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Vesicular sorting controls the polarity of expanding membranes in the *C. elegans* intestine

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Biological tubes consist of polarized epithelial cells with apical membranes building the central lumen and basolateral membranes contacting adjacent cells or the extracellular matrix. Cellular polarity requires distinct inputs from outside the cell, e.g., the matrix, inside the cell, e.g., vesicular trafficking and the plasma membrane and its junctions.[1] Many highly conserved polarity cues have been identified, but their integration during the complex process of polarized tissue and organ morphogenesis is not well understood. It is assumed that plasma-membrane-associated polarity determinants, such as the partitioning-defective (PAR) complex, define plasma membrane domain identities, whereas vesicular trafficking delivers membrane components to these domains, but lacks the ability to define them. In vitro studies on lumenal membrane biogenesis in mammalian cell lines now indicate that trafficking could contribute to defining membrane domains by targeting the polarity determinants, e.g., the PARs, themselves.[2] This possibility suggests a mechanism for PARs' asymmetric distribution on membranes and places vesicle-associated polarity cues upstream of membrane-associated polarity determinants. In such an upstream position, trafficking might even direct multiple membrane components, not only polarity determinants, an original concept of polarized plasma membrane biogenesis[3,4] that was largely abandoned due to the failure to identify a molecularly defined intrinsic vesicular sorting mechanism. Our two recent studies on *C. elegans* intestinal tubulogenesis reveal that glycosphingolipids (GSLs) and the well-recognized vesicle components clathrin and its AP-1 adaptor are required for targeting multiple apical molecules, including polarity regulators, to the expanding apical/lumenal membrane.[5,6] These findings support GSLs' long-proposed role in in vivo polarized epithelial membrane biogenesis and development and identify a novel function in apical polarity for classical post-Golgi vesicle components. They are also compatible with a vesicle-intrinsic sorting mechanism during membrane biogenesis and suggest a model for how vesicles could acquire apical directionality during the assembly of the functionally critical polarized lumenal surfaces of epithelial tubes.

Our findings on GSLs' and clathrin/AP-1's roles in C. elegans intestinal polarity and tubulogenesis are reported in the articles listed in the boxed area at the bottom left of this page.

Here, we introduce the *C. elegans* postmitotic intestine as an in vivo model for polarized membrane biogenesis and comment on the potential impact of our findings generated in this model on concepts of polarity.

The Expanding *C. elegans* Intestine is a Model for the in Vivo Analysis of Polarized Membrane Biogenesis

Membrane-associated polarity regulators such as the Par-, Crumbs- and Scribble-associated protein complexes were first identified in *C. elegans* and *Drosophila* developmental studies.[7-12] It is assumed that they generate and maintain polarized plasma membrane domains via a system

of mutual inhibition of their apical and basolateral components.[11-13] These protein complexes are highly conserved in mammalian epithelia, where they, with certain species- and tissue-specific modifications, also act as general apicobasal domain determinants and possibly operate by a similar mechanism.[1,14] In epithelia, their effect on plasma membrane domain definition is additionally mediated via apical junctions that define domain boundaries. Epithelial polarity defects are typically preceded by junction defects that may result in the expansion of one, often the apical, domain, at the expense of the other, or cause membrane equilibration.[13-16]

These polarity concepts were defined in model systems where polarized membrane biogenesis occurs coincident with complex polarization events such as polarized cell division (e.g., in yeast and in the *C. elegans* zygote) and directed cell migration (e.g., of *Drosophila* and *C. elegans* epidermal sheets). Furthermore, the analysis is typically performed in cells whose apical or anterior (e.g., in the *C. elegans* zygote) membranes or surfaces directly or indirectly face an external rather than an internal cell free space. Such membranes may lack distinctive structural microdomains that discriminate apical/lumenal membranes in tubular epithelia. Moreover, spreading epidermal cells are generally flat, complicating judgments about lateral membranes, apical/lateral boundaries and apical junction depth. Finally, the model may be defined by unique extrinsic or intrinsic characteristics, such as the symmetry-breaking event of sperm entry in the *C. elegans* zygote or the inward growth of membranes during *Drosophila* embryo cellularization.[13]

In vitro polarity models of mammalian cells are similarly complicated by proliferation and migration, and the 2D analysis of cells spreading along tissue culture dish surfaces is also restrained by flatness. This experimental setting and the larger size of mammalian cells do, however, facilitate the analysis of trafficking. Mammalian cell lines used to study polarity are often derived from tubular epithelia, such as renal Madin-Darby-canine-kidney (MDCK) or intestinal cells, and important cues to polarized trafficking, including the few known lipid cues, were first identified here.[17] Tubular epithelia assist in the analysis of polarity by their structurally distinctive apical/lumenal membrane microdomains such as microvilli or canaliculi. Their 3D in vitro analysis, overcoming the problem of flatness, has recently provided novel insights into polarity and lumen biogenesis, particularly from the trafficking point of view.[2,18] Whereas membrane-associated polarity cues defined in vivo in lower organisms (such as the Pars) have been shown to also operate in mammalian cell lines, the reverse is less clear: do polarized trafficking cues define membrane domain identities and morphogenesis in vivo? Although their endocytic roles in polarity is increasingly recognized,[19] comparatively few trafficking molecules have been identified in unbiased screens in invertebrate polarized organ morphogenesis, and deleting critical trafficking and polarity cues in mice may cause early lethality.[20-24]

The transparent *C. elegans* permits tubulogenesis to be observed in the living organism, with tubes simple enough to evaluate single cells and an invariant morphogenesis program allowing each cell's division and migration to be tracked. Its intestine consists of 20 comparatively large epithelial cells (E20) of clonal origin forming an overall bilaterally symmetrical single-layered epithelial tube, after completing one intercalation step from a 4-fold symmetrical tube during mid-embryogenesis[25] (**Figs. 1A and 2A**). From the E20 stage onward, during late embryogenesis and through four larval stages, these postmitotic cells expand the tube by growth alone, without further cell division or migration.

