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Review

Polarized traffic towards the cell surface: how to find the route

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Polarity is the structural and functional hallmark of epithelia. The apical plasma membrane, facing the organism's exterior (the lumen of the gut, renal tubule and glandular duct), differs in many important respects from the basolateral plasma membrane that is apposed to the interior of the organism. The generation and maintenance of epithelial polarity require a highly specialized subcellular machinery to bring proteins to their appropriate sites of action. This is a dynamic process involving the interpretation of sorting signals, vectorial delivery mechanisms, membrane-specific fusion and retention processes. Here, we will provide a review of the field, highlighting recent advances within a historically relevant context.

Introduction

The fundamental feature of cell polarity is that the cell's plasma membrane is divided into discrete domains. Examples include the membranes of axons and dendrites in neurons, the growing bud and mating projection in the yeast *Saccharomyces cerevisiae* and lamellipodia in fibroblasts. However, the best-studied type of cell polarity is found in epithelia, which is the most common type of tissue in animals.

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Key words: apical plasma membrane, endocytosed protein, glycosphyngolipid, influenza haemagglutinin, multidrug resistance protein, neurotrophin receptor.

Abbreviations used: AEE, apical early endosome; AP, adaptor protein; AQP4, aquaporin 4; BEE, basolateral early endosome, CASK, calcium/calmodulin-dependent serine protein kinase; Cdc42, cell division cycle 42; CFTR, cystic fibrosis transmembrane conductance regulator; CRE, common recycling endosome; ER, endoplasmic reticulum; FAPP2, PI(4)P (phosphatidylinositol-4-phosphate) adaptor protein; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; gp, glycoprotein; GPI, glycosylphosphatidylinositol; HA, haemagglutinin; KIF5B, kinesin family member 5B; KIFC3, kinesin family member C3; Kir 2.3, inwardly rectifying potassium channels 2.3; LDL, low-density lipoprotein; LLCPK-1, Lilly Laboratories cell porcine kidney cells; LPH, lactase-phlorizin hydrolase; MAL, myelin and lymphocyte protein (also called VIP17); MCAM, melanoma cell adhesion molecule; MDCK cell, Madin–Darby canine kidney cell; NaPi-Cap2, sodium phosphate C-terminal-associated protein 2; NaPiII, sodium phosphate co-transporter type 2; NCAM, neural cell adhesion molecule; NHERF, Na^+/H^+-exchanger regulatory factor; NKCC2, Na-K-2Cl co-transporter type 2; pIgR, polymeric immunoglobulin receptor; PLAP, placental alkaline phosphatase; PMCA2, plasma-membrane Ca^{2+}-ATPase 2; p75NTR, neurotrophin receptor p75; RE, recycling endosome; SAP97, synapse-associated protein 97; SNAP23, 23 kDa synaptosome-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor; Stx3, syntaxin 3; TGF-β, transforming growth factor-β; TGN, *trans*-Golgi network; TeNT, tetanus-neurotoxin, TJ, tight junction; t-SNARE, target SNARE; VAMP, vesicle-associated membrane protein; VIP36, vesicular integral protein 36; v-SNARE, vesicle SNARE; VSV-G, vesicular-stomatitis-virus glycoprotein.

The formation of specialized intercellular adhesive structures, the TJs (tight junctions), initiates the polarization process by demarcating two biochemically and functionally distinct domains: the apical and basolateral plasma membranes. The protein and lipid compositions of these surfaces are tailored to the particular functions of each domain, which include protecting against invasion by toxins and pathogens, absorption or secretion of nutrients, signalling and ion transport. Once the polarity is established, the maintenance of this polarity requires a highly specialized subcellular machinery to bring membrane proteins to their appropriate sites of action and to recycle them to one or the other domain.

According to the present understanding, polarized protein traffic in epithelial cells is governed by signals embedded within the structures of the proteins themselves. These sorting signals are read, interpreted and acted on by the intracellular trafficking machinery, which segregates and packages these membrane proteins into specialized membrane transport vesicles destined for apical and basolateral delivery. A large number of diseases are now recognized to be associated with failure of normal protein recycling from the membrane, protein mistargeting away from the correct cell surface compartment or to the wrong intracellular compartment.

