi complex [10,14,33], indicating that S80 is immediately phosphorylated on the ER membrane upon synthesis of Cav1. At the Golgi complex, a phosphatase may be encountered, which could bind to and/or is activated by cholesterol or sphingolipids, which dephosphorylates S80 and thereby initiates cooperative coat assembly. One may speculate that the serine/threonine phosphatase PP2A is involved, as it can form a complex with oxysterol-binding protein (OSBP) and cholesterol [34], and with a Golgi complex-localized regulatory subunit (B56gamma) [35]. In addition, PP2A has been implicated previously in membrane trafficking from the Golgi complex [36] and in Golgi complex fragmentation [37]. Again, extensive experimentation will be required to test this hypothesis.

The reverse of the above (i.e. coat disassembly, by depolymerization of cav1 polymers into smaller oligomers) may happen when polymerized caveolar coats encounter an active kinase that phosphorylates Cav1 at S80. This could be mediated by the same kinase that phosphorylates newly synthesized Cav1 on the ER membrane, and might regulate caveolar coat disassembly when caveolar vesicles are targeted to the ER or to ER-derived lipid droplets [28]. Because polymerized Cav1 has a higher affinity for cholesterol than Cav1 as monomers or small oligomers [20], the speculative model described above provides one possible mechanism by which caveolar vesicles could mediate directional cholesterol trafficking to lipid droplets. A polymerized coat sequesters cholesterol to at least a 1:1 molar ratio with Cav1 molecules (100–200 per caveolar vesicle), but probably more because the caveolar coat forms a lipid raft microenvironment in which more cholesterol molecules, together with sphingolipids will partition. When this coat disassembles and Cav1's affinity for cholesterol reduces, at least part of the cholesterol molecules will be released into the surrounding lipid environment. On the surface of lipid droplets, this may be facilitated by cholesterol esterification, as cholesteryl esters do not bind to Cav1, and partition into the neutral lipid interior of lipid droplets. In addition, it may be that the lipid raft microenvironment is lost, leading to even additional release of cholesterol molecules. And, because the single leaflet that surrounds lipid droplets are in regulated continuity with the outer leaflet of the ER membrane, the disassembled caveolar coat subunits can be recycled via the biosynthetic route for a new round of transport. Since the S80A mutant of Cav1 does not cause newly synthesized Cav1 to accumulate in the ER (our unpublished observations), S80 phosphorylation