We found that this expanding postmitotic *C. elegans* tubular epithelium allows polarized membrane biogenesis to be directly analyzed in vivo. In contrast to any of the above-described models, plasma membrane polarization can here be observed and modulated in single cells, independent from cell division- and migration-dependent polarity cues, contingent only on cellular growth. We performed a genome-wide RNA interference (RNAi) screen on animals engineered with ERM-1::GFP-labeled apical/lumenal membranes to examine the requirement of lethal genes for tubular polarity and lumen morphogenesis (a prior chromosome-III screen had determined that > 90% of informative phenotypes were accompanied by lethality). The actin-membrane linker ERM-1, the ancestral *C. elegans* ezrin-radixin-moesin ortholog, is submembraneously enriched at apical/lumenal membranes of tubular organ epithelia throughout development and in the adult.[26] This screen identified, among other molecules, a number of lipid-biosynthetic enzymes, clathrin and several of its AP-1 adaptor subunits, whose knockdowns all caused a similar novel polarity phenotype in the multicellular intestine.[5,6]

In Vivo Conversion of Polarized Membrane Domains and Ectopic Lateral Lumen Formation in a Postmitotic Epithelium

This polarity phenotype was characterized by basolateral ERM-1 displacement and transformation of the contiguous central lumen into multiple ectopic lumens developing along the lateral membranes of single cells in the expanding larval intestine (**Fig. 1**). Multiple-lumen formation was accompanied by the displacement of all examined apical membrane and submembrane components to the lateral membrane or cytoplasm, including transmembraneous proteins, membrane lipids and the polarity determinant PAR-6. Transmission electron microscopy (TEM) revealed a corresponding structural transformation of apical and basolateral domains: microvilli and the terminal web were lost from the apical and acquired at the lateral, ectopic-lumenal membrane. We therefore describe this membrane domain identity change as polarity conversion rather than apical membrane expansion.

No evidence of structural or functional apical-junction-assembly defects preceding or accompanying this polarity alteration was found, further distinguishing it from the typical epithelial polarity phenotype with documented junction defects and membrane-domain-boundary alterations suggestive of perturbations of the apicobasolateral equilibrium.[13,14] Moreover, excess junctions were present at lateral membranes, not, however, as junction-broadening that is typical for junction-assembly defects in flat epithelia, but

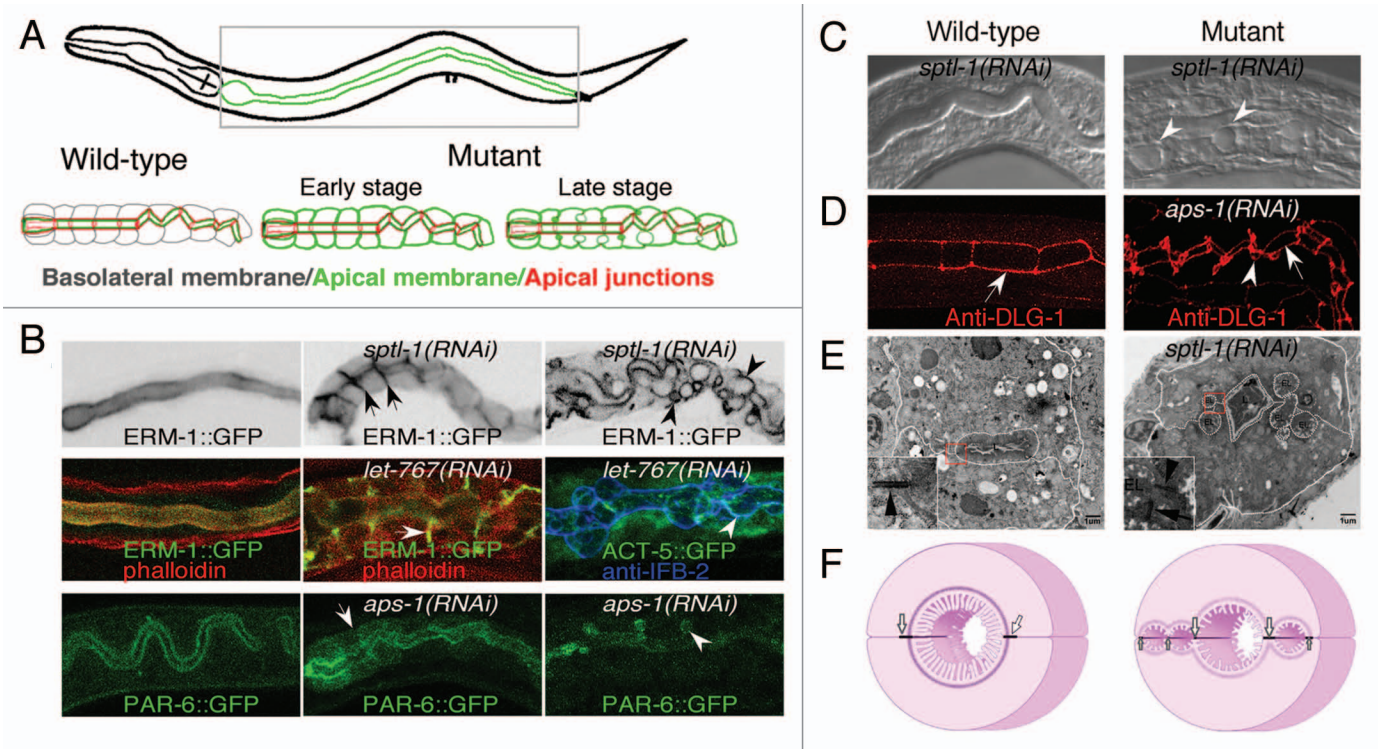

Figure 1. The *C. elegans* postmitotic larval intestine and the polarity-conversion and ectopic-lumen phenotypes. (**A**) Schematics of entire animal (top) and higher magnification of boxed area (bottom) showing postmitotic intestines of wild-type (left column) vs. SL/clathrin/AP-1-depeleted mutant/RNAi animals (middle and right columns). All phenotypes are similar: examples below include *let-767-*, *sptl-1-* (two lipid-biosynthetic enzymes) and *aps-1* RNAi (an AP-1 subunit). Early stage of phenotype development: basolateral displacement of apical membrane components (middle column); later stage: formation of ectopic lateral lumens (right column). Ultimately, the central lumen disintegrates, loses apical membrane components and lateral lumens further enlarge (not shown). (**B**) All tested apical membraneous and submembraneous components are displaced basolaterally (arrows) and/or cytoplasmically before being displaced to ectopic lumens (arrowheads). Here and below confocal images show sections of L1 larval intestines. Apical components: ERM-1, a membrane-cytoskeleton linker; actin (phalloidin: dorsal and ventral lines represent muscle actin); ACT-5, a tube-specific cortical actin; IFB-2, an intermediate filament component; PAR-6, the apical PAR polarity complex component. (**C**) Differential interference contrast (DIC) images of wild-type central lumen (left) and mutant ectopic lateral lumens in L1 intestines (right, arrowheads). The *sptl-1(RNAi)* animal during early-stage polarity conversion, shown on the left, is indistinguishable from wild-type by DIC (the other images below in this column show wild-type intestines). (**D**) All apical junction components tested (DLG-1/Discs-Large shown here) are contiguous (arrows) at apicolateral boundaries in both wild-type and mutant/RNAi intestines, but additional junctions surround ectopic lumens in the latter (arrowheads). (**E**) TEM cross-sections of whole L1 intestines. Membrane boundaries of the two intestinal cells are outlined by dotted lines. Multiple small lumens, surrounded by ectopic junctions, emerge along the lateral membrane in RNAi animal; these and the apicolateral junctions appear ultrastructurally intact (boxed and magnified in insets). Microvilli are lost from the central apical and appear at the originally lateral, membrane, and the submembraneous web dissociates from the central lumen and forms at lateral lumens (compare with schematics in F). Note vesicle paucity in RNAi intestine. (**F**) Schematics corresponding to E, each showing the paired intestinal cells. Loss of microvilli and dehiscence of submembraneous terminal web (dark ring beneath the lumenal membrane) is indicated, with the emergence of both structures at ectopic lateral lumens in RNAi/mutant animal. Large arrows point to apicolateral junctions (wild-type position), small arrows to ectopic lateral junctions. Adapted from references 5 and 6.