Much of what has been learned through the analysis of the polarized trafficking in epithelial cells has provided insight into mechanisms operating during the sorting and trafficking to axonal and

somato-dendritic membrane domains in neurons. However, the mechanisms involved in these fascinating processes are beyond the scope of this review (see reviews by Kim and Sheng, 2004; Groc and Choquet, 2006; Sann et al., 2009).

Here, we provide a review of the polarized trafficking in epithelial cells, highlighting recent advances within a historically relevant context.

Trafficking routes in epithelial cells

To date three different routing pathways have been identified in epithelial cells.

Newly synthesized proteins can follow a *direct* route to their final apical or basolateral membrane destination. In this case, proteins are sorted soon after their synthesis and before reaching the plasma membrane, and thus they follow their biosynthetic pathway directly to their final destination. The TGN (*trans*-Golgi network) is the principal sorting station of this direct biosynthetic sorting pathway in epithelial cells. Within or soon after the TGN these apical and basolateral membrane-destined cargo proteins are segregated and then shipped directly to the appropriate polarized membrane domains.

This pathway was discovered in MDCK (Madin–Darby canine kidney) cells, which form a well-polarized monolayer when cultured on permeable supports (Cereijido et al., 1978). Pioneering work showed that viral glycoproteins such as the influenza HA (haemagglutinin) and the VSV-G (vesicular-stomatitis-virus glycoprotein) are rapidly and directly transported to their target membranes without any transitory stops on opposite plasma membrane domains (Rindler et al., 1984; Rodriguez-Boulan et al., 1984). Most recently, biochemical and live imaging studies have shown that newly synthesized proteins segregate into different vesicles on exit from the TGN and then exit in separate tubulovesicular carriers to pursue the direct route to the plasma membrane (Kreitzer et al., 2003; Christiansen et al., 2005; Paladino et al., 2006).

Although it was initially believed that most of the protein sorting in the direct route was carried out in the TGN, this relatively simple model has been challenged by evidence of sorting in pre-TGN compartments and by the observation that biosynthetic cargo may travel within intermediate compartments en route from the TGN to the plasma membrane (Paladino et al., 2004; Alfalah et al., 2005; Tveit et al., 2005; Vuong et al., 2006; Hein et al., 2009).

For instance, using an assay designed to measure the meeting of newly synthesized membrane proteins with endosomal compartments loaded with horseradish peroxidase, Orzech et al. found that the biosynthetic road travelled by pIgRs (polymeric immunoglobulin receptors) can involve the CREs (common recycling endosomes), suggesting that the CRE might also serve as a polarized sorting station on the direct pathway (Orzech et al., 2000). Evidence accumulated over a decade and consolidated in the most recent studies (Ang et al., 2004; Cancino et al., 2007; Cresawn et al., 2007; Gravotta et al., 2007; Cramm-Behrens et al., 2008) have shown that the biosynthetic route of at least some proteins includes a post-TGN transit through REs (recycling endosomes).

Proteins that arrive at the plasma membrane after either TGN or endosomal sorting may be retained at the membrane via direct or indirect interaction with cytoskeletal elements. Alternatively, they can undergo relatively rapid endocytosis and postendocytic sorting, which can also provide the chief polarizing mechanism for protein sorting. In fact, since nearly 50 % of a typical polarized plasma membrane is endocytosed per hour, this postendocytic sorting ensures that internalized proteins are recycled back to the appropriate plasma membrane.

Most endocytosed proteins are initially found in two spatially and biochemically distinct populations of early endosomes: BEEs (basolateral early endosomes) and AEEs (apical early endosomes). Each population of early endosomes supports a rapid and efficient membrane protein recycling to its cognate membrane without passing through intermediate compartments (Sheff et al., 1999; Rea et al., 2004). For instance, the transferrin receptors may rapidly recycle back to the basolateral plasma membrane through vesicular transport dependent on the small GTPase Rab4a, a marker of BEE (Maxfield and McGraw, 2004; Leonard et al., 2008). Most recently, Hellberg et al. identified protein kinase C as the critical signalling component, which regulates the sorting of the PDGFβ (platelet-derived growth factor β) receptor at the level of the early endosomes (Hellberg et al., 2009).

