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MINIREVIEW

Mosaic Organization of the Endocytic Pathway

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In the era of genomic and proteomic research the biochemical composition of intracellular organelles becomes deciphered very rapidly. However, we still largely do not know how single components, proteins and lipids, are organized into higher order structures. By shifting the research focus toward the analysis of multicomponent complexes rather then individual molecules, it is becoming clear that proteins and lipids building organelle membranes are not evenly distributed throughout the bilayers but are able to segregate and form organized domains. Such compartmentalization ensures a high efficiency of various processes taking place simultaneously and warrants organelle integrity. Membrane domains can be considered modular building blocks and an organelle can be viewed as an unique and dynamic combination of various domains, coupled together spatially and functionally. Here we review some general mechanisms which are responsible for the formation of organized membrane domains. We provide examples of such structures present in the endocytic pathway, including lipid rafts, caveolae, and Rab-domains. Among molecules acting as domain organizers, we emphasize in particular the role of lipids, especially phosphoinositides, and Rab proteins. We further present a novel mechanism whereby different membrane domains can communicate between each other via divalent Rab effectors and discuss the implications of the domain concept for the structure–function relationship and biogenesis of endocytic organelles.

OVERVIEW OF THE ENDOCYTIC PATHWAY

The endosomal system represents an interconnected and dynamic network of organelles which differ in their biochemical composition and localization within the cell. Endocytic organelles exhibit a complex morphological organization in the form of membrane vacuoles, cisternae, tubules, and multilamellar or multivesicular bodies. Molecules internalized at the plasma membrane by means of clathrin-dependent or clathrin-independent endocytosis reach early endosomes from where they are either sorted toward late endosomes and lysosomes for degradation or recycled to the plasma membrane passing through pericentriolar recycling endosomes [1]. In addition, early and late endosomes exchange cargo with the trans-Golgi network (TGN). The complexity of these transport pathways implies that each endocytic compartment must possess specific molecular machineries that enable fusion of different incoming vesicles as well as formation of distinct transport carriers (such as vesicles, tubules, multivesicular or multilamellar bodies) destined to various organelles. Furthermore, dynamic interactions of the endocytic compartments with the cytoskeleton enable the motility of transport vesicles and the positioning of organelles within the cell and likely influence organelle shape [2].

Another function of the endosomal system is to regulate the cellular response to signaling molecules. Recent data demonstrate that endosomes play a role not only in down-regulation of ligand–receptor complexes but also provide a platform for the interaction between signaling molecules [3]. The action of resident enzymes and membrane transporters (e.g., lumen-acidifying proton pumps) enables a spatial and temporal control over the dissociation of ligand–receptor complexes and cargo degradation by progressively lowering the lumenal pH along the endocytic pathway.

It is evident from this description that endocytic organelles have to perform different sets of functions but they also have to dynamically connect with each other. It is difficult in this respect to imagine that the various molecules performing these functions (e.g., Rab proteins, SNAREs, membrane-tethering factors, signaling molecules, microtubule motors) would be randomly distributed and diffuse freely on the surface of the organelle membranes. An alternative possibility is that these molecules self-assemble within biochemically defined membrane territories. The advantage of such organization is that organelles would maintain their structural and functional integrity in time, despite continuous membrane transactions. Furthermore, this view is in agreement with the complex three-dimensional organization of endocytic organelles

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which implies the separation of functional components within distinct membrane subcompartments. In the next section we summarize some emerging principles underlying the segregation of proteins and lipids within defined membrane areas.

MECHANISMS OF MEMBRANE DOMAIN FORMATION

In the classical model of the fluid mosaic, lipids were not recognized to play a significant role in the formation of membrane domains [4]. More recently, however, several lines of evidence have drawn attention to the interactions between lipids, which can lead to phase separations within bilayers. This phenomenon was originally discovered by studies on the behaviour of lipids in artificial systems but has been subsequently observed in biological membranes under physiological conditions. Lipid rafts, for example, are created primarily by the lateral interactions between sphingolipids and cholesterol leading to the formation of liquid-ordered phases within the more fluid liquid-disordered bilayer [5]. These highly dynamic structures are found at the extracellular leaflet of the plasma membrane but they are also thought to circulate between this compartment, the early and recycling endosomes, and the TGN (Fig. 1). The specific lipid enrichment in rafts favors partitioning of some classes of proteins such as GPI-anchored or doubly acylated proteins [5].