interspersed between newly-formed lateral lumenal membranes, identified as such by the presence of microvilli. These observations suggest an unexpected independence of in situ apical membrane morphogenesis from and possibly control over, junction assembly. Perhaps, apical junction placement, critical to confine apical membrane components, occurs concomitant with, or even secondary to, de novo apical membrane biogenesis, relying on some of the same mechanisms required to target apical membrane components.

The polarity alteration appeared to be limited to the postmitotic intestine, with early embryonic polarity undisturbed, at least in knockdowns of sphingolipid(SL)-biosynthetic enzymes and AP-1 adaptor subunits, suggesting that these molecules and the processes they control maintain but do not establish polarity. In these experiments, where dsRNA was fed to animals via bacteria, RNAi is induced in both parents and offspring, resulting in sustained knockdowns during embryonic and larval development of the progeny. Stage-specifically induced larval RNAi was used to demonstrate that apicobasal membrane identities and central lumen contiguity are indeed actively maintained in the postmitotic epithelium through the action of these molecules. However, perturbation of the system only converted polarity in the expanding larval intestine, not in the non-expanding starved larval, or adult, intestine, suggesting a defect in de novo polarized membrane biogenesis rather than in membrane polarity maintenance. In fact, the polarity conversion

was dependent on ongoing growth and involved the misrouting of newly-synthesized membrane components. Thus the same process that "maintains" pre-established polarized domains and lumen positions "establishes" both on growing membranes, an observation made possible by the ability to separate polarized membrane biogenesis from overall cellular polarity in this expanding postmitotic epithelium (whether this process also establishes early embryonic membrane polarity is discussed below in the section describing the clathrin phenotype).

Intriguingly, we found that this membrane polarity alteration was reversible. Membrane domain identities and ectopic lumens could both be reverted back to their wild-type states if the source of perturbation was removed: reactivating SL biosynthesis in *let-767(RNAi)* larvae, for instance, moved apical membrane biogenesis back to the original apical side, restored the central lumen and rescued the lethality. The reversibility of membrane domain identities and lumen positions on growing membranes, by itself, provides a strong argument for an intrinsic ability of membrane biogenesis to define such domains and initiate lumen formation. Furthermore, this observation reveals an amazing plasticity of postmitotic, single-cell and organ polarity: apicobasal polarity can be shifted in situ in individual cells, and the assembly, disassembly and reassembly of one contiguous central vs. multiple ectopic lumens throughout an entire organ can be accomplished without affecting the viability of the animal.

The dynamic nature of postmitotic polarity demonstrated here might reflect a general ability of tubular, or even all, epithelia. This idea is supported by three independent, isolated observations on tubular epithelia, all, however, made in mitotic cells ex situ: MDCK cells can shift their lumen position when transitioned from 2D to 3D cultures; hepatocytes form inter-cellular bile-canalicular lumens, with both renal and liver cells retaining a PAR-1/microtubule-dependent flexibility to change lumen positions[27-29] (intriguingly, bile-canalicular formation was linked to SL biosynthesis[30,31]); uniquely, Caco-2 intestinal cells were shown to be capable of junction-independent single-cell polarization via LKB1/PAR-4.[32] If reversibility of polarity is indeed preserved in human tissues, this might allow its modulation in vivo, suggesting novel ways of intervention in diseases with altered or lost polarity, such as cancer.

Together, the specifics of the here-described polarity phenotype all fit with an underlying trafficking defect interfering with polarized membrane biogenesis, namely: membrane domain conversion; junction-independency; dependency on growth; misrouting of newly-synthesized membrane components; presence in postmitotic and single cells and reversibility. In fact, the polarity alteration was shown to be accompanied by defects in the biogenesis and positioning of apical and presumed lumenal-membrane-forming vesicles and could be suppressed by cold-temperature that foremost inhibits vesicle trafficking. The idea is furthermore supported by the well-defined trafficking nature of the molecules identified in this unbiased screen on tubular polarity: GSLs, clathrin and AP-1 adaptor subunits.

Several recently characterized in vivo and in vitro phenotypes, in both vertebrate and invertebrate tubular epithelia, also support a link between vesicular trafficking defects and ectopic lumen formation. A type Vb myosin gene mutation causes Microvillus Inclusion Disease (MVID), a lethal human intestinal failure syndrome with ectopic intracellular, not lateral, lumen formation, a distinct yet closely related phenotype also observed upon deleting another trafficking molecule, murine RAB8.[33,34] A similar phenotype, recently described in *C. elegans wts-1*/Lats-kinase mutants, could in fact be suppressed by exocytosis defects.[35] Finally, MDCK and hepatoma cells develop intracellular lumen-like vacuolar apical compartments (VACs) or related subapical vacuolar compartments (SACs), respectively, when trafficking is impaired.[36-38]

Sphingolipid (SL) Biosynthesis is Required for Sorting Apical/Lumenal Membrane Components During De Novo Membrane Biogenesis

Our screen on tubular polarity identified different types of molecules whose loss caused this specific polarity phenotype, among them several lipid-biosynthetic enzymes: POD-2, an acetyl-CoA carboxylase; LET-767, a steroid dehydrogenase/3-ketoacyl-CoA reductase; ACS-1, a long chain fatty acid acyl-CoA ligase and SPTL-1, a serine palmitoyltransferase (SPT). We developed follow-on lipid-biosynthetic pathway screens and functional genetic and biochemical assays, which demonstrated that the fatty-acid-biosynthetic enzymes affected polarity via SL synthesis. These experiments ultimately identified ceramideglucosyltransferases (CGTs) as endpoint biosynthetic enzymes and thereby suggested their product GSLs as the common lipid species that mediated the function of these different enzymes in polarity. The additional identification of an SL hydroxylase in these screens, together with the results of added glycosylation screens and mass spectrometry analyses, further characterized the putative underlying compound as a hydroxylated glucosylceramide (GlcCer-OH), with a branched C17 long-chain-base and a saturated long-chain fatty acid of probable C22 length.

