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Review

Rafting through traffic: Membrane domains in cellular logistics

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ABSTRACT

The intricate and tightly regulated organization of eukaryotic cells into spatially and functionally distinct membrane-bound compartments is a defining feature of complex organisms. These compartments are defined by their lipid and protein compositions, with their limiting membrane as the functional interface to the rest of the cell. Thus, proper segregation of membrane proteins and lipids is necessary for the maintenance of organelle identity, and this segregation must be maintained despite extensive, rapid membrane exchange between compartments. Sorting processes of high efficiency and fidelity are required to avoid potentially deleterious mistargeting and maintain cellular function. Although much molecular machinery associated with membrane traffic (i.e. membrane budding/fusion/fission) has been characterized both structurally and biochemically, the mechanistic details underlying the tightly regulated distribution of membranes between subcellular locations remain to be elucidated. This review presents evidence for the role of ordered lateral membrane domains known as lipid rafts in both biosynthetic sorting in the late secretory pathway, as well as endocytosis and recycling to/from the plasma membrane. Although such evidence is extensive and the involvement of membrane domains in sorting is definitive, specific mechanistic details for raft-dependent sorting processes remain elusive.
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Contents

1. Introduction

The self-organization of cellular macromolecules into structurally and functionally distinct subcellular compartments is one of the most intriguing and complex questions in biology. Most eukaryotic cell organelles are delimited by a lipid and protein membrane, which separates the cytoplasm from a topologically disconnected aqueous lumen. Because these membranes are the interface between the organelles and the rest of the cell, their molecular identities (i.e. protein and lipid composition) play a key role in defining the function of a given compartment as a whole. Therefore, the efficient and accurate sorting of membrane molecules between organelles underlies much of subcellular organization.

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Recent decades have seen the discovery and characterization of the extensive proteinaceous machinery responsible for intracellular membrane transport. One general theme is that membranes are sorted and transported by small (i.e. less than 100 nm diameter) vesicular intermediates produced by the action of a "coat protein", which are actually large, multiprotein complexes. The classic examples of such "coatomers" are the COat Protein complexes (COPI and COPII) that mediate vesicle formation and transport between the endoplasmic reticulum (ER) and early Golgi, and the polymerized clathrin cages in the late secretory pathway and endocytosis. An alternative vesicle-producing apparatus – that does not utilize a coated intermediate – is the ESCRT machinery responsible for lumen-directed vesicle fission in endocytosis [1], virus budding [2], and membrane repair [3]. These machines are responsible for the generation and fission of transport vesicles, but all require additional factors for targeted, vectorial vesicle transport to the appropriate cellular compartment and fusion with the target membrane. These functions are served in part by the famous SNARE (Soluble N-ethylmaleimide-sensitive-factor Attachment protein REceptor) proteins [4], which determine where and when a given transport vesicle will fuse. In addition to this core machinery, hundreds of ancillary proteins regulate subcellular traffic, including the vital sorting function served by Adapter Proteins (AP1-4 [5]) to selectively recruit specific proteins into the transport carriers, and the Rab GTPases that act as "address labels" for the various organelles [6].

In contrast to the wealth of information available for subcellular *protein* sorting, a mechanistic understanding of how *lipids* are distributed in cells remains elusive. Organellar membranes have distinct lipid compositions (though clean, detailed data to this point are scant) that are likely required for their function and cannot be accounted for solely by enzymatic production/turnover [7–9]. However, how the cells achieve this steady-state heterogeneity despite active and rapid exchange of lipids between compartments remains unclear, in part because the rules for sorting lipids and proteins are quite different:

(1) lipids are not sorted absolutely, i.e. all membranes contain broadly similar lipid classes (e.g. phosphatidylcholine), but the specific molecular identities and, more importantly, relative concentrations of the lipids vary between compartments
(2) lipids are not covalently linked into membranes, but are organized by weaker intermolecular interactions which give rise to the fluid matrix of the bilayer
(3) whereas proteins are often sorted by specific intermolecular coupling between a cargo protein and the coatomer, lipids are generally too numerous and too small for such one-by-one selection (although there are important exceptions [10–12]).

Because of these unique features, specific mechanisms are required for intracellular lipid sorting. Membrane lipid monomer transport through the cytoplasm does not occur on cell-relevant time scales (though cholesterol transport may be an exception [13]), because despite lipids not being covalently incorporated into the bilayer, the entropic penalty for hydrating their aliphatic regions makes spontaneous diffusion of lipid monomers into the cytoplasm extremely unfavorable [14]. In some cases, carrier proteins facilitate monomeric lipid transport by providing a hydrophobic cavity [10–12]. Additionally, physical contact sites between the ER and other organellar membranes may provide channels for direct lipid transfer between organelles [15]. However, these mechanisms are insufficient for the whole lipidome sorting observed in subcellular organelles; thus, membrane lipids, like proteins, are generally trafficked by vesicular intermediates, begging the question of how such vesicles "choose" the lipids destined for a particular compartment.

Lateral membrane domains of distinct compositions provide an ideal platform for lipid sorting. The archetype of such domains are membrane rafts, defined as lipid and protein assemblies whose formation is dependent on the preferential interactions between specific lipids (sterols, glycosphingolipids, and saturated lipids) that drive the formation of a liquid-ordered membrane state that coexists with a relatively disordered state as fluid, lateral domains [16]. It is important to emphasize that the conception of rafts as long-lived, large, stable domains is imprecise and probably incorrect — rafts in the PM are believed to be small and highly dynamic [17], and there remains active controversy about the physical properties, compositions, and mechanistic consequences of raft domains. However, several recent observations – most notably, microscopically observable liquid–liquid phase separation in biological membranes [18–22] – have provided strong evidence for their existence and biological relevance. Moreover, the proposed size of such domains (tens to hundreds of nm [17]) and their capacity to sequester both lipids and proteins provide rafts with ideal features for acting as sorting mechanisms in membrane trafficking.

