Epithelial cells carry out key vectorial functions for host multicellular organisms that depend on the polarized distribution of plasma-membrane proteins into apical and basolateral domains. The first observations of the vectorial properties of epithelial cells date back to the 1940s: physiologists, using radioactive tracers in combination with electrophysiological procedures, showed that epithelial cells could transport nutrients vectorially against steep electrical and chemical gradients (reviewed in REF. 1). In the following three decades (see the TIMELINE), electron microscopists described the complex asymmetric structure of epithelial cells, including the polarized organelles in the secretory pathway and the junctional complex (TIGHT JUNCTIONS, ADHERENS JUNCTIONS and DESMOSOMES) at the border of the apical and lateral regions of the cell (the apical–lateral border). In the late 1970s, the fundamental question of how epithelial cells establish and maintain their polarized phenotype became experimentally approachable when Cereijido, Misfeld and co-workers made the groundbreaking discovery that Madin–Darby canine kidney (MDCK) cells develop an electrically tight epithelial monolayer when plated on a permeable substratum (BOX 1). The MDCK model was refined to study polarized protein targeting after it was shown that influenza virus assembles from the APICAL SURFACE and vesicular stomatitis virus (VSV) assembles from the BASOLATERAL SURFACE (reviewed in REF. 1).

The experimental advantages that were provided by virus-infected MDCK cells (such as high biosynthetic levels of envelope glycoproteins and no interfering pool of endogenous plasma-membrane proteins) led to the identification of the TRANS-GOLGI NETWORK (TGN; the distal region of the Golgi complex) as the sorting compartment in the biosynthetic route for apical influenza HA and basolateral VSVG (BOX 1). In the late 1980s, epithelial cell biologists characterized the biosynthetic, endocytic, recycling and transcytotic routes of MDCK cells and other epithelial cell lines using sensitive protein-targeting assays (BOX 1). This knowledge led to the concept of the flexible epithelial phenotype — that is, that different epithelial cell types can change the final localization and transport routes of apical and basolateral proteins to carry out tissue-specific vectorial functions (reviewed in REF. 1). Insights into the mechanisms that are responsible for this variation first emerged in the late 1980s and early 1990s with the discovery of apical and basolateral sorting signals (reviewed in REF. 1). In the 1990s, the identification of cellular components that regulate polarized vesicular trafficking (BOX 2) and motors in the organization of post-Golgi trafficking (reviewed in REF. 1) provided a real-time view of sorting processes in epithelial cells, including key roles for actin, microtubules and motors in the organization of post-Golgi trafficking.
**REVIEWS**

**Timeline | Discoveries in the field of epithelial polarity: the impact of the MDCK model.**

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
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<tbody>
<tr>
<td>1940</td>
<td>Cell fractionation discovered.</td>
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<tr>
<td>1946</td>
<td>Electron microscopy developed.</td>
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<tr>
<td>1949</td>
<td>Secretory pathway in exocrine pancreas.</td>
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<td>1952</td>
<td>Polarized MOCk cells grown on monolayers and cultured on permeable substrates.</td>
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<td>1963</td>
<td>Sorting of apical and basolateral proteins in Golgi complex.</td>
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<tr>
<td>1974</td>
<td>Ca^2+^-switch protocol developed.</td>
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<tr>
<td>1978</td>
<td>Transcytotic routes in MDCK, intestinal and liver cells.</td>
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<tr>
<td>1984</td>
<td>Zona occludens-1 identified as first tight junction component.</td>
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<tr>
<td>1985–1987</td>
<td>Common recycling endosomes, immediately at the exit of the TGN (routes 1, 2).</td>
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<tr>
<td>1985–1990</td>
<td>Different epithelial cells vary widely in the use of direct and transcytotic routes for apical proteins.</td>
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<tr>
<td>1986</td>
<td>Until recently, it was assumed that most apical proteins are transferred to the apical surface of MDCK cells directly from the TGN, but a recent paper has introduced lively controversy on this issue. The authors found that addition of tannic acid, a mild fixative, to the basolateral medium of live MDCK cells prevented the delivery of newly synthesized glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) to the apical surface, but did not interfere with the transport of another apical protein, the p75 neurotrophin receptor (p75NTR). Apical GFP-tagged GPI-APs and basolateral VSVG were present in common tubular transporters, which indicates that some apical proteins might be sorted after arrival at the basolateral surface. These latest results indicate that the transcytotic mode of apical transport might be more widespread among epithelial cells than was previously thought, a proposition that needs to be further tested by the field.</td>
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**Analysis of genetically tractable model organisms:**

Saccharomyces cerevisiae, Caenorhabditis elegans and Drosophila melanogaster — led to the discovery of a growing list of 'polarity genes,' the products of which are clustered around the junctional complex. Recent work has provided fascinating details of their complex interactions and roles in epithelial morphogenesis but their role in polarized vesicular trafficking remains largely unknown. In the late 1990s and the new millennium, the advent of green fluorescent protein (GFP) and its derivatives, combined with high-resolution optical-imaging techniques (Box 1), started to provide a real-time view of the sorting processes that occur in the Golgi complex and recycling endosomes of polarized cells. This provided an increased understanding of the fundamental role that is carried out by the cytoskeleton in the organization of the delivery and fusion of transport vesicles to the plasma membrane.

Here, the mechanisms that have a role in vesicular trafficking to apical and basolateral membranes are reviewed, with a predominant focus on MOCk cells, in which most of this work has been carried out. A historical perspective, which highlights the main discoveries in the field, the current status, and the remaining open questions, is provided. We focus sequentially on the mechanisms that: first, carry out the initial segregation of apical and basolateral proteins into different transport intermediates; second, mediate the formation and movement of these transport intermediates to the cell surface; and, third, determine docking and fusion at specific sites in the plasma membrane. Because of the restrictions in reference numbers and length, the review focuses on vesicular trafficking and omits exciting parallel discoveries on cell–cell junctions or epithelial morphogenesis and the role of cell–cell adhesion, unless the findings are directly relevant to vesicular-trafficking mechanisms.