Certain integral membrane proteins can additionally stabilize lipid rafts resulting in the formation of morphologically recognizable stable structures such as caveolae [6]. The major structural component of these flask-shaped cell surface invaginations are caveolins (caveolin-1 and -2), integral membrane proteins capable of forming oligomers and binding cholesterol. Although originally proposed to function in endocytosis, caveolae seem to play an important role in signal transduction and cell proliferation. Caveolin-1 knockout in mice abolishes the formation of caveolae, underscoring the structural role of this family of proteins in maintaining these domains [7]. Moreover, caveolin-1 knockout animals show a severe dysfunction of vasculature resulting from the deregulation of the endothelial NO synthase which normally localizes to caveolae, as well as foci of uncontrolled proliferation in lungs. Similar to rafts, caveolae are highly enriched in cholesterol and sphingomyelin as well as ceramide and ganglioside GM1. They can be thus considered a specialized form of cholesterol-glycosphingolipid rafts which are additionally stabilized by structural proteins and regulate the activity of signal transduction molecules.

Various mechanisms of increasing complexity can determine the selective accumulation of proteins and lipids in a defined membrane region. These include oligomerization of integral membrane and peripheral proteins, localized production of lipids, and, as a consequence, localized membrane recruitment of cytosolic proteins binding these lipids. SNARE complexes represent an example of integral membrane proteins that cluster in a cholesterol-dependent manner by oligomerization and lateral interactions with other membrane components (such as synaphin; [8–10]). Another mechanism is based on the localized production of lipids in a certain membrane area (e.g., in Rab5 domain, see below). This can be achieved by local recruitment or activation of an appropriate lipid-modifying enzyme. There are several examples of cytosolic proteins that are recruited to defined membrane areas via specific lipid-binding domains. Several such domains have been described, especially for phosphoinositides (various PH domains binding PI(4)P, PI(4,5)P_2, PI(3,4)P_2 or PI(3,4,5)P_3; FYVE and PX domains binding PI(3)P; ENTH domain for PI(4,5)P_2) but also for other lipids (C1 domains binding diacylglycerol; C2 domains binding acidic phospholipids in a calcium-dependent manner; sterol-binding domains; for a recent review see [11]). In the next section we describe how the Rab5 GTPase, by using a combination of these mechanisms, is thought to generate a membrane domain of specific protein and lipid composition.

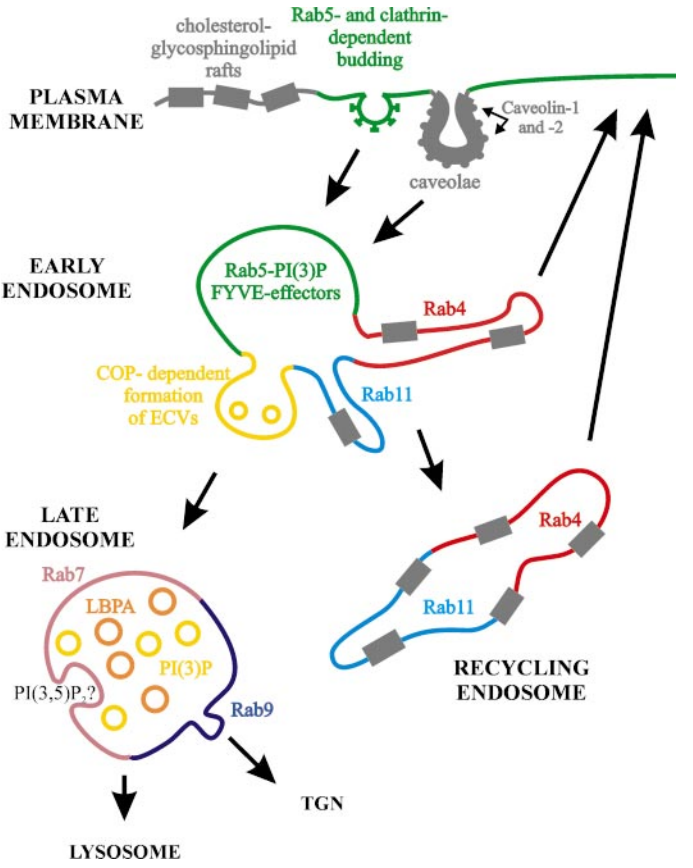

FIG. 1. Domain organization of the endocytic pathway. Abbreviations: ECV, endosomal carrier vesicles; LBPA, lysobisphosphatidic acid; TGN, trans-Golgi network; PI(3)P, phosphatidylinositol 3-phosphate; PI(3,5)P_2, phosphatidylinositol 3,5-diphosphate.