This review focuses on evidence supporting the central role of raft domains in subcellular membrane sorting. We start with a discussion of the genesis of the raft hypothesis to explain the distinct membrane compositions of apical and basolateral plasma membrane (PM) domains in polarized epithelia, and expand on the general utilization of these domains in secretory sorting and trafficking to the PM in both polarized and non-polarized cells. Next, we summarize the robust literature describing raft domains in endocytosis and sorting in the endocytic system, before briefly reviewing the scant information on the protein machinery that supports raft-mediated trafficking. As with much of the raft field, there remain more questions than answers. However, this review is intended to provide a primer to the topic, while highlighting the abundant evidence in support for the hypothesis that rafts are a key mediator of the specific membrane compositions of several subcellular organelles.

2. Rafts in secretory traffic

The Golgi Apparatus (GA) is the intermediate between the site of most lipid synthesis (ER) and other membrane-bound organelles, thus making it a major membrane sorting station. More specifically, while the "cis" portions of the GA (i.e. those associated with bi-directional membrane exchange with the ER) are responsible for the post-translational modification and refinement of membrane proteins, the most "trans" cisterna of the organelle, termed the trans-Golgi network (TGN), is the site of selection for export of membrane (and luminal) components via newly assembled transport carriers destined for their final cellular location (Fig. 1). In some cases (e.g. the mannose-6-phosphate receptor for lysosomal delivery [23]), the sorting determinants are simple and clear. However, for most membrane lipids and proteins, the mechanisms of distribution remain unresolved. In epithelial cells, this problem is further complicated by the existence of a highly specialized PM domain, the apical PM, coexisting contiguously with a basolateral PM. These domains must be compositionally distinct because their functional requirements are very different: the apical PM must often provide a robust barrier between the cell and a harsh and inhospitable environment (e.g. gut or kidney lumen), whereas the basolateral is responsible for exchanging information and nutrients with the rest of the body.

2.1. Genesis of the raft hypothesis

An early observation in epithelial cell biology was that not only the protein, but also the lipid composition of the apical PM is highly differentiated, most notable in the enrichment of glycosylated sphingolipids (GSLs) and cholesterol, and relative depletion of glycerophospholipids (see studies cited in Ref. [24]). The sorting of both glycosylated proteins [25] and lipids [26] to the apical surface seemed to occur simultaneously in the TGN, suggesting a membrane-mediated mode of action, which was confirmed by the isolation of distinct vesicle subtypes originating at the TGN and containing either apical or basolateral cargo [27]. To explain these observations, a model based on the known propensity for

Table 1
Involvement of raft domains in various endocytic pathways and their cargo.

Endocytic pathway	Clathrin coated pits	Caveolae	CLIC/GEEC	Flotillin	Arf6 dependent
Raft involvement?	No (a)	Yes (b,c) Cav1 binds cholesterol	Yes (d,e) GPI-AP found in lipid rafts	Likely	Unclear (f)
Cholesterol dependence	Unclear (g) Affected by cholesterol depletion, but might be due to cellular toxicity	Yes (b,c)	Yes (d,e)	Not known	Yes (h)
Implicated proteins	Clathrin (i), epsin (j), intersectin (k), dynamin (l), Arf6 (m), PKC (n), Rac1 (o), cdc42 (p)d, RhoA (o)	Caveolins (b,c), PKC (q), SRC (q), cdc42 (r), intersectin (r), dynamin (s)	RhoA (t), GRAF (u), cdc42 (d), Arf1 (v), cortactin (w), Arf6 (v)	Flotillin (x)	Arf6 (h)
Known cargo	GPCR (y) transferrin receptor (j) anthrax toxin (z), cadherin (aa), LDL (bb), influenza (l)	GP60 (cc), CTX (ee), SV40 (ff), cadherin (aa), GPI-AP (ff), LacCer (gg), IL2 (hh)	IL2, SV40 (e), GPI-AP (d,e)	CD59, proteoglycans (ii)	MHC I (h), CD59

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sphingolipids to self-associate via their intramembrane hydrogen bonds [28] posited that membrane lipids and proteins separate into microdomains representing the composition of their eventual target compartments (i.e. apical and basolateral PM) [24]. A critical validation of this model came with the observation that the "apically-directed" microdomains could be specifically isolated by their relative resistance to non-ionic detergents (usually Triton X-100), and that they gain insolubility late in the secretory pathway, i.e. in the TGN [29,30]. Components of these detergent-resistant membranes (DRMs) were similarly observed to be involved in PM-directed traffic in non-polarized cells [31], suggesting that the glycosphingolipid-enriched entities are a general feature of TGN-to-PM sorting (Fig. 1). The physicochemical mechanism by which