**Trafficking routes in epithelial cells**

The biosynthetic, endocytic, recycling and transcytotic routes of MDCK cells are summarized in Fig. 1. Although it was initially believed that most of the protein sorting in the biosynthetic route of MDCK cells was carried out in the TGN (routes 1 and 2 in Fig. 1), recent work indicates that some apical–basolateral sorting in the biosynthetic route could occur in common recycling endosomes, immediately at the exit of the TGN (routes 3, 4 and 5 in Fig. 1). Common recycling endosomes are also the main sorting site for membrane proteins that are internalized from the apical and basolateral surfaces. Different epithelial cells vary widely in the use of direct and transcytotic routes for apical proteins (Box 2). Until recently, it was assumed that most apical proteins are transferred to the apical surface of MDCK cells directly from the TGN, but a recent paper has introduced lively controversy on this issue. The authors found that addition of tannic acid, a mild fixative, to the basolateral medium of live MDCK cells prevented the delivery of newly synthesized glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) to the apical surface, but did not interfere with the transport of another apical protein, the p75 neurotrophin receptor (p75NTR). Apical GFP-tagged GPI-APs and basolateral VSVG were present in common tubular transporters, which indicates that some apical proteins might be sorted after arrival at the basolateral surface. These latest results indicate that the transcytotic mode of apical transport might be more widespread among epithelial cells than was previously thought, a proposition that needs to be further tested by the field.
A second group of apical signals comprises N-glycans\textsuperscript{37} and O-glycans\textsuperscript{28,29} — the latter are usually present in juxtanemembrane regions of transmembrane proteins. Removal of N- or O-glycans results in the non-polarized release of luminal secretory proteins in MDCK cells\textsuperscript{27,32}. Removal of N- and O-glycans blocks the exit from the Golgi complex of two apically targeted plasma-membrane proteins\textsuperscript{91}. N- and O-glycans usually function as recessive signals relative to basolateral sorting signals (described below); their wide distribution in plasma-membrane and secretory proteins explains the frequent finding that removal of basolateral signals from a basolateral protein results in its apical localization\textsuperscript{10,28,32}.

A third group of apical sorting signals encompasses proteinaceous motifs in the exoplasmic, transmembrane or cytoplasmic domains of the protein. Complete deletion of N-glycans does not alter the apical secretion of some soluble glycoproteins\textsuperscript{33}, endogenous plasma-membrane proteins\textsuperscript{44} or exogenous glycoproteins\textsuperscript{90}, which implies that there are also proteinaceous apical targeting motifs. The transmembrane domain of influenza HA contains apical sorting information, which mediates incorporation of the protein into lipid rafts (discussed below). The cytoplasmic domain of the light-sensitive protein rhodopsin\textsuperscript{35} has binding sites for the microtubule motor protein cytoplasmic dynein, which mediates translocation of rhodopsin to the apical membrane in MDCK cells. The cytoplasmic domains of megalin\textsuperscript{36,37} and other apical proteins contain apical sorting signals that have not yet been fully characterized\textsuperscript{38}.

Apical sorting receptors? Apical sorting signals that mediate association with dynein are thought to function by promoting microtubule-mediated directional transport to the apical surface (discussed later in this review). By contrast, the sorting mechanisms that are used by other apical signals are still poorly understood. They could function as conventional sorting signals — for example, by interacting with an apical sorting receptor that promotes incorporation into an apical transport vesicle. The existence of apical sorting receptors was implied by experiments showing that the apical secretion of some soluble non-glycosylated proteins became saturated at high expression levels and could be outcompeted by the overexpression of a second apical protein\textsuperscript{39}. However, these receptors have not yet been identified. N-glycans have been proposed to sort apically by interacting with a lectin sorting receptor\textsuperscript{32}. A candidate lectin, VIP36, localizes to the TGN when it is overexpressed. However, endogenous VIP36 is present mainly in the cis-Golgi, and its possible role in apical protein sorting is now considered doubtful\textsuperscript{40}. Finally, there are many exceptions to the ability of N-glycans to function as conventional apical sorting signals, which implies that they might have a different, unconventional role in apical protein sorting.

The lipid-raft hypothesis. An alternative to the conventional sorting-receptor model for apical sorting is presented by the lipid-raft hypothesis, which was put forward by van Meer and Simons in 1998\textsuperscript{(REFS 41,42)}. The lipid-raft hypothesis postulates that many proteins are sorted apically because they have an affinity for microdomains of glycosphingolipids and cholesterol (lipid rafts) that are assembled in the Golgi complex. According to this hypothesis, lipid rafts and their associated proteins form sorting platforms that, on recognition by specialized machinery in the TGN, recycling endosomes or plasma membrane, become incorporated into apical transport intermediates that deliver them to the apical membrane (FIG. 1). The experimental evidence supporting the lipid-raft hypothesis is substantial and is discussed in BOX 3. Examples of proteins that show affinity for lipid- rafts are GPI-APs and influenza HA; the association of these proteins with lipid rafts is mediated by the GPI anchor and by the transmembrane domain, respectively, and occurs at the level of the Golgi complex (BOX 3).

However, full acceptance of the lipid-raft hypothesis for apical sorting has been compromised by inconsistencies that have, so far, been difficult to explain. For example, several GPI-APs are found on the basolateral surface of MDCK cells, even when they are associated...
with lipid rafts according to the usual criterion of insolubility in non-ionic detergents at 4°C (discussed in REF. 43; see BOX 3). Some GPI-anchored proteins also require the presence of N-glycans before they can localize apically, even though the presence of N-glycans does not always promote additional association with lipid rafts. Finally, lipid rafts are difficult to visualize by optical microscopy; indeed, recent studies indicate that lipid rafts are small and highly dynamic structures with a capacity to accommodate a maximum of 3–5 GPI-APs.