ORGANIZATION OF Rab DOMAINS

Based on the analysis of the downstream effectors of Rab5, we have recently proposed that endocytic Rab proteins can act as membrane domain organizers and can locally change their membrane environment [12–14]. In our view, the Rab5 domain on the endosome constitutes one of the structural and functional modules building this organelle. In its GTP-bound state Rab5 interacts with a variety of effectors, including hVPS34/p150, the type III phosphatidylinositol 3-kinase (PI(3)K) [12]. Localized production of PI(3)P enables the recruitment of Rab5 effectors which bind this lipid through their FYVE domains, such as the endosome-tethering protein EEA1 and Rabenosyn-5 [15, 16]. This illustrates an important example of cooperativity between Rab5 effectors, since the enzymatic activity of PI(3)K generates the specific lipid (PI(3)P) required for the binding of EEA1 and Rabenosyn-5 to endosomal membranes. Furthermore, the Rab5 effectors EEA1 and Rabaptin-5 together with the components of the SNARE machinery, syntaxin 13 and NSF, form dynamic oligomeric complexes on the endosomal membrane [17]. Due to the spatial coordination of Rab and SNARE machineries, the Rab5-PI(3)P domain seems to act primarily in the processes of endosome docking and fusion. However, Rab5 regulates also the motility of early endosomes along microtubules. The Rab5-dependent, minus-end directed motility of endosomes is also dependent on lipids, specifically PI(3)P [18]. Therefore, it is conceivable that the same Rab5-PI(3)P domain regulating fusion would also incorporate microtubule-associated and/or motor proteins which would further mediate endosome attachment to microtubules and motility.

The formation of a Rab5 domain depends on the cooperative action of several membrane constituents. On one hand, the localization of a Rab GTPase and its exchange factor(s) provides one of the first spatial and temporal cues for a domain formation. The molecular basis for the targeting of Rab proteins to their specific site of action remains still to be elucidated. In the case of Rab5 and possibly other Rab proteins, the binding of effectors and the presence of exchange factors in the effector complexes [19] determine a positive feedback loop that amplifies the recruitment and the activation of the GTPase. On the other hand, Rab5 effectors require multiple interacting partners on the membrane. For example, in addition to Rab5 EEA1 binds to PI(3)P and syntaxin 6 and 13 and forms oligomers on the endosome membrane with other Rab5 effectors and NSF [17, 20]. The combination of these interactions therefore imposes a restricted localization of EEA1 to a subcompartment of the early endosomes.

Another important element for the formation of the Rab5 domain is the targeting of the PI(3)K hVPS34 to the early endosome membrane. The mechanism is at present unclear, although it most likely depends on the regulatory subunit p150. Rab5 interacts directly with p150 [12] but this interaction may not be sufficient to determine the kinase localization and other interacting partners may be required. In fact, in compartments devoid of hVPS34, such as clathrin-coated vesicles (CCVs) or plasma membrane, the FYVE-finger containing Rab5 effectors are not recruited despite the presence of active Rab5 [21]. On the other hand, another Rab5 effector, type I PI(3)K, p110β/p85α, is selectively enriched on CCVs in comparison to the early endosomes [12]. Since hVPS34 and p110β/p85α produce different types of 3-phosphoinositides (PI(3)P versus PI(3,4)P_2 and PI(3,4,5)P_3, respectively), Rab5 may coordinate the production of distinct phosphoinositide species in a compartment-specific manner. Similar to Rab5 on CCVs and early endosomes, Rab1 has been proposed to interact with a different set of effectors on ER and cis-Golgi membranes [22], providing another example of a Rab protein regulating the directionality of transport through the differential recruitment of effector proteins. Moreover, there are different requirements for the accessory molecules catalyzing GTP exchange and hydrolysis on Rab5 in regulating constitutive and ligand-induced endocytosis on the plasma membrane. The newly identified exchange factor for Rab5, RIN1, regulates the ligand-induced endocytosis of EGFR but not the constitutive internalization of transferrin [23]. Although Rab5-GAP, RN-tre, is necessary for both processes, the regulation of its activity in the case of EGFR internalization depends on the interacting protein Eps8 which is not required for transferrin endocytosis [24]. Cumulatively, these data underscore the idea that the final set of proteins interacting with Rab proteins in different cellular locations depends on the membrane context, providing an additional compartment-specific layer of regulation.