GSLs are ubiquitous obligate membrane lipids residing on endo- and plasma membranes.[39] In the absence of clear apical sorting receptors and cargo signals, they take center stage as vesicular apical sorting signals in mammalian epithelia.[17] However, evidence for this sorting function rests entirely on in vitro analyses constrained by: the inability to genetically delete specific lipids, redundant lipid functions, the non-physiological behavior of labeled lipids and the non-specific effects of the toxins used to inhibit their biosynthesis. Moreover, GSLs' in vivo function in vertebrate polarity is unclear given the early lethality of the non-redundant murine CGT gene knockout.[40,41] Our findings therefore provide the first evidence for the physiological relevance and developmental role of GSLs in polarized morphogenesis, while the in vitro findings obtained in vertebrate epithelia provides support for the role of *C. elegans'* GSLs in apical sorting.

The in vivo perturbation of *C. elegans* GSL biosynthesis produces all the above-described aspects of polarity conversion, including the effects on apical vesicle

biogenesis and placement, consistent with a GSL role in vesicular sorting. Moreover, GlcCer and ceramide (Cer) were also found to reside on *C. elegans* intestinal endo- and apical/lumenal plasma membranes and cooperate with the vesicular coat and adaptor components clathrin and AP-1 in apical sorting (as discussed in the next section on clathrin/AP-1 and GSLs). Nevertheless, potential alternative GSL actions in *C. elegans* polarity are not excluded, since GSLs have documented roles in multiple signaling processes at the plasma membrane, and in both structural apical membrane and junction biogenesis in other systems.[42,43,47,76] However, any proposed sorting-unrelated GSL mechanism in apicobasal polarity in the *C. elegans* intestine must account for the fact that their depletion causes de novo apical membrane biogenesis at the lateral side of the epithelium.

GSLs' role as membrane domain determinants has been hypothesized based on their requirement to sort single apical plasma membrane components, particularly glycosylphosphatidylinositol(GPI)-anchored proteins and lipids, in mammalian cell lines.[17,42,44-48] The *C. elegans* polarity phenotype of GSL-depleted animals, where a full apical membrane domain emerges on the original lateral membrane, now provides the first evidence for such a role. Moreover, the ability of SL biosynthesis to restore wild-type domain polarity on expanding membranes of epithelia with inverted polarity defines GSLs as bona fide membrane domain identity determinants.

Their function in polarity is, however, strictly dependent on growth. SL biosynthesis seems dispensable for the polarity of non-growing larval and adult intestines, and also for the polarity of early embryonic intestines where some growth still occurs. Why are SLs not required for polarity in the early embryonic intestine if they regulate polarized membrane biogenesis? Barring possible distinct stage-specific programs for polarized membrane biogenesis, the effect of SL depletion on membrane growth might only be revealed in non-dividing, non-migrating postmitotic cells whose growth far exceeds that of embryonic cells that must divide within the confined space of the egg. Alternatively, intact early polarity could simply reflect insufficient interference with the lipid compound, as opposed to its biosynthetic enzymes. Maternal lipids, in contrast to maternal RNAs, are not depleted by RNAi, nor absent in balanced mutant strains. Supporting their early function in polarity, *C. elegans* GSLs were recently shown to function in oocytes and embryos, in spite of their restricted CGT expression pattern that had initially been thought to limit their function to only a few cells.[49,50] Furthermore, *C. elegans pod-2* and *fasn-1*, both required for GSL biosynthesis via fatty acid synthesis, were independently shown to affect polarity at the first cell stage.[51]

Mammalian GSLs' apical sorting ability is believed to be linked to their lateral assortment, with or without cholesterol, into nanoscale membrane microdomains (rafts) and their in vitro functional analysis largely depends on isolating such "detergent-resistant" membrane microdomains.[47,52,53] While *C. elegans* is thought to contain rafts,[54,55] its membranes may lack cholesterol, and its rafts may therefore consist exclusively of GSLs.[56] The strict dose-dependency of GSLs' effect on *C. elegans* intestinal polarity is more consistent with their membrane-dependent, raft or other, function in polarity rather than a signaling-mediated function for which a threshold response would be more typical.[57]

The lipid raft theory on polarity proposed that polarization is driven by the lateral self-organization of saturated lipids (such as GSLs) on endomembranes, providing platforms for the partitioning of apical membrane components and thereby maturing apical endo- into plasma membranes.[4] No conclusive evidence for such a process has yet been demonstrated in metazoan epithelia, although there is evidence for a role of SL biosynthesis in plasma-membrane-directed biosynthetic trafficking in plants and fungi.[58-60] A dedicated "apical vesicle" has also not yet been identified, but such a vesicle may be elusive if its constituents change en route to the membrane. Intriguingly, it was recently shown that different populations of yeast secretory vesicles are generically enriched for SLs.[61]

The here-demonstrated SL-dependent in vivo polarization of expanding *C. elegans* membranes could now support the idea that metazoan biosynthetic traffic has the ability to constitutively polarize the plasma membrane. This ability, however, does not need to depend on raft formation. Indeed, our subsequent findings suggest that adaptors (AP-1) and vesicle coat components (clathrin) are additionally required to target these vesicles to the apical membrane. Our identification of GlcCer-OH as the polarity-affecting lipid compound furthermore suggests a possible alternative mechanism of SLs' role in the polarization process: GlcCer, uniquely positioned at the cytoplasm-directed membrane leaflet, is well-placed to directly recruit adaptors, vesicle coat components or other polarity regulators. In any case, whether or not these elements are raft-dependently or independently recruited, their requirement suggests a new model for such a vesicle-intrinsic sorting process that implicates both SLs and coat components in the process.