**Box 1 | The Madin-Darby canine kidney (MDCK) cell model**

**MDCK monolayers**

MDCK cells were the first epithelial cells that were used to form electrically tight monolayers on permeable filters in vitro (see figure, part a). MDCK cells that were grown on filters were also used to develop all of the techniques that are available to study polarized molecular targeting. These include:

- Biotinylation and protease assays to study steady-state polarity and domain-specific biosynthetic protein delivery.
- Assays that use mechanically or biochemically (streptolysin-O-) permeabilized cells to experimentally manipulate post-Golgi exocytosis.
- Assays to study the establishment of polarity, such as the Ca²⁺ switch, collagen embedding or the collagen overlay of MDCK cells.

**Polarized viral assembly**

The envelope glycoproteins of viruses that bud from either the apical (influenza haemagglutinin) or basolateral (vesicular stomatitis virus G protein) domains of MDCK cells are still widely used apical and basolateral markers. They provided the first evidence of polarized biosynthetic routes and sorting at the Golgi complex (see figure, part b). IS, intercellular space.

**Live-cell-imaging of polarized post-Golgi exocytosis**

Apical or basolateral cargo proteins that are tagged with green fluorescent protein (GFP) accumulate in the trans-Golgi network of polarized MDCK cells at 20°C (REFS 23,88). Their exit from the Golgi complex and delivery to the cell surface is visualized using time-lapse fluorescence microscopy, total-internal-reflection microscopy (see figure, part c) or confocal microscopy (see figure, part d).
A recent report, together with older data, provides some clues to the mystery of how lipid rafts might contribute to apical sorting: Zurzolo and co-workers showed that GPI-anchored GFP is targeted to the apical membrane of MDCK cells, but is missorted to the basolateral membrane when mutations are introduced into GFP that prevent its natural tendency to oligomerize. These experiments agree with decade-old data by Hannan et al. that show that newly synthesized GPI-APs arrive at the apical surface of MDCK cells in large immobile clusters (as determined by FRAP), which progressively disperse into small mobile clusters (as determined by FRET). That the formation of large clusters of GPI-APs is required for their apical sorting is shown by studies with lectin (concanavalin A)-resistant MDCK cells that prevent the ability of GPI-APs to form larger clusters in the apical route. These GPI-APs are missorted to the basolateral membrane. Parallel experiments imply that clustering is important in the general endocytic sorting of GPI-APs. Crosslinking of GPI-APs by antibodies that are directed against their ectodomains promotes their incorporation into pre-existing caveolae in fibroblasts and into newly formed caveolae at the apical surface of MDCK cells. (Like most epithelial cells, MDCK cells have only basolateral caveolae, but they develop apical caveolae after crosslinking of GPI-APs.)

The experiments that are described above indicate that the lipid-raft hypothesis might be refined as follows: apical proteins are sorted by association with small lipid rafts that are converted into functional apical sorting platforms by a ‘clustering event’ (see FIG. 1 and insets; also discussed in REF. 43). This modified lipid-raft hypothesis gives rise to secondary hypotheses, which could be tested using available experimental data or future experiments. The first secondary hypothesis is that any feature of a lipid-raft-associated protein that promotes oligomerization could promote apical targeting. This could be tested using approaches that are similar to those used in REF. 43. The next secondary hypothesis is that the formation of clustered lipid rafts might be promoted by a luminal lectin (FIG. 1, insets). On the basis of available data with lectin-resistant MDCK cells, a luminal lectin with affinity for mannos-rich N-glycan cores could participate in such clustering. The third secondary hypothesis is that the formation of functional lipid rafts could be promoted by proteins that promote the coalescence of small lipid rafts into larger rafts. Indeed, there is evidence that caveolin-1, a cholesterol-binding protein that resides on the cytoplasmic side of lipid rafts, forms large homo-oligomers in the apical route (FIG. 1, insets). Luminal and cytoplasmic clustering of lipid rafts could facilitate apical sorting of GPI-APs not only at the TGN but also at other sorting stations in the biosynthetic or recycling routes (FIG. 1).

So, a luminal lectin with an affinity for the mannos core of GPI-APs might promote their clustering and sorting into the apical route, in cooperation with oligomerized caveolin on the cytoplasmic side of the membrane (FIG. 2, insets). There is no evidence yet that a similar mechanism could operate for other lipid-raft-associated apical proteins. In the case of influenza HA, the evidence supports a role for the proteolipid MAL (myelin and lymphocyte protein) in apical targeting, but whether this protein participates in lipid-raft clustering is not known.

Basolateral sorting mechanisms
Basolateral signals. Basolateral signals are usually located in cytoplasmically exposed regions of the protein; their participation in biosynthetic and recycling trafficking in MDCK cells is shown in FIG. 1. Mostow and co-workers showed in 1986 that deletion of the cytoplasmic domain of polymeric immunoglobulin-A receptor prevented its basolateral localization and in 1991 that the signal was transplanted to other proteins. Other two groups showed in 1991 that basolateral signals could be created by introducing mutations that generate endocytic motifs in the cytoplasmic domains of the apical proteins influenza HA and p75NTR.

The finding that basolateral proteins had sorting signals was surprising, as it was then thought that transport to the basolateral plasma membrane occurred by default — an extension of an earlier hypothesis on biosynthetic transport to the cell surface in non-polarized cells. Many studies have since confirmed that basolateral signals are localized in the cytoplasmic domain of basolateral proteins and consist of tyrosine65,57 or dileucine69 motifs, which are often in the vicinity of patches of acidic amino acids, as well as other characteristic motifs (recently reviewed in REF. 60). More recent work has uncovered a new type of basolateral signal that consists of a single leucine motif61. Mutations in basolateral signals might result in disease — for example, a form of familial hypercholesterolaemia is caused by a mutated signal in the LDL receptor.