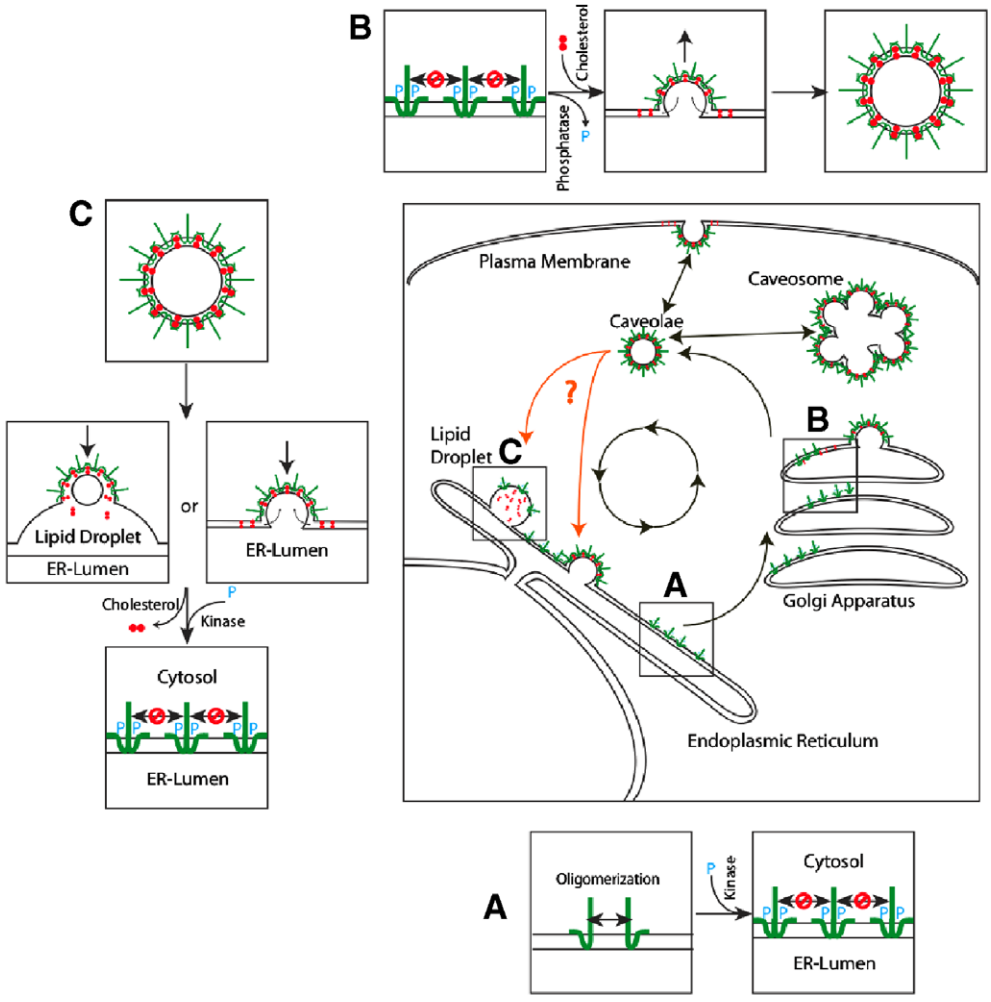

Fig. 3. Suggested cycle of caveolar coat assembly and disassembly. The speculative model presented in this figure relies on three key steps. (A) The first step occurs directly after synthesis of new Cav1 molecules, or after the completion of one cycle. An unknown serine/threonine kinase phosphorylates Cav1 at S80, which prevents it from forming larger polymers. For newly synthesized Cav1 this is suggested to occur on the ER membrane. After the completion of one cycle it may occur on the surface of a lipid droplet, which might be regarded as an extension of the ER, or on the ER itself. Alternatively, it may also occur at other subcellular locations, which are not included in this model. (B) Presumably upon arrival at the Golgi complex, Cav1 is dephosphorylated at S80 by an unknown serine/threonine phosphatase, which now allows Cav1 polymerization, which results in the sequestration of cholesterol into the polymeric complex, and finishes with the formation of a caveolar vesicle. (C) At some point during the trafficking of caveolar vesicles (which may have come from the cell surface, intracellular endosomal compartments, or even directly from the Golgi complex) they are targeted to a compartment where they encounter an unknown serine/threonine kinase that phosphorylates Cav1 at S80. In this model, it is suggested that this occurs on lipid droplets and/or the ER, but other locations are certainly also possible. This would result in disassembly of the cav1 polymers into smaller oligomers, leading to a reduced affinity for cholesterol and reduced lipid raft formation capacity. Lipids might be released into the surrounding bilayer, or in the case of lipid droplets, deposited onto the single leaflet surface and droplet interior. The kinase in (A) might well be the same as in (C), especially if these reactions occur on the same organelle, like the ER or ER-associated organelles. This establishes a closed cycle, which supports cyclic lipid sequestration at the location where the caveolar coat is assembled, and lipid release where the coat is disassembled.

seems not to function as an ER export signal, but rather as a signal to initiate coat disassembly or to prevent unwanted assembly, perhaps to guarantee that cholesterol is sequestered and released at the right place. In adipocytes, where the surface is literally covered with caveolae and Cav1 expression is very high, vectorial transport of a large number of cholesterol molecules could be achieved in this way. However, it must be stressed that the above model is highly speculative. It would for instance imply that caveolar vesicles would be able to hemi-fuse with lipid droplets, for which there is currently no evidence at all. In addition, after depolymerization of the caveolar coat and release of cholesterol, the hemi-fused vesicle must somehow be able to pinch off from the lipid droplet again, or fuse completely with the ER membrane if lipid droplets were in continuity with that organelle. All these speculations remain to be tested.

Although it is likely that the subcellular locations and the particular kinases and phosphatases in the above-described model will be different for reggie/flotillin proteins and reticulons, the principle itself might well be similar. Indeed, all proteins have putative phosphorylation sites close to the membrane, and the possibility to oligomerize, which suggests a general phosphorylation-dependent regulation of cooperative assembly and lipid binding. For reggie/flotillins and reticulons, it is however not known to which lipids they have high affinity, dependent on polymerization state.

5. Diseases related to this type of membrane scaffolds

As is often the case with diseases caused by perturbations of membrane trafficking systems, the symptoms are difficult to trace back to the underlying molecular machinery. The reason is that the vesicles formed by membrane scaffolds sequester and transport many signaling molecules, and in this way participate in maintaining the spatial and temporal network of signal processing in mammalian cells. A perturbation of one type of scaffold does often not lead to a complete block of a certain trafficking and/or signaling route, but to an altered equilibrium of the trafficking and signaling networks in which it participates. To be able to predict these changes requires a systems approach to membrane trafficking in mammalian cells, which first careful steps are being taken only now [31,32].