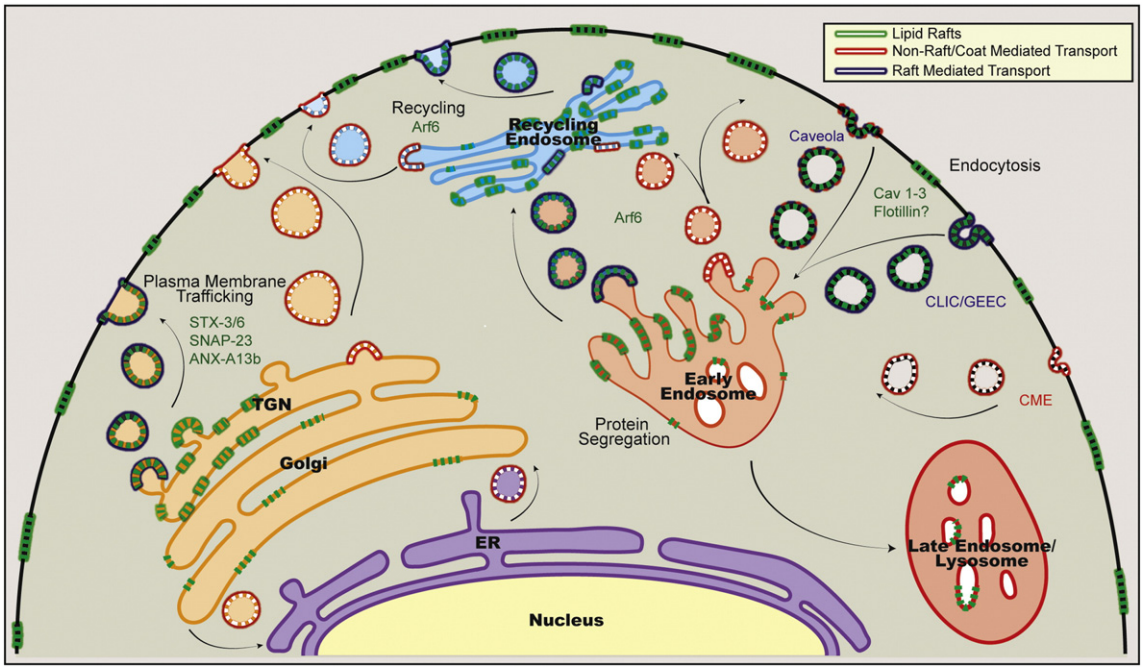

Fig. 1. Involvement of raft domains in membrane traffic. Lateral membrane domains aid in sorting of protein and lipid components between the membranes of subcellular compartments. Membrane rafts (green striped regions) are likely present in the latter stages of the secretory pathway (i.e. the TGN and PM) and early stage of the endosomal pathway (early and recycling endosomes). Rafts recruit components for coordinated exit from a source compartment and traffic to a donor compartment via a raft-enriched vesicular carrier (blue shading around membranes). Such vectorial raft transport includes TGN-to-PM sorting, specific endocytosis at the PM, and recycling from the endosomal system in the EE and RE. The raft pathway coexists with a number of coat/adapter-mediated pathways (red shading). The protein machinery mediating raft trafficking is incompletely characterized, but includes caveolins and flotillins, lectins (e.g. Gal9), the small GTPase Arf6, Annexin 13b, and specific SNARE proteins (Syntaxin 3/6 and SNAP-23).

cholesterol-driven, sphingolipid-rich domains mediate lateral membrane sorting coalesced from the convergence of cell biological studies with a wealth of biophysical observations in synthetic model membranes. In these (incl. Langmuir monolayers [32], supported lipid bilayers [33], and Giant Unilamellar Vesicles [34]), large-scale equilibrium separation of a cholesterol-dependent liquid ordered domain – the synthetic analog of in vivo rafts – is observable across a physiologically appropriate range of lipid mixtures.

The model combining these observations to explain post-Golgi sorting by lateral, lipid-driven membrane domains is known as the lipid raft hypothesis [35]. Because much of the data in support of this hypothesis came from DRM isolations, this preparation became synonymous with lipid rafts. However, it is vital to emphasize the crucial (previously made [36–40]) point that DRMs should not be equated with in vivo rafts, or any structure existing in the cell prior to detergent treatment. Cold detergent extraction is unlikely to reflect the specific underlying reality of a live cell, and the interactions between detergents and native membranes are complex, unpredictable, and difficult to control, making cellular extrapolations from DRM experiments inherently speculative. Although recent experiments in intact isolated membranes have confirmed some of the DRM-based inferences into raft composition and function [18,20], the volume of such studies pales in comparison to those relying on detergent resistance. Because it would be obtrusive to point out the artifactual nature of DRMs for every study referenced here, the rest of the text will conform to the inferred relationship between inclusion in DRMs and in vivo raft association; however, the reader is cautioned to take a skeptical view.

2.2. Polarized sorting in epithelial cells

The seminal observations in epithelial cells touched off a flurry of investigations that defined the lipid and protein compositions of raft subdomains and the mechanisms by which they mediate sorting and trafficking. Some of the earliest proteins found to be associated with the DRMs – and by implication, rafts – were membrane proteins anchored to the lumenal side of the PM by glycophosphatidylinositol (GPI-AP) [29,30,41]. These observations suggested a solution to the riddle of how to sort proteins without cytosolic features that can be recognized by canonical sorting machinery. Both glycosylation and lipidation were also identified as raft-mediated apical PM sorting determinants for transmembrane proteins (TMPs), including those with N-glycosylations [42,43], O-glycosylations [44], and S-acylations (i.e. palmitoylation) (reviewed in Ref. [45]). A key finding was that the efficient apical delivery of raft-associated proteins was interrupted by inhibition of either cholesterol [46] or sphingolipid [47] synthesis. Recently, these in vitro inferences were confirmed by an exciting in vivo study, which showed that organogenesis in *Caenorhabditis elegans* – which requires assembly of a polarized epithelium – was critically undermined by perturbation of in situ GSL biosynthesis [48].