Relative to single-span basolateral proteins, such as those discussed above, less information is available on the sorting signals of hetero-oligomeric membrane proteins. Many ion or nutrient transporters are non-glycosylated multispan proteins that require chaperoning by a glycosylated single-span protein for transport to the cell surface. Among the most well-studied examples are the Na+/K⁺-ATPase, which is found basolaterally in all mammalian epithelia (with the exception of the enteric epithelia, the retinal pigment epithelium and choroid plexus) and the H⁺-ATPase, which is found apically in the parietal (hydrochloric-acid-secreting) cells of the stomach and in other cells. In these two proteins, the apical and basolateral exocytic-trafficking signals are contained in the multispan non-glycosylated α-subunit. The glycosylated β-subunit in the H⁺-ATPase contains a tyrosine-based endocytic motif that mediates re-incorporation into a regulated secretory compartment that, when the cell is stimulated, is exocytosed to the apical plasma membrane. However, trafficking signals do not solely account for the basolateral-surface localization of the Na+/K⁺-ATPase. The α-subunit also has binding sites...
for ankyrin — a spectrin-binding protein that is linked to the basolateral actin cytoskeleton — which mediates its selective retention at the basolateral membrane. Finally, the Na+/K+-ATPase might be retained at the lateral membrane through its β-subunits, which have adhesive properties. Alternative mechanisms for basolateral- (and apical-) domain-selective retention include interaction with scaffold proteins, such as proteins that contain PDZ domains (reviewed in Refs 38,60).

A recognized role of both apical and basolateral trafficking signals is to promote the sorting of cargo proteins into different vesicles at the Golgi complex, plasma membrane or recycling endosomes. Removing sorting signals from certain basolateral proteins by recombinant-DNA techniques causes their retention at the Golgi complex. Adding peptides that contain the tail of VSVG to the cytoplasm of MDCK cells blocks the vesicular release of the VSVG protein from both the Golgi complex and the endoplasmic reticulum but does not affect the exit of the epidermal growth factor receptor (which has a different basolateral signal) from these organelles. This indicates that basolateral-like signals operate at various levels of the secretory pathway. Similar peptide-blocking experiments in fibroblasts, which lack apical–basal polarity, showed that basolateral signal peptides selectively abrogated the exit of exogenous basolateral proteins from the TGN, but did not affect the exit of exogenous apical proteins.

Figure 1 | Trafficking routes and sorting mechanisms in epithelial cells. Apical exocytic routes (1 and 4): glycosylphosphatidylinositol (GPI) anchors, N-glycans and O-glycans sort apical proteins at the trans-Golgi network (TGN), common recycling endosomes (CREs) and apical recycling endosomes (AREs). This sorting might involve clustering small lipid rafts into larger functional lipid rafts, a process that might be promoted by a luminal lectin, caveolin oligomers and MAL (inset). Basolateral exocytic routes (2 and 3): basolateral signals interact with adaptors of the clathrin (Adaptor protein-1 (AP1), AP3, GGA (Golgi-localized, γ-ear-containing, Arf-binding protein)) or non-clathrin (AP4) type at the TGN or CREs. AP1B operates at the level of CREs (route 5; inset); the participation of other adaptors in basolateral sorting is hypothetical. AP4 might mediate basolateral sorting through microtubule motors. Newly synthesized lysosomal membrane proteins (LAMPS) seem to be transported to the lysosome via the basolateral membrane155 (route 2 followed by route 7 to basal sorting endosomes (BSEs), late endosomes (LEs) and lysosomes (LYS)), although some authors believe they follow a direct intracellular route (3c)152. Endocytic routes (6 and 7): endocytosed apical (route 6) or basolateral (route 7) membrane proteins are internalized into apical sorting endosomes (ASEs) or BSEs by AP2, mixed in CREs15,22,153 and are sorted into apical and basolateral exocytic routes by sorting signals that are similar to those used in the biosynthetic route68,70 (routes 4 and 5). Soluble proteins are sorted from membrane proteins in ASEs and BSEs, mixed in LEs and degraded in LYS. Biosynthetic route through endosomes (routes 3a–c): some newly synthesized basolateral proteins reach CREs directly from the TGN15,22,76–78 (routes 3a and 3b; via unknown adaptors) from where they are sorted to the basolateral membrane via AP1B (route 5). Manose-6-phosphate receptors and their ligands (lysosomal hydrolases) move through clathrin-coated vesicles, possibly into LEs (route 3c) from where they are transported back to the TGN. Recycling and transcytosing apical membrane proteins transfer from CREs to AREs (route 4). Some GPI-anchored proteins seem to use a transcytotic route (route 8) in Madin–Darby canine kidney cells23.
Retromer complex
A protein complex that consists of Vps35, Vps26, Vps29, Vps17 and Vps8, which was discovered through genetic screens in Saccharomyces cerevisiae. It functions in the retrieval of proteins from the prevacuolar compartment and transport to the Golgi.

Accordingly, some biochemical properties that are associated with polarized pathways (for example, lipid-raft association) are preserved in fibroblastic cell lines\(^7\), which implies that vesicular-trafficking pathways with ‘apical’ and ‘basolateral’ features might be present in non-epithelial cells that are normally thought to be ‘non-polarized’.

Adaptors
A striking realization that came from the discovery of basolateral sorting signals was their remarkable resemblance to the signals used by receptors that are endocytosed through coated pits from the plasma membrane, or to signals that are used for endocytosis and transport to lysosomes\(^6,15\).

Early experiments had indicated that similar basolateral sorting signals operate in the recycling and biosynthetic routes\(^7\). These findings are now understood, as endocytic, recycling and basolateral sorting signals belong to a family of peptide signals that interact with a family of organelle-specific adaptor protein (AP) complexes\(^5\). The interaction of basolateral signals with organelle-specific APs (which can be thought of as sorting receptors) explains the finding that the basolateral sorting of the LDL receptor can be saturated by overexpression of the protein, which results in apical transport of the excess protein\(^56\).