Nevertheless, some clinical symptoms suggest being explainable directly by the malfunctioning of membrane scaffolds, but this is highly speculative. For caveolar coats, explainable clinical symptoms may be those caused by mutations in Cav3 linked to muscular dystrophy. This may be manifested through the improper generation and/or maintenance of T-tubules in striated muscle cells. In addition, levels of Cav1 expression have been linked to obesity, which might be explained by an improper cholesterol and lipid transport to lipid droplets in adipocytes [28]. For reggie/flottilin proteins, links with protein aggregate-induced neurodegenerative diseases such as Alzheimer's disease and prion diseases have been described [22]. Explanation is not straightforward, but may be caused by a malfunctioning of reggie/flottilin-dependent clustering of the aggregate-forming membrane-associated proteins, according to the hypothetical models presented in this review. This could be occurring in neuronal cells, but also in immune cells (especially for prions) that transport aggregates of misfolded proteins around the body. Indeed, reggie/flottilin proteins are highly abundant in both cell types [22]. Reticulons have been implicated in neuronal regeneration and also in neurodegenerative diseases [38,39]. This might be explained by a recent finding that the ER expands very long peripheral tubules into neurite outgrowths that seem to functionally resemble smooth ER [40]. Considering that reticulons are important for ER tubule formation, neuronal disorders might be explained through inappropriate assembly of ER tubules in neuritis. This is however highly speculative, since the role of peripheral ER tubules in neuronal cell functioning is currently not understood.

6. Outlook

While the wealth of, sometimes contradictory, information on caveolins asks for structural studies that can clarify remaining uncertainties, the situation for reggie/flotillin proteins and reticulons is different. Classical biochemical analysis is needed to study scaffold assembly, oligomerization, polymerization, phosphorylation/dephosphorylation and lipid binding. According to the hypothetical framework provided here, rapid progress will hopefully be booked. It will be very interesting to see if these protein scaffolds indeed behave according to similar principles. For a full structural understanding, comparable to other membrane coats, we will require methods that can reveal the structure of large protein complexes within a membrane at atomic resolution, and develop in vitro systems that can study assembly and disassembly of these coats within the plane of lipid bilayers. We predict that these coats are significant determinants of organelle shapes, and a better understanding of their molecular assembly and disassembly mechanisms will allow us to formulate models on how particular organelle shapes are created.

Another important aspect is to integrate these scaffolds into the emerging complex network of membrane trafficking. Do they interact with classical membrane trafficking machinery such as SNAREs, are they controlled by RabGTPases, and how are they capable of ligand sequestration and release? Some of the scaffolds (caveolins, reggie/flotillins) appear to constitute coats of emerging membrane trafficking routes that are independent of classical coats. Perhaps several, currently unknown membrane trafficking routes exist, based on the number of proteins that can form this type of scaffolds. How do these routes integrate with other routes, and how do they together form a complex network in space and time. Perhaps, the 'classical' coats determine only a modest fraction of all existing membrane trafficking mechanisms and routes in mammalian cells. An integrated understanding of membrane trafficking will require a custom-tailed systems approach. In part, this can build on emerging systems approaches in cellular signaling, but will also require the development of new physical and chemical concepts, in order to describe reactions that, on a small and local scale, take place in two dimensions (lipid bilayers), but whose integrated action determines the three-dimensional surface of complex organelles (e.g. tubules, ribbons, grapes, reticuli) with dynamic shapes and changing locations.

Finally, it will be crucial to elucidate the underlying regulatory network of coat assembly and disassembly, and to link this to cellular physiology. Which are the kinases and phosphatases involved, how are they regulated, and in which signaling networks do these participate? Are the molecular (dis)assembly mechanisms of these scaffolds linked to systems that sense specific lipids, such as cholesterol? This may reveal how particular cellular needs regulate and control the shaping of organelles, and how particular organelle shapes can contribute to the cellular response. Besides a better understanding of the basic mechanisms of organelle assembly in an 'average' mammalian cell, such insights may also contribute to understanding the process of ER expansion in maturing B lymphocytes, ER-mediated phagocytosis in macrophages, ER tubulation in developing neurons, tubulation of late endosomes in maturing dendritic cells, the expansion of grape-like caveolar assemblies and their lipid-stimulated internalization to lipid droplets in differentiating adipocytes, etc.

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