A number of exceptions challenge the central role of rafts in polarized PM sorting in simple epithelia (reviewed in Ref. [49]). For example, not all GPI-APs are apically directed, even when these are associated with DRMs [43,50]. Further, raft lipid perturbations do not always lead to mis-sorting, even when they induce disruption of DRMs [51]. To explain these discrepancies, the model was amended to include the stabilization/coalescence of small and dynamic raft domains into practically useful platforms by protein-mediated clustering of raft components. The key evidence for this hypothesis was the observation that apically directed GPI-APs clustered into large, multimolecular, cholesterol-dependent aggregates, whereas basolateral GPI-APs remained as monomers [50]. These clusters, which dramatically inhibit the lateral mobility of their constituent proteins, seem to disperse upon arrival at the PM [52], into homodimeric "unit rafts" [53] or homoclusters [54]. At the moment, it is unclear whether clustering of raft components stabilizes the domains themselves [33,55], or instead increases the affinity of proteins for pre-existing domains (as has been shown in model membranes [20,56]). It is most probable that both effects synergize to underlie the important function served by clustering.

Intriguingly, these raft-mediated trafficking mechanisms also seem to be involved in more complex epithelial tissues like hepatocytes, which rely on basolateral-to-apical transcytosis for differentiation of PM domains. In these, clustering of GPI-anchored GFP mediated efficient apical PM transcytosis, with an unclustered point mutant of the same protein accumulating in an endosomal compartment [57]. Also, like in simple epithelia, the flow of cargo to the apical PM can be interrupted by depletion of raft lipids (i.e. cholesterol and sphingolipids) [58].

Because stabilized raft domains appear to be a major mediator of secretory trafficking to the apical PM, it stands to reason that this membrane would be a raft-enriched compartment. Consistently, the apical PM was shown to be a "percolating" raft domain, i.e. one in which the raft phase is the continuum interrupted by non-raft inclusions [59]. This contrasts with non-polarized cells, whose PMs are believed to present a continuous non-raft state with dissolved raft domains [60,61]. However, it is important to emphasize that the relative fraction of raft versus non-raft phases in any membrane is not well established, and arguments for a majority raft phase have recently been forwarded in cellular biophysical literature [21,62–64].

The site of separation for apically directed raft components has traditionally been understood to be the TGN. Dramatic images of apical cargo separating from basolateral/non-raft cargo confirmed separation of these membranes in the Golgi prior to generation of a carrier vesicle [65]. However, it is also possible that the appearance of raft lipid enriched domains happens earlier in the biosynthetic pathway. The cholesterol concentration in the ER is low (<5 mol% [7]) and almost certainly insufficient to support cholesterol-mediated domains; however, the relative fraction of cholesterol increases progressively through the Golgi stacks [66] peaking in the PM [67], which can be as high as 40–50 mol% cholesterol [68]. Similarly, glycosylation of sphingolipids occurs throughout the GA. Together, these effects lead to a thickening of the membrane in the late secretory pathway [69], potentially reflective of increased raft-like membrane content. It is difficult to estimate the minimum (or maximum) cholesterol and sphingolipid fractions at which robust separation of raft domains would be expected, although model membrane studies can provide a guide. In these, liquid–liquid domains can be observed with as low as 10% cholesterol [21], suggesting that domains may exist and mediate sorting also in the cis Golgi regions, and maybe even as early as the ER [70,71]. Fully consistent with this proposal, a recent combination of modeling and experimental observations suggested that a two-phase membrane system best explains Golgi maturation [72].

2.3. Raft-mediated sorting in non-epithelial cells

Although most of the attention in membrane-mediated subcellular traffic has been paid to polarized sorting in epithelial cells, non-polarized cells also require accurate and efficient membrane distribution, and despite the relative (compared to the apical PM) paucity of glycosphingolipids in the PMs of non-polarized cells. For example, as in epithelial cells, perturbation of sphingolipid organization inhibits transport carrier formation in the GA in HeLa cells, leading to defective secretion [73]. Similarly, cholesterol is required for efficient PM localization of proteins that localize to the apical PM in epithelia (e.g. influenza hemagglutinin [46] and GPI-APs [74]). Although non-polarized cells do not obviously require a mechanism for spatially distinct PM delivery, there remain many advantages to having parallel routes to the cell surface. Consistently, two such routes have been observed in both BHK and CHO cells [75], with one mediated by raft domains. Moreover, non-polarized cells clearly have mechanisms to specifically sort cargo that would be apically-directed in polarized cells [75,76]; however, the eventual destination of such cargo seems to depend on cell and cargo type.

The raft-mediated GA-to-PM sorting track in non-epithelial cells was directly demonstrated in a series of methodological *tour de force* experiments in the budding yeast, *Saccharomyces cerevisiae* [77–80]. By a combination of yeast genetics, sophisticated immunoisolation of intact subcellular compartments, and quantitative shotgun mass spectrometry, it was demonstrated that lipid composition of the ~100 nm trafficking intermediates leaving the TGN for the PM was wholly distinct from the source compartment. These vesicles were clearly enriched in sterol and glycosphingolipids, in addition to being significantly more ordered [78]. With regard to almost all lipid categories and features, these carriers proved to be intermediate between their source and destination compartments (i.e. the TGN and PM), definitively confirming lipid-based secretory sorting [77].

2.4. Sorting in neuronal plasma membrane analogous to epithelial cells

Neuronal PMs represent a curious hybrid between the sharply defined PM domains of polarized epithelia and the undifferentiated membrane of non-polarized cells. Despite the absence of the lipid-impermeable tight junctions that act as diffusion barriers to maintain the extreme lipid compositional heterogeneity that characterizes epithelial PM domains, the compositional uniqueness of axonal versus somatodendritic regions of neurons underlies their functional specialization [81]. Raft-mediated protein sorting in neurons was observed to be highly analogous to epithelial cells, with the axonal delivery of proteins that define apical PM domains being dependent on intact raft domains [82]. By constraining the delivery of axonal proteins and lipids, raft-mediated trafficking appears to be required for the maturation of the axon [83], and thus functional neuronal maturation.