Available evidence indicates that two members of a family of heterotetrameric APs — AP1B\(^7\) and AP4 (REF. 73) — are involved in the sorting of basolateral membrane proteins (FIG. 2). AP1B shares subunits \(\beta, \gamma\) with AP1A, but has an epithelial-specific medium subunit (\(\mu1B\)). This subunit is absent in LLC-PK1 cells (a porcine epithelial cell line), which explains the apical mis-sorting of transferrin receptors and LDL receptors in these cells\(^72\). Transfection of the \(\mu1B\) subunit into LLC-PK1 cells enables the AP1B complex to assemble and reverse the targeting defect\(^7\). AP1B promotes the

Figure 2 | Machinery that controls polarized vesicular trafficking in epithelial cells. The vesicular-trafficking routes correspond to those in FIG. 1. Sorting signals promote the incorporation of apical proteins at the trans-Golgi network (TGN) or in common recycling endosomes (CREs) into clustered lipid rafts that recruit microtubule motors (such as kinesin) directly or through unknown adaptors; in some cases (rhodopsin) the motor (dynein) is recruited through direct interactions. The motors generate tubular elements that move along microtubules (inset). Basolateral proteins are incorporated into clathrin-coated or into uncoated tubules (which are pulled by unidentified microtubule motors that are presumably different from those used by apical proteins). Cdc42 stimulates the exit of apical proteins and inhibits the exit of basolateral proteins from the TGN through unknown downstream effectors and might have similar functions at CREs. Fusion of apical transporters is mediated by the GTPase dynamin\(^1\), protein kinase D (PKD) promotes vesicle release for the basolateral route\(^7\) (inset). Dynamins 1 and 2, the adaptor DAB2 and the GTPase ARNO regulate specific endocytic steps\(^6\). Other regulators of protein trafficking include phosphatidylinositol 4-kinases\(^1\), heterotrimeric G-proteins\(^4,12\) and retromer\(^5\). Microtubule and actin motors (myosins I, II, Vb and VI) probably participate at several stages of endocytic and biosynthetic trafficking\(^1\). Hip1R, a linker between actin and clathrin, promotes the release of clathrin-coated vesicles that contain mannose-6-phosphate receptors from the TGN\(^2\). The production of a vesicle from a donor compartment is coordinated with delivery to the acceptor compartment by various compartment-specific Rab proteins\(^4,13\). The exocyst, together with RaA and Rab8, coordinates basolateral exocytic routes, whereas Rab25 and Rab11 coordinate apical recycling\(^1\). ARE, apical recycling endosome; ASE, apical sorting endosome; BSE, basal sorting endosome; LE, late endosome; LYS, lysosome.
basolateral sorting of the LDL and transferrin receptors at recycling endosomes rather than at the TGN, as has been established by recycling assays and colocalization with endosomal, rather than TGN, markers. Recent work indicates that VSVG protein might transit through recycling endosomes en route to the plasma membrane (see routes 3a and 3b in Figs. 1, 2), in agreement with previous data for other basolateral proteins. Experiments with antibodies that block the function of AP1B indicate that AP1B might sort basolateral proteins in a post-TGN compartment at the crossroads of both the recycling and the biosynthetic routes. This could depend on the different microtubule motor, interacts with the C-terminal end of the cell surface. These different processes are coordinated with their incorporation into vesicular and tubular transport intermediates and the transport of these intermediates across the cytosol to specific areas of the cell surface. These different processes are coordinated with various factors including organelle-specific Rab proteins, elements of the actin and microtubule cytoskeletons and a particle known as the exocyst (Fig. 2).

The microtubule cytoskeleton. Extensive experiments with microtubule-disrupting agents showed a selective requirement for microtubules in the transport of apical membrane proteins (reviewed in Ref. 82) but did not determine at what step microtubules were required. Recent live-cell imaging experiments indicate important roles for microtubules and microtubule motors in the exit of apical proteins (and possibly some basolateral proteins) from the TGN and endosomes (Fig. 2, inset) and in their transport across the cytosol. In non-polarized cells and in fully polarized MDCK cells, GFP-tagged apical and basolateral markers were shown to leave the Golgi complex in long, tubular transport intermediates and small vesicles that move along microtubule tracks.

Three lines of evidence imply that specific microtubule motors participate in the sorting/transport of apical proteins. First, antibodies that were directed against the motor protein kinesin blocked the production of tubules that contained the apical marker p75NTR-GFP; they also blocked transport from the Golgi area to the cell surface of small vesicles containing p75NTR-GFP, which were presumably released from the Golgi by an independent mechanism. These antibodies cause basolateral mis-sorting of apical markers in polarized MDCK cells (G.K., unpublished data). Second, cytoplasmic dynein, a minus-end-directed microtubule motor, interacts with the C-terminal end of rhodopsin through the dynactin light chain TCEX, and is crucially involved in the apical transport of rhodopsin in MDCK cells. Third, the kinesin-family member KIFC3, another minus-end-directed microtubule motor, has been associated with the apical delivery of influenza HA and annexin-13B (Ref. 90); however, the motor function of KIFC3 in this process has still not been shown. These experiments are consistent with the observation that microtubules orientate their negative ends towards the apical pole of polarized...
epithelial cells (discussed below). Microtubules also form arrays of mixed polarity underneath the apical membrane, which implies that plus-end-directed motors might also participate in apical delivery.