Rab DOMAINS AND OTHER MECHANISMS OF ENDOSOME MEMBRANE COMPARTMENTALIZATION

Rab Domains on Endosomes

The proposal that Rab proteins and their effectors can be restricted within specialized membrane domains received further support from the morphological analysis of the distribution of Rab proteins in early and recycling endosomes [13] (Fig. 1). Despite a significant degree of colocalization within the same organelle, Rab proteins are not intermixed on the plane of the membrane. Instead, each of them occupies a separate area on the membrane, forming domains that exhibit different pharmacological sensitivity and are in a dynamic equilibrium with each other. Early endosomes appear mainly composed of Rab5 and Rab4 domains, with a smaller proportion of Rab11-positive regions. In contrast, recycling endosomes are enriched in Rab11 and

Rab4 domains [13]. Several other Rab proteins have been localized to compartments of the early endocytic pathway, such as Rab15, Rab18, Rab20, Rab22, and Rab25 [14, 25]. It will be interesting to determine whether these proteins also exhibit a domain-specific localization. Late endosomes are characterized by the presence of Rab7 and Rab9 [25]. Rab7 regulates transport between early and late endosomes, whereas Rab9 governs the formation of carriers destined for the TGN. Remarkably, it has been recently shown that the Rab9 effector TIP47 can bind mannose 6-phosphate receptors (MPR) and its affinity for MPRs is increased by Rab9-GTP. On the basis of these observations TIP47 has been proposed to act as a cargo selection device to ensure the incorporation of Rab9-GTP and cargo into the vesicles generated from late endosomes [26]. Rab9 and its effectors may therefore constitute a distinct sorting platform for cargo destined to be recycled to the biosynthetic pathway, away from the degradative route to lysosomes.

The distribution of Rab domains is not stochastic and cargo progressing along the endocytic-recycling pathway traverses Rab5, Rab4, and Rab11 domains sequentially [13]. This implies a mechanism whereby the ability of two contiguous Rab domains to communicate can be tightly regulated. This regulation involves divalent Rab effector, such as Rabaptin-5 and Rabenosyn-5. Both proteins were originally identified as Rab5 effectors but were later shown to bind also the active form of Rab4 [27]. Upon overexpression, Rabenosyn-5 and Rabaptin-5 increase the association of Rab5- and Rab4-domains, and concomitantly, decrease the fraction of Rab4+Rab11 endosomal structures. Consequently, internalized cargo recycles more efficiently from early endosomes to the surface and accumulates less efficiently in perinuclear Rab11-positive recycling endosomes [27]. We propose that divalent Rab effectors are central components of the machinery that regulates the communication between contiguous Rab- domains (e.g., from Rab5- to Rab4-domains and from Rab4- to Rab11-domains) and thereby ensures the sequential transport of cargo from one intracellular compartment to another.

Role of Lipids in Membrane Shape

Given that several other molecular mechanisms could result in membrane compartmentalization, it will be interesting to see how these principles could be integrated with the activity of Rab proteins and their effectors to generate the three- dimensional organization, functional properties, and dynamics of endocytic organelles. These can be viewed as a unique and dynamic combination of various domains, coupled together spatially and functionally. Different biophysical properties of various domains (such as fluidity or bilayer curvature) likely contribute to determine the shape of an organelle. This aspect must be of a particular importance for the endocytic compartments, which exhibit a complicated vesiculo-tubular (early endosomes) or multivesicular/multilamellar (late endosomes) morphology. Indeed, experiments with fluorescent lipid analogues demonstrated a differential partitioning of lipids between the tubular and the vesicular parts of early endosomes, depending on their fatty acid chain properties [28]. Another interesting example of lipid compartmentalization is provided by the selective accumulation of lysobisphosphatidic acid (LBPA) in the internal membranes of late endosomes. These organelles are unique in their multivesicular and multilamellar morphology, with the inner involutions and vesicles constituting a distinct membrane domain from the outer organelle membrane. These internal membranes accumulate, in addition to LBPA, PI(3)P, long-chain saturated glycophospholipids as well as certain proteins absent from the limiting membrane [29, 30]. The exact mechanisms governing the inward membrane budding in the late endosomes remain unclear. However, in yeast vacuoles $PI(3,5)P_2$ was shown to be essential for sorting of membrane proteins into the intralumenal vesicles [31]. Further studies are required to unravel the mechanisms by which these lipids function in protein sorting and in the biogenesis of late endosomes.