3. Rafts in endocytic traffic

Classically, intracellular membrane traffic has been separated into outward and inward routes referred to as biosynthetic and endocytic pathways, respectively. In reality, there is extensive cross-talk between these cellular highways, with direct transit from the biosynthetic machinery to late endosomes and/or lysosomes [84] as well as shuttling of endocytosed components back to the Golgi for processing and re-sorting. Although they are treated here separately, it is important to emphasize the extensive integration of the membrane trafficking system and thus the critical organizing role that membrane domains play in both pathways.

3.1. Rafts in endocytosis from the plasma membrane

Endocytosis and vesicle formation by polymerization of the clathrin triskelion is one of the most architecturally striking and functionally important aspects of eukaryotic cell biology, and so unsurprisingly, clathrin-mediated endocytosis (CME) is one of the most well-studied and thoroughly characterized cellular mechanisms [85]. However, complete deletion of clathrin function does not abrogate all endocytosis [86], suggesting multiple parallel routes for internalization of surface membranes and extracellular material. A significant observation is the reciprocal exclusion between clathrin and membrane rafts — i.e. proteins endocytosed by CME, and clathrin itself, are generally excluded from DRMs, while raft-associated proteins are often excluded from clathrin-coated pits [87] (though there are exceptions [88]). Moreover, whereas all raft functions are believed to be sensitive to depletion of PM cholesterol, CME is not [89,90]. This observation suggests a simple (likely over-simplified) dichotomy for endocytosis: CME mediated by specific protein-protein interactions is responsible for internalizing non-raft PM components, while rafts mediate the alternate route for those proteins with specific affinity for ordered membrane domains (Fig. 1).

Possible analogs for clathrin-coated pits in this raft-mediated endocytic route are caveolae — small tapered invaginations of the plasma membrane observable under electron microscopy [91]. These caveolar invaginations appear to be intermediates in raft-associated (but notably clathrin-independent) trafficking pathways, especially endocytosis from the PM [92] (for discussion of mechanisms of caveolar

trafficking, see Section 4.1). The two key pieces of evidence for the relationship between caveolae and lipid rafts are the enrichment of raft components in caveolae and the sensitivity of caveolae-mediated traffic to perturbation of raft lipids. Both cholesterol and sphingolipids are highly enriched in caveolae, even compared to the relatively cholesterol-/SL-rich PM [93]. Similarly, several raft-associated proteins were shown to be included in caveolae [94], and more importantly, to induce the genesis of caveolar trafficking intermediates upon their cross-linking (which, as described below, stabilizes and coalesces raft domains) [95]. This crosslink-mediated raft coalescence is the mechanism that might be responsible for the internalization of pathogens or bacterial toxins that target cellular glycosphingolipids like cholera and SV40 for GM1 [96] and *Shigella dysenteriae* for Gb3 [97].

Despite the central role of caveolae in raft-associated endocytosis, robust clathrin-independent endocytosis can be observed even in cells without caveolae [86]. These observations led to the characterization of non-clathrin/non-caveolar endocytic routes (Fig. 1), which remain better defined by what they are *not*, than what they *are* and how they *work*. The most prominent example is the CLIC/GEEC system — i.e. Clathrin-Independent Carriers (CLIC) and GPI-Enriched Endocytic Compartments (GEEC) [86,98]. This pathway is largely defined by its internalization of proteins anchored to the extracellular leaflet of the PM, including GPI-anchored proteins and the GSL-binding toxins described above, as well as its independence for caveolin, clathrin, and dynamin, the necessary scission factor in CME [99]. It is tempting to speculate from (1) the cholesterol-sensitivity of the CLIC/GEEC pathway; (2) the significant overlap between its cargo and that of caveolin-mediated endocytosis; and (3) the coordinate regulation of caveolin and GEEC by Cdc42 [99,100], that the core molecular machinery for both of these pathways is linked to ordered membrane domains; however, a clear molecular description of that machinery remains elusive. A similar case can be made for Arf6-mediated trafficking, which is poorly characterized but appears to be a route for both endocytosis and recycling of raft-associated cargo to the PM [101] (See Table 1).

A surprising recent set of observations supports raft-associated endocytosis via a novel endocytic mechanism known as Massive ENDocytosis (MEND). In MEND, up to 50% of the PM can be endocytosed in a matter of minutes, induced by large intracellular calcium transients. The hypothesized cause for this effect is an unregulated coalescence of raft domains leading to endocytosis [102].

3.2. Deeper down: raft-associated sorting in endosomal progression, recycling, and degradation

Although most of the attention in endocytic trafficking has been on internalization of specific components from the PM domains are likely integrated into all aspects of endocytic traffic (Fig. 1). In analogy to the vectorial flow of the secretory system (i.e. ER-to-PM), the endocytic system can be conceptualized as a two-way highway, with bulk traffic proceeding towards the cellular waste bin – i.e. the lysosome – and a robust pathway for recycling internalized components back to the PM (note: while the various endocytic compartments are typically referred to as discrete cellular locations, it is probably more accurate to conceptualize them as a maturing continuum, from small, neutral endocytic vesicles to larger, more acidic degradative compartments). Moreover, in polarized epithelia, these endocytic pathways are complicated by the presence of two different "source" compartments — i.e. the apical and basolateral PMs. These are associated with specialized apical and basolateral recycling endosomes (ARE and BRE, respectively), which converge/mature into a common recycling endosome (CRE). The divergence of lipid composition in those compartments has not been clearly delineated, but it is a reasonable prediction that they reflect the specific membrane compositions of their sources/destinations.