**The actin cytoskeleton.** The actin cytoskeleton has an increasingly recognized role in the assembly of vesicular transport intermediates at the Golgi complex and on recycling endosomes, and in their translocation across the cytoplasm. Actin, actin-associated proteins (such as spectrin, ankyrin and myosins I, II and VI) and actin-regulatory proteins (Cdc42 and several of its downstream effectors, and dynamin) are present in the Golgi complex (reviewed in REF. 91). Interfering with the function of Cdc42 using constitutively active or inactive mutants slows the exit of the basolateral protein N-CAM (neuronal cell-adhesion molecule) but accelerates exit of the apical protein p75NTR from the TGN. Inhibiting actin polymerization causes missorting of basolateral proteins from recycling endosomes and promotes their transcytosis to the apical surface.

How do actin filaments work in apical–basolateral sorting? They could function conventionally as tracks for the myosin-driven movement of vesicles by Rab — as has been suggested for myosin VI and Rab11 in apical recycling. In vitro assays indicate that a myosin activity promotes the release of basolateral, but not apical, vesicles from the TGN of MDCK cells, but the identity of this myosin activity remains controversial.

Alternatively, by analogy with its role at the plasma membrane, actin might participate in a less conventional role — that is, in the fission of vesicles from the Golgi complex in response to dynamin or downstream effectors of Cdc42 (REF. 97) (Fig. 3, inset). Dominant-negative dynamin inhibits the release of p75NTR from the TGN of MDCK cells and the release of VSVG–GFP in non-polarized cells. Finally, actin could negatively regulate the release of some proteins from the TGN by forming a physical barrier that must be removed for vesicular budding to occur.

In summary, the microtubule and the actin cytoskeletons have important regulatory roles in sorting apical and basolateral proteins in the TGN, recycling endosomes and at the plasma membrane. The mechanisms underlying this regulation and the roles of specific actin and microtubule motors in the generation of transport intermediates are still largely unknown. The microtubule and actin cytoskeletons are also involved at specific steps in the transport of vesicular and tubular transport intermediates across the cytosol (Fig. 2). Future work needs to elucidate in detail the participation of these motors in the transport of specific cargos and their regulation.

**Polarized exocytosis of transport intermediates**

The release of transport intermediates that carry apical and basolateral proteins from the TGN and endosomes must be coordinated with their transport across the cytoplasm and their insertion in the plasma membrane at specific sites. Work over the past 10 years has identified various components of the exocytic machinery that transport apical and basolateral proteins and has started to identify the mechanisms that regulate their localization and function (Fig. 3).

Apical exocytosis is mediated by a tetanus-toxin-insensitive vesicle membrane (v)-SNARE (ti-V AMP) and by the target membrane (t)-SNAREs syntaxin-3 and SNAP23 (REFS 99–101). Epithelial-specific Munc18-2 regulates the assembly of the apical SNARE complex.

Annexin-2 and annexin-13B might promote docking of

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**Box 3 | Sorting by lipid rafts**

The influential lipid-raft hypothesis postulates that many apical proteins are sorted through their affinity for microdomains of glycosphingolipids and cholesterol (lipid rafts) that are initially assembled in the Golgi complex. This proposal is supported by strong experimental evidence:

- First, many apical proteins, such as influenza haemagglutinin (HA) and glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs), become insoluble in non-ionic detergents at 4°C as they reach the Golgi complex. Detrimental insolubility reflects lipid-raft association, as raft lipid components are insoluble in non-ionic detergents at 4°C.
- Biophysical techniques such as fluorescence recovery after photobleaching (FRAP), which measure lipid-raft association more accurately than detergent insolubility does, show a good correlation between apical targeting for HAs and mutations in the transmembrane anchor that disrupt association with lipid rafts.
- Second, epithelial cell types that fail to incorporate GPI-APs into detergent-insoluble complexes missort these proteins to the basolateral membrane.
- Third, depletion of glycosphingolipids or cholesterol results in the missorting of GPI-APs and influenza HA on their way to the apical plasma membrane of Madin–Darby canine kidney (MDCK) cells. Additional work is necessary to show that missorting results from the disruption of lipid rafts in the sorting compartment rather than indirectly from defective trafficking of key apical sorting factors or from increased signalling by ceramide precursors in cells that have been exposed to inhibitors of glycosphingolipid synthesis.
- Finally, lipid rafts are enriched in the apical membrane. There is strong evidence for the presence and sorting of lipid rafts in the endocytic and recycling pathways but there is no definitive evidence that raft lipids have the intrinsic ability to be sorted apically in the trans-Golgi network (TGN). Early results in MDCK cells have been contested by later evidence. Furthermore, it remains unclear whether lipid sorting drives protein sorting or vice versa. Progress in this area has been slowed by technical difficulties with available raft lipids tagged with fluorescent dyes; these lipids frequently lose their association with lipid rafts, which makes the interpretation of live-cell-imaging studies difficult.
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Figure 3 | The exocytic machinery of MDCK cells. At the apical surface: apical vesicles in the direct route contain a tetanus-insensitive v-SNARE (ti-VAMP) that heterotrimersizes with the apical syntaxins SNAP23 and syntaxin-3 (REF. 98), a process that is regulated by Munc18-2. Apical syntaxins are restricted apically by microtubules, using unknown mechanisms. Unknown SNAREs mediate the fusion of transcytotic and recycling apical vesicles with the apical membrane. At the basolateral surface: basolateral vesicles carry a tetanus-sensitive v-SNARE (ts-VAMP) that interacts with syntaxin-4 and SNAP23, which are components of the basolateral docking/fusion machinery. The syntaxins localize to the lateral membrane independently of microtubules. Many proteins that are involved in regulating basolateral exocytosis (indicated by a green background) are orthologues of regulators of the secretory pathway in Saccharomyces cerevisiae. These include Cdc42, exocyst, Rab8 (Sec4 in yeast) and Lethal giant larva (Lgl; Sro7/Sro77 in yeast). Lgl interacts with syntaxin-4 but a vesicular-trafficking regulatory role has not been shown. The recruitment of exocyst to the junctional area is promoted by nectin (REF. 107) and is a key determinant of the fusion of vesicles in this area. The products of three sets of physically and/or genetically interacting polarity genes (Crumbs–Pals1–Par1; apical protein kinase C (pPKC)–Par3–Par6; scribble–discs–large–Lgl) along with PAR1 and PAR4 are also likely to have important vesicular-trafficking regulatory roles. These gene products are restricted to either the apical or lateral domain, and are often concentrated just above or below the apical junctional complex. Their crosstalk ensures the maintenance of distinct epithelial surface domains. SNARE, soluble NSF (N-ethylmaleimide-sensitive fusion protein) accessory protein (SNAP) receptor; VAMP, vesicle-associated membrane protein.

SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein (SNAP) receptor). SNARE proteins are a family of membrane-tethered coiled-coil proteins that regulate fusion reactions and target specificity in vesicle trafficking. They can be divided into v-SNAREs and t-SNAREs on the basis of their localization.

The exocyst. Further factors are required, in addition to the SNARE machinery, to promote translocation of transport intermediates to the apical or basal poles of cells. Basolateral vesicle delivery requires tethering of the vesicles by a highly conserved complex of eight proteins that is known as the exocyst (FIG. 3). A tethering factor for vesicles in the apical route has not been identified yet. Exocyst antibodies selectively block the transport of basolateral proteins in M DCK cells but do not inhibit the delivery of apical proteins (REF. 108). Interestingly, the exocyst, has a second role at sorting organelles — it probably participates in the formation of basolateral transport vesicles from a perinuclear compartment (REF. 109) — and a third role at the endoplasmic reticulum, where it selectively modulates the synthesis of basolateral proteins (REF. 107).

Consistent with these different functions, the exocyst localizes not only at the junctional complex (REF. 108), but also at a perinuclear compartment (REF. 109), which is more likely to comprise recycling endosomes than the TGN (REF. 88). Both of these localizations might be regulated by AP18 (REF. 110), whereas the junctional localization of the exocyst is promoted by E-cadherin and nectin (REF. 108). The small GTPases RaA (REF. 110) and Rab8 (REF. 111; the yeast orthologue of which (Sec4) interacts genetically with the exocyst) have activities that are consistent with the regulation of the vesicular-trafficking roles of the exocyst. In M DCK cells, dominant-negative Rab8 inhibited basolateral exocytosis (REF. 112), and constitutively active Rab8 promoted missorting of basolateral proteins (REF. 113). The dual regulation of both production and tethering of basolateral vesicles by the exocyst resembles the dual trafficking role of Rab proteins and probably contributes to the homeostasis of the basolateral membrane.

The cytoskeleton and polarized trafficking. Growing evidence indicates that exit from the sorting organelles (TGN and recycling endosomes) and targeting to the plasma membrane are also coordinated by the actin and microtubule cytoskeletons, which show a division of function in this regard. Whereas microtubules seem to be important in the organization of apical exocytosis, the actin cytoskeleton seems to be an organizer for basolateral exocytosis.

Microtubules and apical delivery. The organizing role of the microtubule cytoskeleton in apical exocytosis is shown by live-cell-imaging experiments in non-polarized and polarized M DCK cells (FIG. 4). TOTAL-INTERNAL-REFLECTION FLUORESCENCE MICROSCOPY, which allows high-resolution analysis of cell–substrate contact areas, shows extensive fusion of post-Golgi transport intermediates that contain p75NTR–GFP with the basal plasma membrane of non-polarized M DCK cells (REF. 88) (and also, presumably, with the rest of the plasma membrane, which cannot be imaged with this technique). By contrast, post-Golgi transport intermediates that contain p75NTR–GFP fuse with neither the basolateral nor the lateral membrane in polarized M DCK cells; therefore, they must fuse with the apical surface, although this event has been difficult to image so far (REF. 88) (FIG. 4). The restriction of fusion sites to the apical membrane...
cells, syntaxin-4 shifts from a random localization to a lateral membrane distribution. That syntaxin-4 has a broader localization than just basolateral exocytic sites indicates that additional factors (such as the exocyst) contribute to the localized fusion of basolateral transport intermediates near junctional areas of the plasma membrane.

The basolateral delivery routes to the lateral membrane and the lateral localization of syntaxin-4 are not antagonized by microtubule disruption; instead, evidence is accumulating that the actin cytoskeleton and its regulators are involved in this process (FIG. 3). Several orthologues of gene products that control the docking and fusion of post-Golgi vesicles in *S. cerevisiae* regulate basolateral exocytosis. Paramount in both yeast and MDCK systems is Cdc42. Expression of dominant-negative or activated Cdc42 disrupts both post-Golgi targeting and endosomal recycling of basolateral proteins in MDCK cells115,116. Consistent with this observation, depolymerizing the actin cytoskeleton disrupts basolateral targeting of recycling basolateral proteins93,94.

**Epithelial lumen formation**

**The role of PAR proteins.** Simple (non-stratified) epithelial cells position their lumen at the apex in columnar epithelia (which is therefore known as the apical domain, for example, in the kidney and intestine) or at the lateral

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**Figure 4 | Microtubules organize vesicular trafficking to the apical pole.**