Transient and localized changes of lipid composition must take place in membrane regions involved in budding and tubulation processes, resulting in an alteration of membrane curvature allowing the formation and subsequent fission of vesicles or tubules. Interestingly, phospholipase A2 activity, resulting in the production of inverted-cone shaped lysophospholipids, has been implicated in the tubulation of Golgi membranes [32]. Such mechanism could operate also in endosomes. Moreover, certain proteins, such as dynamin-1 and amphiphysin-1 [33], or peptides, such as amphipathic Hel 13-5 [34], have been shown *in vitro* to convert spherical liposomes into narrow tubules. The driving forces inducing a positive curvature of lipid bilayer and resulting in vesicle budding remain largely obscure but the local production of cone-shaped lipids such as phosphatidic acid promoting the negative curvature appears to be required for fission of synaptic vesicles [35]. Several types of vesicles and transport carriers are formed in the endocytic pathway, although the corresponding molecular mechanisms are still poorly understood. Clathrin-coated vesicles, distinct from those formed at the plasma membrane and at the TGN, bud from the tubular extensions of the early endosomes, likely participating in the recycling processes [36]. Interestingly, Rabaptin-5 has been found to interact specifically with γ-adaptin, a component of clathrin coat adaptor complex [37]. This suggests that Rab proteins and their effectors could also regulate budding processes at the early endosome. Although the localization

of bud sites with relation to Rab4 has not been directly addressed, it is interesting to envisage that Rab4 domains present in the tubular subcompartments of the early endosome could coordinate the formation of recycling vesicles. In contrast, this activity may be excluded from Rab5 domains in the vesicular part of the endosomes. Similar to the biosynthetic route, small GTPases of the ARF family seem to coordinate at least some of the endocytic budding events, such as COP-I-dependent formation of endosomal carrier vesicles (ECVs) at the early endosomes (Fig. 1) [38]. Like Rab proteins, ARF GTPases are capable of local membrane remodeling by activating the PI(4)P 5-kinases and phospholipase D [39]. Increased production of $PI(4,5)P_2$ by the PI(4)P 5-kinases can result in the recruitment of several PH domain-containing proteins involved in signaling at the plasma membrane and actin cytoskeleton rearrangements [40]. Identification of further ARF effectors should help in understanding more precisely the role of this class of proteins in the formation of membrane domains.

Phosphoinositides and Membrane Compartmentalization

Another class of molecules emerging as key players in achieving membrane compartmentalization are phosphoinositides. This class of lipids is particularly well suited to forming membrane domains since both their generation and turnover can be precisely regulated. A growing number of specific kinases and phosphatases involved in phosphoinositide metabolism have been identified [41, 42]. By their coordinated action phosphoinositides can be interconverted into several distinct species. Different phosphoinositide molecules are involved in a variety of protein–lipid interactions, mediated by specific lipid-binding motifs (see above, [11]). Furthermore, phosphoinositide metabolism can be spatially and temporally modulated by several factors, including signaling molecules in addition to Rab and ARF GTPases, resulting in a high degree of compartmentalization within a membrane bilayer. Indeed, PI(3)P is present in distinct patches on early endosomes as well as on internal vesicles of late endosomes [29]. A transient, localized, and coordinated production of $PI(3,4,5)P_3$ and $PI(4,5)P_2$ as well as diacylglycerol has been visualized at the sites of phagocytosis on the plasma membrane [43, 44]. Different phosphoinositide-modifying enzymes present in the neighboring domains could be functionally linked in a way that a lipid product made in one domain would be a substrate for an enzyme in another. In this manner coordinated steps of phosphoinositide metabolism would provide a basis for communication between membrane domains required for the completion of a membrane trafficking step. This could indeed be the case for the transport between early and late endosomes which are marked by the presence of PI(3)P and $PI(3,5)P_2$, respectively [29, 31, 45]. It has been previously reported that wortmannin inhibits transport from early to late endosomes, unraveling the importance of 3-phosphoinositides in this transport step [46]. It is possible to imagine that PI(3)P produced in Rab5 domains at the early endosomes would be subsequently converted to $PI(3,5)P_2$ at the level of late endosomes. Indeed, the PI(3)P 5-kinase (PIKfyve in mammals [47] and Fab1p in yeast [48]) is itself a FYVE finger protein. Therefore, the presence of the lipid substrate (PI(3)P) allows for an efficient recruitment of the enzyme that modifies it. Clearly, other specificity requirements have to be fulfilled to confine the $PI(3,5)P_2$ production to late endosomes.