The main location for raft involvement in these processes is the central sorting compartment of the endocytic system, the early endosome (EE) (Fig. 2). The EE is the primary destination of most endocytosed material, and is also the site of bifurcation for the degradative and recycling pathways. Its structure contains both reticulated tubules characteristic of the recycling endosome (RE — the intermediate for endosomal recycling back to the PM) and a multivesicular vacuole resembling the late endosome (Fig. 2). Both EEs [103] and REs [104] are enriched in cholesterol, sphingomyelin, and phosphatidylserine (PS) [105], as well as caveolin [104]. In striking contrast, these same components are largely depleted from late endosomes (LEs) [103,105,106], though there is evidence for raft domains in LEs [107]. These findings prompt the hypothesis that rafts mediate one route for recycling of specific membrane components from the EE to the PM (via the RE) (Fig. 2).

This hypothesis has recently received striking experimental confirmation. Our group generated panels of mutants of several single-spanning PM proteins to yield variants with experimentally defined raft affinities ranging from completely raft-excluded to raft-enriched. Rather than relying on detergent resistance (which can be artifact-prone [38,39] and intractable for quantification [40]), we quantified raft association using Giant Plasma Membrane Vesicles [18,20,108] (Fig. 2) and observed a clear relationship between raft affinity and PM localization. While all raft-partitioning variants were enriched at the PM, a variety of unrelated mutants with abrogated raft affinity accumulated in late endosomes and lysosomes, via transit through EEs [109]. These observations were explained by a model wherein PM proteins are constitutively endocytosed, then recycled from the EE via their association with raft domains. If raft association was abrogated, proteins not equipped for an alternative recycling route (e.g. coat/adapter protein recognition sequences), were degraded through a bulk endosomal maturation pathway (Fig. 2).

Although these findings were consistent across different cell lines [109], raft-mediated recycling has previously been shown to be cell-type dependent. A dramatic example is the trafficking of GPI-APs between CHO and BHK cells, two common, fibroblastic cell lines. In CHO cells, internalized GPI-APs are recycled from EEs to REs to the PM [110], via their association with rafts [111]. Interestingly, when raft association is perturbed by modifying the lipid anchor (to bear non-raft preferring unsaturated acyl chains), proteins also recycled to the PM, but at a different rate, comparable to other non-raft cargo [111,112]. In contrast, in BHKs, both raft and non-raft variants of lipid-anchored proteins were targeted for the LE, and degradation in the lysosome [110,112].

An alternative, non-exclusive mechanism to explain the depletion of raft components from late endosomes and lysosomes is that cholesterol is selectively enriched in the intraluminal vesicles (ILVs) that give LEs their characteristic multivesicular body (MVB) appearance [113]. The ILVs are destined for degradation or secretion, suggesting a specific mechanism for removal of raft lipids from the endocytic cycle. ILV formation is mediated by the ESCRT complex, suggesting that this machinery may have specificity for recruiting and deforming raft domains, both in the LEs and otherwise. Remarkable recent observations in yeast have definitively demonstrated the potential for large-scale lateral domain formation in degradative endosomes, specifically the yeast vacuole. A variety of metabolic challenges induced swelling of the vacuoles such that they were observable by light microscopy and a clear, sterol-dependent coexistence of two liquid domains was observed in the vacuolar membranes [22]. We speculate that this dramatic effect was observable because the cellular stresses prevented the selective removal of raft lipids from LEs and lysosomes causing phase separation.

An intriguing hypothesis [114] is that the specific recruitment of raft material into the internal vesicles of LEs results in some diseases characterized by dysfunctional lysosome catabolism. Such lysosomal storage diseases (LSDs) comprise several dozen disorders, all characterized by the accumulation of undegraded lysosomal material, resulting in lysosomal proliferation and hypertrophy, and often-severe neuronal dysfunctions [115]. Canonical raft components (sphingolipids, glycolipids, cholesterol) are often over-represented in the accumulated, undegraded material [116,117], particularly in sphingolipidoses, which comprise a