Membranes contain per protein ~100 lipid molecules, whose diverse roles may be increased by a potentially huge number of specific modifications.[62] The importance of membrane lipids for polarity could be dramatically underestimated given the current technical constraints for their analysis and their inaccessibility in simple genetic screens. Membrane lipids, present on both endo- and plasma membranes, are well placed to integrate trafficking and membrane-associated polarity cues, as exemplified by the critical polarity function of phosphoinositides, the only vesicle-based sorting signals shown to determine both polarized trafficking and polarized plasma domain identities.[63,64] Like GSLs, they are required for lumen morphogenesis in 3D-MDCK cell-culture systems, although little is known about their function in in vivo morphogenesis.[18]

Since GSLs represent only a minor portion of saturated membrane lipids, other saturated membrane lipids may play similar roles in the same or in other polarized tissues. Such a role would be of particular interest in the highly polarized, SL-rich nervous system. Indeed, mutations in the rate-limiting serinepalmitoyltransferase SPT, the human *sptl-1* ortholog, cause

hereditary sensory and autonomic neuropathy type I (HSAN1), a disease of the nervous system whose pathogenesis is still unclear and that includes morphogenesis defects (F. Eichler, pers. comm.).[65,66]

CHC-1/Clathrin and Its AP-1 Adaptor Cooperate with GSLs in Apical Sorting During De Novo Membrane Biogenesis

Our tubulogenesis screen also identified the clathrin heavy chain (*chc-1*) and two clathrin/AP-1 adaptor subunits genes (*apb-1* and *aps-1*) with polarity/ectopic-lumen mutant phenotypes that largely copied the defects generated by interference with SL biosynthesis (please also see ref. 67).

This finding was unexpected, since neither clathrin nor AP-1 was previously implicated in apical biosynthetic trafficking, nor in defining apical membrane domain identities or in promoting lumen morphogenesis. The post-Golgi vesicle coat component clathrin is best known for its role in endocytosis and signaling at the plasma membrane.[17,68] Its recently identified role on trafficking routes toward the plasma membrane was thought to be limited to basolateral trafficking in epithelial cells, partially dependent on AP-1B, and, more recently, also on AP-1A, adaptor subunits.[69,70]

The characteristics of the *C. elegans* SL depletion phenotype also apply to the clathrin/AP-1-dependent phenotypes: displacement of all tested apical molecules, including PAR-6; ectopic lateral lumen formation; seemingly intact apicolateral sealing junctions but excess junctions around lateral lumens; dependency on growth; reversibility. One significant difference, however, is that *chc-1* RNAi additionally generates early embryonic intestinal polarity defects in dividing and intercalating cells (**Fig. 2A–C**). Interference with each of three out of four AP-1 subunits results in a later emerging milder phenotype, although the severity of triple or quadruple null mutants has not yet been evaluated. Since clathrin, like GSLs, is maternally required and essential in oocytes, complete loss-of-function conditions cannot be examined for either.[50,71] However, although RNAi cannot deplete maternally-derived lipids, it should effectively—if not entirely—destroy maternal *chc-1* RNA. Several lines of evidence suggest that SLs, clathrin and its AP-1 adaptor disrupt the same process but to a different degree: (1) the similarity of the polarity defects generated by any one molecule; (2) the severity of phenotypes being proportional to the degree of interference with either; and (3) the mutual enhancement of the phenotype in all reduction-of-function combination conditions tested, including transheterozygotes. Therefore, the *chc-1(RNAi)* early polarity phenotype may represent the most severe, although still not complete, loss-of-function phenotype and all these molecules may also contribute to polarized membrane biogenesis in the early embryo.

The independent identification of CHC-1 and two of its AP-1 adaptor subunits, three classical vesicle components, in an unbiased screen on apical membrane biogenesis strongly implicates vesicular trafficking in this process, strengthening GSL's proposed role in polarity via trafficking while in turn supporting clathrin's role on an apical route. Interference with clathrin/AP-1, like interference with SL biosynthesis, also reduced the biogenesis and subapical placement of several post-Golgi vesicle subpopulations, particularly RAB-11+-vesicles with documented functions in apical/lumenal membrane biogenesis in vertebrates and invertebrates, including *C. elegans*.[2,72,73] Furthermore SL-biosynthetic enzymes and clathrin/AP-1 genetically interacted in apical sorting; CHC-1- and GSL-rich vesicles partially co-localized, particularly in the vicinity of Golgi membranes and their biogenesis and asymmetrical localization to polarized membrane domains depended on each other and on AP-1 (**Fig. 2D**). It therefore appears that these molecules could directly converge on a directional trafficking path or even vesicle membrane, another unexpected finding given the distinct roles of clathrin and lipid rafts in the biogenesis of endocytic carriers.[74,75] Collectively, our findings suggest that SL-rich endomembranes recruit clathrin via its AP-1 adaptor at Golgi membranes and/or endosomes to promote the biogenesis of an apically-destined vesicle, a scenario compatible with the presence of an intrinsic vesicular sorting mechanism.

Among a number of trafficking components recently implicated in apical membrane and lumen biogenesis, SLs/clathrin/AP-1 seem to be the only bona fide sorting molecules. As such they could be positioned upstream of the RAB-11/RAB-8-dependent trafficking pathway required for in vitro lumenal membrane biogenesis in MDCK cells.[2] However, the *C. elegans* and the MDCK tubulogenesis models are not directly comparable: MDCK lumens form in mitotic cells that move into an initial 4-fold symmetry, thus corresponding to the early embryonic pre-intercalation, not the postmitotic, *C. elegans* intestine that was examined here. Knockdowns of RAB-11 and RAB-8 in MDCK cells also result in the formation of ectopic lumens. However, these lumens emerge in the cytoplasm rather than on the lateral membrane, suggesting interference with apical transport, not sorting per se (the same was observed for *rab-11* RNAi in the *C. elegans* intestine[67]). Although both sets of molecules displace the polarity regulators CDC-42/PAR-6, it remains to be demonstrated whether this is the underlying mechanism for their membrane-polarizing function in either the in vitro mammalian or in the in vivo *C. elegans* system. In a recent screen on apical RAB-11-associated vesicle and transmembraneous PEPT-1 placement in the *C. elegans* intestine, numerous trafficking components were identified whose losses caused cytoplasmic, however not basolateral-membrane, displacement of apical components. The failure of this screen to recover sorting molecules could be due to its high-throughput/low-resolution approach and its focus on the adult rather than the larval intestine.[73] It will be interesting to examine whether some of these components cooperate on a trafficking route that depends on the here-described apical sorting mechanism.