Rab DOMAINS IN POLARIZED CELLS

We predict that Rab domains are also important in the complex endocytic circuits of polarized cells such as neurons and epithelial cells. Epithelial cells, for example, contain distinct early endosomal populations (basolateral and apical) which are in communication with each corresponding plasma membrane domain. By a process termed transcytosis, cargo can nevertheless be transported in an apical-to-basal and basal-to-apical direction [49]. Along these routes, cargo is sequentially transported to different endocytic compartments. Communication between the apical and the basolateral endocytic pathways has been orginally proposed to involve the apical recycling endosome. However, with the recent advances of light microscopy analysis and multicolor imaging techniques, the sorting of transcytosed cargo appears to be more complex and include several discrete compartments [49, 50]. It is possible that, similar to the organization of early endosomes and recycling endosomes in nonpolarized cells, these structures reflect different arrangements of Rab domains, which are in constant dynamic communication. Certain Rab proteins are specifically expressed in polarized cells, such as Rab17 in epithelial cells [51] or Rab3 family members in neurons or cells performing regulated secretion [52]. These Rab proteins may form novel domains and participate in cargo sorting and polarized transport events. Moreover, Rab domains in polarized cells may incorporate cell type-specific molecules, e.g., transcytotic cargo receptors in epithelial cells. By analogy to the epithelial cell-specific heterotetrameric adaptor complex AP-1B required for basolateral sorting of membrane proteins at the TGN [53], it is likely that epithelial-specific coat adaptors may exist to mediate the formation of different types of vesicles at the apical and basolateral endosomes and at recycling endosomes. Such interactions may involve also Rab effectors, by analogy to Rab9 and TIP47 [26]. Although certain Rab proteins (Rab11, 17, or 25) are present specifically on apical endosomes of epithelial cells or

apical dense tubules of kidney tubule epithelial cells (Rab18 and Rab20), others, such as Rab5, localize to both apical and basolateral structures [14, 25]. Again, Rab5 is seemingly capable of recruiting different sets of effectors depending on the compartment identity. EEA1 is localized to somatodendritic early endosomes but is absent from the axonal endosomes in neurons [54]. Conflicting data have been reported concerning the localization of EEA1 to the apical endosomes in MDCK cells [50, 54]. However, it remains possible that the Rab5-PI(3)P domain is predominantly formed basolaterally. Apical-specific Rab5 effectors are currently unknown but their identification will likely advance our knowledge of mechanisms establishing polarity at the organelle and cellular level.

CONCLUSIONS AND FUTURE DIRECTIONS

The coordination of numerous different processes within the endocytic system appears to be achieved by a high degree of spatial organization and compartmentalization of membrane bilayers. Several types of domains, such as cholesterol-glycosphingolipid rafts, caveolae, Rab, and phosphoinositide domains formed and maintained by a combination of molecular interactions, exist within the membrane of a single organelle. Since we have just begun to unravel the complexity of organelle organization, several important aspects need further investigation. It will be essential to continue mapping proteins and lipids and examine their subcellular localization. However, this will have to be accomplished at the level of organelle subdomains. Lipid-binding domains can further serve as a useful tool to assess the distribution of lipid species [29, 43, 44]. Consequently, the function of various proteins and lipids in determining the membrane curvature and compartmentalization needs to be mechanistically dissected in more details. Moreover, we still largely do not know how various machineries, for example, those regulated by Rab, Rho, and ARF GTPases, relate to each other and coordinate different functions in time and space. The question of how different membrane modules communicate with each other to build an intracellular compartment is crucial for our understanding of organelle biogenesis. To address this problem we need to elucidate the complex network of interactions between membrane components and develop new methods for visualizing and manipulating membrane domains.

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