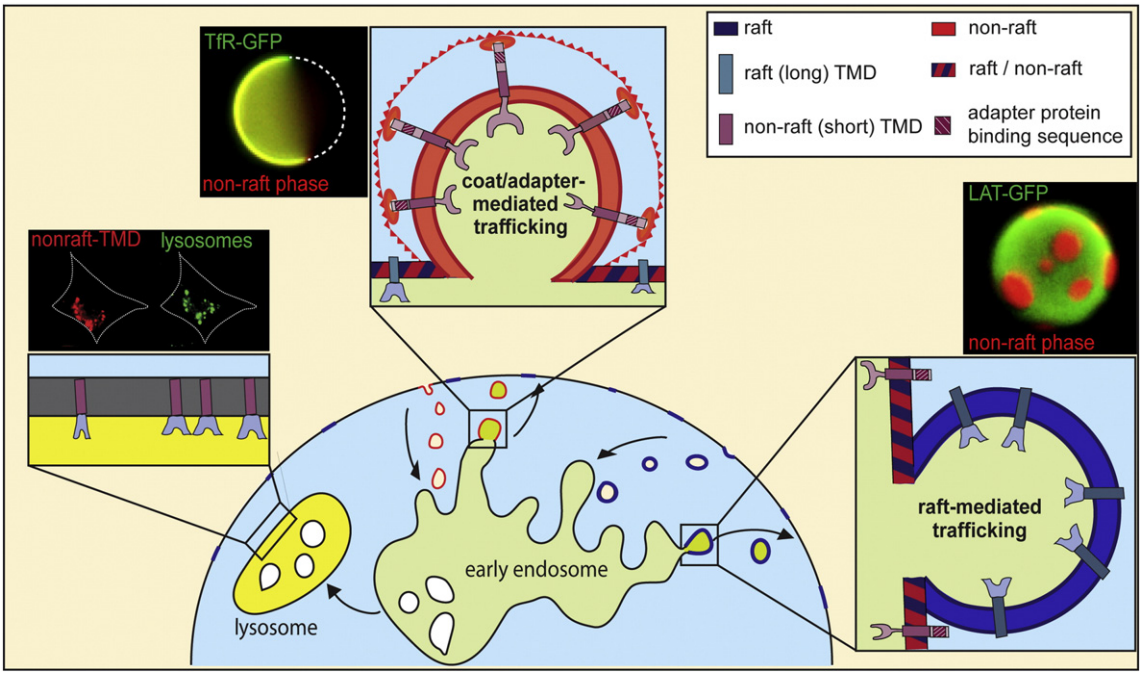

Fig. 2. Transmembrane domain-dependent raft association and endosomal recycling. Early endosomes are key sorting stations for internalized membrane cargo. Endocytosed membrane proteins are recycled to the PM by two parallel routes. For non-raft proteins (red), specific amino acid motifs in their cytosolic domains recruit coat/adapter machinery, which both sort components and aid in the generation of a transport vesicle. Proteins requiring raft association for recycling (blue) partition into ordered membrane domains due to their transmembrane domains (TMD) [109] and post-translational modifications [20,45], and are packaged into a raft-enriched recycling carrier destined for the PM (possibly via recycling endosomes). Proteins with neither recycling signal (red TMD but no adapter protein recognition sequence) are retained in the endosomal system, for eventual degradation in the lysosomes. Raft association can now be directly quantified by observing isolated plasma membrane vesicles [108,109], which separate into coexisting domains that selectively recruit membrane proteins. The right image is of a plasma membrane vesicle from a cell expressing a raft protein (linker for activation of T-cells — LAT-GFP), stained with a non-raft marker lipid. The image at the top is a similar preparation, but the tagged protein is the non-raft Transferrin Receptor (TfR-GFP). Images at the left show colocalization between LAT with a non-raft TMD and a lysosomal marker. For details, see Ref. [109].

relatively large group of lysosomal storage diseases including Fabry, Niemann–Pick, and Tay–Sachs diseases. Moreover, raft lipids seem to be inseparable travel partners, as failure to degrade one type (e.g. cholesterol) often leads to accumulation of other types (e.g. sphingolipids) without a specific defect in their catabolism [117,118]. Although the molecular pathophysiology of these sphingolipidoses remains unresolved, it is possible that altered cholesterol/sphingolipid homeostasis resulting from the co-recruitment of raft lipids into lysosomes leads to wide-ranging deleterious effects [114].

4. Molecular machinery for raft-mediated sorting

Although the raft concept emphasizes lipid-driven membrane self-organization as a key mechanistic contributor to cellular logistics, these functions clearly require extensive proteinaceous molecular machinery for efficient and accurate trafficking. A hypothesized exception to this requirement is the budding of raft-enriched vesicular carriers from their host membranes driven by the line tension (two-dimensional analog of surface tension) present at the interface between unlike membrane regions [119]. This edge energy – the same force that produces spherical droplets in three-dimensional emulsions and circular domains in planar membranes – is dependent on the length of the domain interface, which could be reduced by deforming the planar domain into a bud in order to minimize edge length. In theory, such a mechanism could potentially lead to complete vesicle budding as the edge length is driven to zero [119]. This drive to minimize the line tension is opposed by the energy necessary to bend a planar bilayer into a curved vesicle, with the competition between these two forces determining the extent of budding. Unfortunately, direct estimation for membrane parameters like line tension, bending rigidity, and intrinsic curvature is difficult for biological membranes, especially the membranes of internal organelles where much trafficking occurs. The current understanding is that a strictly domain-induced mechanism for vesicle budding is unlikely because the difference between coexisting domains, and thus the tension between them, is likely to be small in biomimetic membranes [120], or even non-existent, as would be the case for a critical system [21]. Small tensions would require micrometer-sized domains to induce domain budding, and such domains are generally not observed in living cells, even under raft-coalescing conditions. Thus, as is the case for most cellular functions, proteins play a primary role in raft dependent sorting (Fig. 1).

4.1. Caveolin, flotillin, and caveolae

The protein most associated with lipid rafts in cellular traffic is caveolin. Originally identified as a phosphorylation target of v-Src and potential mediator of oncogenesis [121], VIP21/caveolin was soon described as the core component of caveolae [122] and a major constituent of apically directed transport vesicles [123]. It has since been shown to be widely expressed and required for inducing caveolae in a variety of cell types (reviewed in Ref. [91,94]), remarkably including prokaryotes [124]. Caveolin binds cholesterol directly [125] and its function is sensitive to cholesterol depletion [122]. Correspondingly, caveolin itself is highly enriched in DRMs — indeed it is widely used a raft marker, though it likely defines a subset of raft domains involved in caveolin-mediated traffic. Importantly, caveolin only associates with the raft-enriched membrane fractions after its oligomerization, and this oligomerization is stabilized by raft lipids present in PMs and the late Golgi [126,127]. These observations suggest caveolin oligomerization (likely mediated by co-factors including the other caveolin family members Cav2 and Cav3, as well as cavins [91]) as one possible driving force for raft coalescence and functionalization, and feedback regulation between this oligomerization and raft lipids.