A Model for an Intrinsic Vesicular Sorting Mechanism Defining Domain Identities on Growing Plasma Membranes

Could the SL/CHC-1/AP-1-dependent apicobasal polarity alteration be caused by

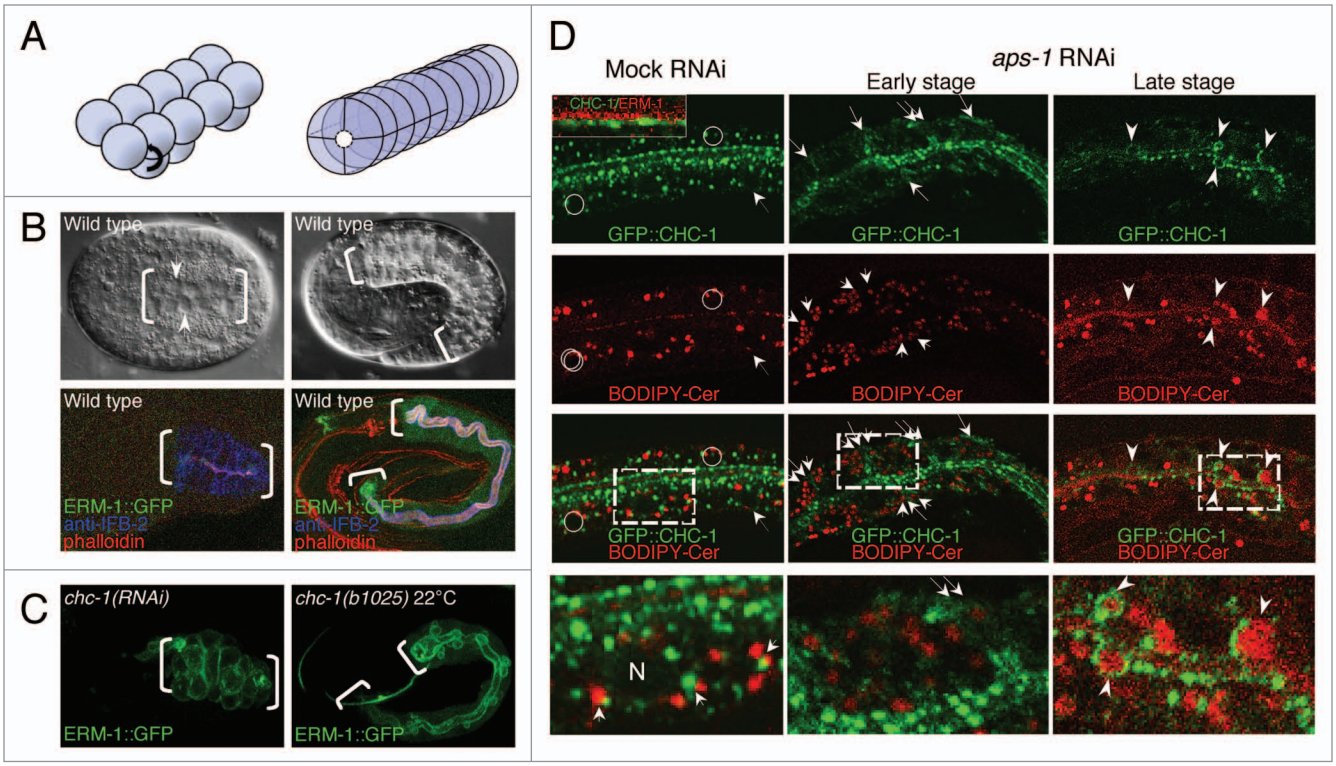

Figure 2. Polarity conversion in the early embryonic intestine and dynamics of clathrin-coated and ceramide-rich vesicles during polarity conversion. (**A**) Schematic of the pre- and post-intercalation wild-type embryonic *C. elegans* intestine. Left: E16 stage: 10 dorsal and 6 ventral cells (3 ventral cells obscured in this view); cells move in direction of the arrow during intercalation (2 cells will still divide). Right: E20 stage: postmitotic cells fixed in a bilaterally symmetrical tube of 9 INT rings (4 cells in first INT, 2 in all others); this tube is rolled up within the 3-fold embryo in the egg (as shown in B and C). (**B–C**) Corresponding stages (pre-intercalation left, post-intercalation right) of (**B**) wild-type and (**C**) CHC-1-depleted embryonic intestines (bracketed); DIC images top, confocal images below (see **Figure 1** legend for markers). (**B**) Two rows of intestinal nuclei in upper left image (corresponding to the upper layer of cells shown in A above) are indicated with arrows. Triple staining in confocal images renders lumenal ERM-1/actin/IFB-2 overlay pink (compare with displaced green ERM-1::GFP in mutant [C]). Phalloidin also stains pharyngeal actin (left side of right image) and muscle. (**C**) Lateral displacement of ERM-1::GFP in a *chc-1(RNAi)* intercalating intestine and ectopic lateral lumen formation in the post-intercalation intestine of a *chc-1(b1025ts)* mutant animal at the restrictive temperature (compare with pink lumenal ERM-1::GFP in wild-type images above). ERM-1::GFP staining of excretory canals is also visible on the left of the right image. (**D**) Distribution of GFP::CHC-1 and BODIPY-Cer in *mock(RNAi)* (left) vs. *aps-1(RNAi)* intestines during early (middle) and late (right) polarity conversion. Confocal images of L1-larval intestinal sections are shown, GFP::CHC-1 top row, BODIPY-Cer second row, merged images in third row with boxed areas magnified in fourth row. Left column: wild-type pancytoplasmic GFP::CHC-1 and BODIPY-Cer vesicles assemble perinuclearly (examples of nuclei are indicated by circles, long arrows or N); CHC-1::GFP also forms a pearls-on-a-string pattern below the lumenal cytoskeleton (inset [lumen top, cytoplasm bottom] shows relation to submembraneous ERM-1 [red]) and BODIPY-Cer labels the lumenal membrane. High magnification image shows partial association (short arrows) and partial overlap (yellow) of GFP::CHC-1 and BODIPY-Cer vesicles around nuclei. Middle column: basolateral displacement of GFP::CHC-1 (long arrows; placement on both sides of lateral membrane indicated by double arrows) and BODIPY-Cer vesicles (short arrows) during early-stage polarity conversion. Note the dissociation and lateral (cytoplasmic) position of BODIPY-Cer vesicles relative to lateral GFP::CHC-1 vesicles. Right column: GFP::CHC-1 and BODIPY-Cer assemble at ectopic lumens (arrowheads) during later-stage polarity conversion. Note that GFP::CHC-1 vesicles are now on the lateral (cytoplasmic) side surrounding the lateral ectopic lumenal BODIPY-Cer. Also note overall reduction of vesicle number in *aps-1(RNAi)* animals. Adapted from reference 6