- **a** | Total-internal-reflection fluorescence microscopy shows fusion of apical and basolateral post-Golgi transport intermediates with the basal surface in subconfluent Madin–Darby canine kidney (MDCK) cells. These fusions are not observed in polarized MDCK cells88. In polarized MDCK cells, basolateral post-Golgi transport intermediates (which contain low density lipoprotein (LDL) receptors) fuse with the upper third of the lateral membrane as determined by spinning disc confocal microscopy88 (see also BOX 1). The restriction of basolateral fusion sites to the lateral membrane correlates with a restricted localization of fusion (syntaxin-4) and tethering (exocyst) machinery (see FIG. 3). In polarized MDCK cells, post-Golgi transport intermediates that contain the apical marker p75 neurotrophin receptor (p75NTR) tagged with green fluorescent protein fuse directly with the apical surface. Microtubule depolymerization results in a dispersed Golgi complex and the fusion of apical, but not basolateral, post-Golgi transport intermediates with the basal membrane; these fusions are inhibited by antibodies to syntaxin-3. Microtubule depolymerization does not disrupt the fusion of basolateral post-Golgi transport intermediates with the lateral membrane.
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Figure 5 | PAR1 controls epithelial microtubule organization and lumen morphogenesis. a | Madin–Darby canine kidney (MDCK) cells cultured at 5 μM Ca2+ lack cell–cell contacts127; show a centrosomal microtubule array with the negative ends emanating from a juxtanuclear MICROTUBULE-ORGANIZING CENTRE (MTOC) and accumulate luminal (apical) markers (green) in large intracellular vacuoles that are known as vacuolar apical compartments (VACs)121; b | After addition of Ca2+, MDCK cells establish E-cadherin-mediated cell–cell contacts. VACs are exocytosed, forming intercellular lumina (green) that are surrounded by tight junctions (TJ; red) — these are reminiscent of liver bile canaliculi122. Microtubules reposition to a horizontal position, with the MTOC immediately under the lumen123 — such an arrangement is typical of hepatocytes125. c | Several hours after the addition of Ca2+, the lateral lumen is displaced to the apical surface122 and the microtubules acquire a non-centrosomal vertical arrangement that is typical of columnar epithelial cells121,129, PAR1 promotes the formation of lateral lumina and inhibits the progression to columnar morphology.

BILE CANALICULI
Tiny channels on the surface of liver cells that collect the bile that they produce.

14-3-3 PROTEIN
A scaffold protein that regulates the localization of other proteins by binding to conserved phosphotyrosine-containing motifs in a phosphorylation-dependent manner.

MICROVILLI
Small, finger-like projections (1–2-μm long and 100-nm wide) that occur in the exposed surfaces of epithelial cells to maximize the surface area.

MICROTUBULE-ORGANIZING CENTRE (MTOC). Also called the centrosome or spindle-pole body, this structure nucleates and organizes microtubules.

Non-polarized MDCK cell
Liver polarity stage
Columnar MDCK cell

Protein that, when prevented from binding 14-3-3 proteins by mutation of a potential PAR1 phosphorylation site121, interferes with MDCK lumen formation in a similar manner to PAR1 (REF. 128). In some systems, PAR4 (another serine/threonine kinase, also known as LKB1) activates PAR1, which implies that there are possible common polarizing signalling routes that are mediated by PAR1, PAR3 and PAR4 (REF. 129). Recent work130 has shown that PAR4, under the influence of the adaptor protein STRAD (STE20-related adaptor protein), promotes polarization of intestinal epithelial cells with polarized apical MICROVILLI and luminal proteins, in the absence of cell–cell contacts — that is, in single cells.

PAR1, however, seems to be unique in that it promotes not only lumen formation per se, but it also regulates lumen position. Overexpression of PAR1 locks MDCK cells in a ‘liver polarity phenotype’, with lateral lumina that resemble bile canaliculi121 (FIG. 5) and, interestingly, promotes a transcytotic route for apical proteins, which is also a characteristic of liver epithelial cells131. These experiments imply that levels of PAR1 activity might control the developmental decision between the establishment of columnar or hepatic epithelia, a hypothesis that needs to be tested experimentally. The lumen polarity and traffic-organizing functions of PAR1 might be mediated by its known ability to regulate microtubules. Inhibition of PAR1 function prevents the change in the organization of microtubules from the radial centrosomal array that is found in non-polarized MDCK cells (and in other non-polarized cells such as fibroblasts) to the non-centrosomal, vertical microtubule arrays that are characteristic of polarized epithelia (FIG. 5). Overexpression of PAR1 in MDCK cells organizes microtubules in horizontal arrays with their minus ends facing lateral lumina, which is similar to the organization that is found in liver cells121.

Concluding remarks
The study of the generation and maintenance of epithelial polarity has been, and will continue to be, a challenging endeavour, as polarization involves the coordination of all of the main subcellular systems in...
response to cues that are provided by cell–cell and cell–substrate contacts. The establishment of polarized trafficking requires precise coordination between mechanisms that are involved in the sorting of plasma-membrane proteins into post-Golgi and post-endosomal transport vesicles, and the delivery of these vesicles to restricted sites at the cell surface. Several apical and basolateral routes exit the Golgi complex and fuse randomly with the cell surface in non-polarized cells. The reorganization of the microtubule cytoskeleton that occurs during epithelial polarization under the control of signaling proteins such as PAR1 and PAR4 leads to the apical delivery of luminal markers, probably by restricting specialized docking and fusion machinery to the apical surface. The organization of basolateral delivery to the apical third of the lateral membrane is, instead, under the control of Cdc42, some of its downstream effectors, and the actin cytoskeleton — the same protagonists that are involved in the secretory pathway from the mother cell to the budding daughter cell in S. cerevisiae.

Future research will uncover important details of the machinery that is involved in Golgi and endosomal sorting, including the roles of lipid rafts, sorting signals, adaptins and the cytoskeleton. Individual microtubule- and actin-based motors that participate in the exit of various classes of proteins from the TGN must be identified. The molecular interactions that underlie the control by the microtubule cytoskeleton and the actin cytoskeleton of the establishment of luminal and basolateral docking sites for post-Golgi routes will be elucidated.

MDC2 cells will continue to be an important tool in this task, as they are the most well-studied mammalian epithelial cell and have provided the context for the development of the most incisive cell-biological technology with which to test hypotheses and candidate genes in polarized vesicular trafficking.

The first demonstration that basolateral sorting information exists in the cytoplasmic domain.

69. Reports a key role for exocyst in basolateral targeting.

75. Reports a key role for exocyst in basolateral targeting.
A balance between microtubule-associated proteins is a key organizer of microtubules and lumen polarity in epithelial cells.