A second protein family involved in functional raft coalescence is the flotillins (aka reggie1 and 2). Both family members are highly DRM-

enriched [128], though unlike caveolins, they are not integral membrane proteins and therefore require lipid modifications for stable membrane attachment. Like caveolins, flotillins tend to oligomerize [128], with oligomerization required for endocytosis [129]. In polarized epithelia, the internalization of raft markers GPI-APs and cholera toxin is strongly dependent on flotillin expression [130,131], a fact made more remarkable considering that neither set of proteins crosses the membrane, thus precluding any direct protein–protein interaction with the intracellular flotillin [130].

Finally, there remain several mysterious partners involved in raft-mediated vesicle generation, implicated through crude proteomic characterization of apical transport vesicles in MDCK cells [27]. Among these is VIP17/MAL [132], a small proteolipid that can oligomerize, induce membrane domains [133], and affect trafficking [132,134]; annexin 13b, an epithelial specific isoform [135] responsible for raft-mediated traffic in MDCKs [136]; and galectin-9, an extracellular lectin that binds and crosslinks raft glycosphingolipids [137].

4.2. Arf6 and other machinery

In addition to the core machinery associated with raft trafficking, there is a plethora of supporting players that are necessary to mediate specific targeting, identification, and fusion to destination compartments [138]. One of the best characterized of these is the Ras superfamily small GTPase Arf6 [139]. Small GTPases, like Rho, Rac, and the many Rab family proteins, are ubiquitous regulators of cytoskeletal and membrane dynamics, relying on switchable activity mediated by GTP hydrolysis for their rapid and dynamic regulation. Although they are not themselves protein coats, several members of the Arf family recruit clathrin and potentially other coat/adaptor proteins [140]. Unlike the other Arf proteins, Arf6 is localized to the PM and early endosomes, where it appears to be directly involved in both endocytosis and recycling of raft-like membranes [101]. To some extent, Arf6 appears to mediate its own distinct non-clathrin/non-caveolar pathway that also does not enrich for GPI-APs [141]. This pathway has been shown to be important in processing of the amyloid precursor protein (APP), the source of the amyloid peptides accumulated in Alzheimer's disease, where it facilitates the endosomal rendezvous between APP and the beta-secretase (BACE1) required for peptide generation [142]. Such amyloidogenic processing has previously been associated with lipid rafts [143], further implicating Arf6 as a key mediator of raft traffic. Microtubules are proposed to be the cellular track taken by Arf6/raft vesicles [101,144], with the minus-end directed motor KIFC3 as the carrier [145]. Finally, several SNAREs have been implicated in raft vesicle fusion and targeting, including syntaxin 3, SNAP-23, and VAMP7 [146], as well as VAMP4 and syntaxin 2 [147].

5. Perspective

As with much of our current understanding of cellular functions that rely on collective properties rather than individual molecular components, membrane sorting and trafficking between cellular organelles remain largely mysterious. Rafts clearly play an important role in these processes; however, the paltry list of molecular players in Section 4 and the lack of structural insight into their mechanisms of action suggest that the details of how rafts are coalesced, budded, trafficked, and targeted are unresolved. Much of the difficulty arises from the inherent interconnectedness and redundancy of membrane traffic. Although Fig. 1 suggests step-wise, discreet pathways, in reality, there is extensive crosstalk between the secretory and endosomal systems, with direct membrane exchange possibly occurring between all compartments. Moreover, the plethora of trafficking options in the cell allows cargo to take “alternate” routes when a given pathway is abrogated. It remains an open question whether all of the endosomal routes associated with raft domains (described in Section 3) share core machinery, or whether each is truly distinct.

An underappreciated consideration is that almost all studies aimed at elucidating raft properties have focused on the only easily accessible membrane of the cell (the PM), despite there being no a priori reason to believe that intracellular membranes should have similar rafts to the PM. Indeed, rafts are almost certainly different in each compartment of a cell because of the unique protein/lipid composition of any given organelle. Thus, it is probably more accurate to conceptualize lipid rafts as a mechanism for membrane organization, rather than specific entities of strictly defined compositions and physical properties (e.g. size) [16]. The impact of such raft diversity on subcellular sorting can only be speculated, but it is possible that the compositions of the major membrane sorting stations in the cell (TGN and early endosome) may promote a more robust separation of raft from non-raft domains than would be observed at the PM.

Although caveolin is almost certainly a key raft-trafficking mediator, there is no comparison between it and the level of mechanistic insight into clathrin-mediated endocytosis. Caveolin-mediated membrane budding and specific selection of raft components into in vitro caveolae have not been reconstituted (though demonstrated in a cell-free assay [148]), thus there is no evidence for whether caveolin comprises the minimal required machinery for raft sorting. Moreover, the specific recruitment of proteins into raft domains underlies the functional relevance of rafts, but the structural determinants by which transmembrane proteins are recruited into raft domains are only now starting to become elucidated [45,109,149,150].

The evidence presented above makes a strong case for lipid-mediated, lateral membrane domains in subcellular sorting and trafficking. Moreover, the ubiquity of this involvement suggests a simple organizing principle for cellular traffic, wherein lipid-driven domains interface with protein machinery. However, a mechanistic description of this simple principle remains elusive, with tangled, interconnected pathways often sharing cargo and machinery both spatially and temporally. We hope that this confusion will be resolved by a combination of nanometer-resolution imaging methods [151,152], advances in functional membrane protein reconstitution [153], and a more accurate understanding of what rafts are, what they are not, and what they do in the cell.

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