a trafficking defect that does not involve sorting? One alternative possible cause, more in agreement with the prevailing view of polarity regulation, is a trafficking defect affecting apical junction integrity. SLs and clathrin/AP-1 could deliver or recycle molecules to the apicolateral junctions, thereby preventing leakage of apical membrane components to the basolateral membrane. Although neither we nor others[67] were able to detect junction defects in mutant/RNAi animals, the loss of minor but essential junction material—material not yet defined or below detection level—might precede and cause the polarity alteration. An SL and clathrin requirement in junction recycling is documented in other systems.[76,77] For example, some intestinal VACs (pathologic ectopic lumens of mammalian epithelia, see above) arise together with, and are thought to be caused by, junction integrity defects, but, in contrast to our findings, appear to involve endocytic clathrin/AP2-, but not clathrin/AP-1- or raft-dependent, mechanisms.[78,79] Analyzing these intriguingly similar apical-membrane-domain changes in parallel should be informative and could lead to different interpretations of findings in either system. For instance, junction components in both the mammalian and the *C. elegans* system could be linked to a vesicle-intrinsic apical sorting process and as such be displaced concomitantly with, or subsequently to, but not prior to, the polarity alteration.

Among possible sorting defects causing the apicobasal polarity conversion, a defect on a membrane-directed apical biosynthetic route is the simplest explanation. Supporting this viewpoint: (1) SLs/CHC-1/AP-1 are required to apically target several post-Golgi vesicle populations that not only include RAB-11+ presumed lumenal-membrane-forming vesicles, but also a string of clathrin-coated vesicles that we found located beneath, not above, the lumenal cytoskeleton and thus unlikely to represent endocytic coated pits; (2) clathrin-coated and SL-rich vesicles preferentially associate near the Golgi and endocytic recycling compartment,[75] documented sorting stations for membrane-directed transport;[17] and (3) AP-1 loss decreases the number and association of clathrin-coated and SL-rich vesicles and disrupts their apical localization while displacing them laterally during the initial phase of polarity conversion. This view is also supported by SLs' roles in other systems, including their in vitro function in apical sorting in mammalian cells and their in vivo functions on cell-surface-directed routes in plants and yeast. Intriguingly, several of the SL-biosynthetic enzymes identified in our *C. elegans* screen were also identified in a genome-wide screen in yeast as being required for the proper plasma membrane placement of a GFP marker.[59]

Disruptions of other trafficking routes could, however, also explain the SL/CHC-1/AP-1-dependent apicobasal polarity conversion. For example, a defect on an endocytic route could interfere with the removal of apically-destined components from the basolateral membrane. Clathrin is best known for its role in endocytosis although this role typically requires its AP-2 adaptor.[68,80] However, neither disrupting endocytosis by knocking down dynamin resulted in apical proteins appearing at basolateral intestinal membranes,[67] nor did we find any evidence for an AP-2 involvement in polarity (although this latter possibility was not rigorously excluded). Still, other components of the endocytic machinery might help in removing apical components from the basolateral membrane toward lysosomes, thereby placing clathrin/AP-1 on well-established endocytic and endosome-to-lysosome-directed routes, respectively.[68,81] Consistent with this scenario, AP-1 is thought to act on recycling endosomes in *Drosophila* sensory organ precursors, where it prevents endocytosed Notch and Sanpodo from trafficking back toward apical junctions.[72]

If we include membrane domain inhibitors in this discussion—a concept consistent with the prevailing idea of mutually inhibitory components of apical and basolateral membrane domains—the variety of trafficking defects that could explain the polarity conversion phenotype is further extended. For instance, disrupting a basolateral membrane-directed route could prevent molecules that inhibit apical membrane formation from reaching basolateral membranes. Defects on such a route are consistent with the recently identified role of clathrin/AP-1 in basolateral trafficking in mammalian epithelia (see above) and could also explain AP-1's role in sorting basolateral membrane components in the *C. elegans* intestine.[67] However, any model that considers polarity exclusively determined by domain inhibitors and endocytic routes makes additional assumptions on membrane biogenesis, such as the supposition of an initial default, for instance non-polarized, transport of membrane components that must be modified to achieve the final polarized state. Otherwise it has to explain by some other mechanism how "non-inhibited" membrane domain components (in our model, the apical membrane components) arrive at the other membrane domain (here, the basolateral membrane) during the process of polarity conversion.

Taking these considerations into account, our findings could support an intrinsic apical sorting model for polarized membrane biogenesis. This model combines aspects of lipid-dependent endomembrane self-organization with the requirement for vesicle coat components, two elements previously thought to define the major alternate modes of vesicle biogenesis (**Fig. 3**): (1) trans- or post-Golgi membranes laterally form SL-rich microdomains/rafts or simply accumulate specific lipids, e.g., GlcCer at the outer membrane leaflet, that (2) recruit the clathrin adaptor AP-1 which in turn (3) recruits clathrin to assemble a vesicle coat at Golgi and/or at several post-Golgi locations for the biogenesis of an apically-destined vesicle. Possible means for the apical transport of this vesicle are suggested in **Figure 3A**. The formation of SL-rich endomembranes is in the most upstream position in this model. Given their pleiotropic trafficking roles, clathrin and AP-1's specific sorting function could be generated context-dependently: for instance, while the GSL-AP-1/clathrin configuration confers apical directionality (as demonstrated in the *C. elegans* intestine), the configuration PtdIns(3,4,5)P3-AP-1/clathrin promotes basolateral targeting (as demonstrated in mammalian cell lines).[82] During membrane expansion, apical sorting may be the clathrin/AP-1 function that is most sensitive to interference.

Polarized transport to the apical membrane could occur on a direct apical-, an indirect apical-, an apical recycling and/or a transcytotic route (**Fig. 3B**). During the SL-depletion-initiated slow process of polarity conversion, ectopic lateral lumen formation proceeds in an apical-to-lateral direction and is preceded by a conspicuous accumulation of apical molecules directly beneath (lateral to) apical junctions. This could point to a defect in either the short apical arm of a transcytotic passage from the equivalent of a targeting patch,[83] or in an apicolateral endosomal recycling compartment that sorts apical from basolateral cargo.[84] Even if this sorting process is exclusively required for recycling during membrane biogenesis, it must, however, sort apical membrane components to rather than from the apical membrane. Apical cargo could be recruited at any step of this sorting process. We observe that not only apical membrane components, but entire vesicle populations are laterally displaced during polarity conversion and that this displacement occurs during the initial stages of this process. More than single apical polarity determinants (e.g., apical PARs via CDC-42[85]) or other apical membrane effectors (perhaps ERM-1) may thus be sorted, raising the possibility that vesicular sorting confers directionality to the process of membrane biogenesis itself, as originally proposed by early models of polarity.[3,4]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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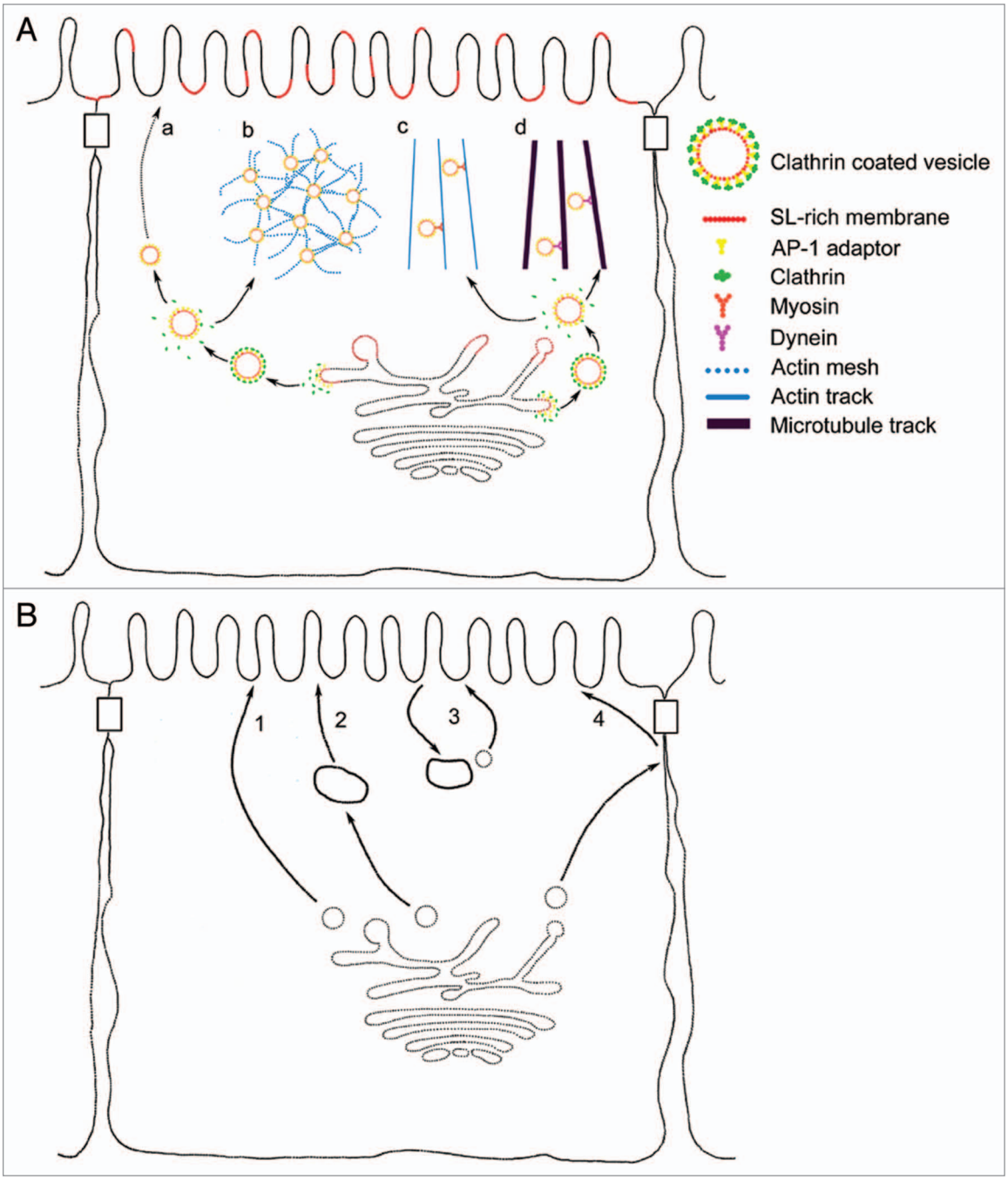

Figure 3. A model for a vesicle-intrinsic apical sorting process. Schematics of a tubular epithelial cell each are shown, with apical membrane and microvilli (top), separated by apical junctions (box) from basolateral membranes (bottom). Golgi membranes are indicated as a possible launch pad for apically-destined vesicles. (**A**) Apical vesicle biogenesis and possible modes of directional transport to the apical/lumenal membrane. Golgi membranes laterally form SL-rich microdomains/rafts or accumulate GlcCer (red); SL-rich rafts/GlcCer recruit the clathrin adaptor AP-1 (yellow), which in turn recruits clathrin (green) to assemble the coat for a vesicle that, likely after shedding its coat, moves apically (this process of vesicle biogenesis, magnified in this image for clarity, could also occur at other post-Golgi membranes, not shown). Apical movement of this "apically-primed" vesicle could occur either via: (a) self-maturation by accumulating further intrinsic endomembrane-organizing or extrinsic directional cues, the latter exemplified by RAB conversions shown to directionally shift vectors of endocytic and exocytic routes;[86,87] or by employing directional tracks, for instance (b) an actin mesh (blue dotted lines) nucleating on vesicle membranes, as demonstrated for the formin-dependent apical transport of RAB-11+-vesicles in mouse oocytes;[88] (c) an actin/myosin track (blue/orange), a mechanism supported by the involvement of atypical myosins in MVID (see text); (d) a microtubule (MT) track, for instance via a minus-end directed motor such as dynein (purple/pink) that promotes vesicle transport to the apical membrane in polarized epithelia; precedence for an AP-1/MT connection promoting vesicle transport to the cell periphery is provided by AP-1's link to the plus-end directed kinesin motor KIF5 via Gadkin in HeLa cells.[89] (**B**) Possible trafficking routes to the apical/lumenal membrane. Directed transport to the apical membrane could occur on: (1) a direct apical route, (2) an indirect apical route via specific endosomes, (3) an apical recycling route, (4) a transcytotic route via lateral membranes. See text for further discussion.

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