Proteins internalized in AEEs and BEEs can be directed to the CRE, which is characterized by a perinuclear localization and tubular morphology. Because

the CRE compartment recycles internalized proteins coming from both the apical and basolateral compartments, it is generally believed to be a major polarized sorting station (Brown et al., 2000; Wang et al., 2000; Thompson et al., 2007).

For instance, the CFTR (cystic fibrosis transmembrane conductance regulator) is rapidly endocytosed from the plasma membrane into the CRE compartment and undergoes efficient recycling back to the plasma membrane in human airway epithelial cells, with >75 % of endocytosed wild-type CFTR recycling back to the plasma membrane (Picciano et al., 2003; Bomberger et al., 2009).

In some polarized systems, it has been shown that resident apical proteins may also be sorted in an 'indirect' or transcytotic pathway. In this case, they are first sent from the TGN or endosomal compartment to the basolateral surface, from where they are endocytosed and subsequently transported to the opposite apical surface [for a review, see Mostov et al. (2000)].

Hepatocytes had been suggested to lack the direct route for the apical delivery of apical proteins and to rely only on the transcytosis pathway for apical protein delivery. However, a direct pathway for the apical delivery of the polytopic apical plasma membrane ABC transporter (ATP-binding-cassette transporter) proteins, MDR1 (multidrug resistance protein 1) and SPGS (sister of p-glycoprotein) has been proposed in liver cells, indicating that there are important exceptions to the long-standing model of an exclusive indirect transfer of resident apical proteins in liver cells (Sai et al., 1999; Kipp and Arias, 2000).

Until recently, it was assumed that the transcytosis was a typical feature of hepatic and intestinal cells, whereas the apical proteins were sorted to the apical surface of MDCK cells only following the direct route.

However, when expressed by transfection in MDCK cells, the pIgR and its ligand, IgA, are endocytosed from the basolateral membrane, sorted to apical endosomes and successively to the apical cell surface (Mostov et al., 1995; Oztan et al., 2007; Verges et al., 2007).

Interestingly, it has been shown that addition of tannic acid, a mild fixative, to the basolateral membrane of live MDCK cells prevented the delivery of newly synthesized GPI (glycosylphosphatidylinositol)-anchored proteins to the apical surface, suggesting that certain proteins that were thought to be sorted at the level of the TGN of MDCK cells (e.g. GPI-associated proteins) are now thought to follow a transcytotic route via the endosomal compartment (Polishchuk et al., 2004). In addition, using the same experimental approach, Chmelar et al. demonstrated for the first time that a G-protein-coupled receptor achieves its apical localization in MDCK cells via transcytosis (Chmelar and Nathanson, 2006), suggesting that the transcytosis pathway might be used more commonly by a variety of epithelial cells than was previously believed.

Alternatively, proteins can be randomly targeted to both membrane domains and achieve their asymmetric distribution by selective stabilization or retention at one cell surface following the so-called 'random' route (Wozniak and Limbird, 1996; Matter, 2000).

For instance, the CFTR is randomly sorted to the apical and basolateral membranes in MDCK cells and specifically retained in the apical membrane via interaction with a PDZ protein (Swiatecka-Urban et al., 2002). Like CFTR, when stably transfected in MDCK cells, the human $\alpha 5\beta$-1 integrin was integrated into both cell surface domains following its biosynthesis. The apical pool of the integrin was subjected to rapid degradation, whereas the basolateral pool was stabilized, leading to the typical basolateral localization observed in the steady state (Gut et al., 1998). The authors suggested the fascinating hypothesis that $\beta 1$ integrins participate in the determination of the spatial orientation in MDCK cells; thus they are constitutively transported to both cell surface domains acting as sensors that signal the presence of matrix to the interior of the cells. Thus selective retention can add another level of regulation of the polarized distribution of membrane components (Figure 1).

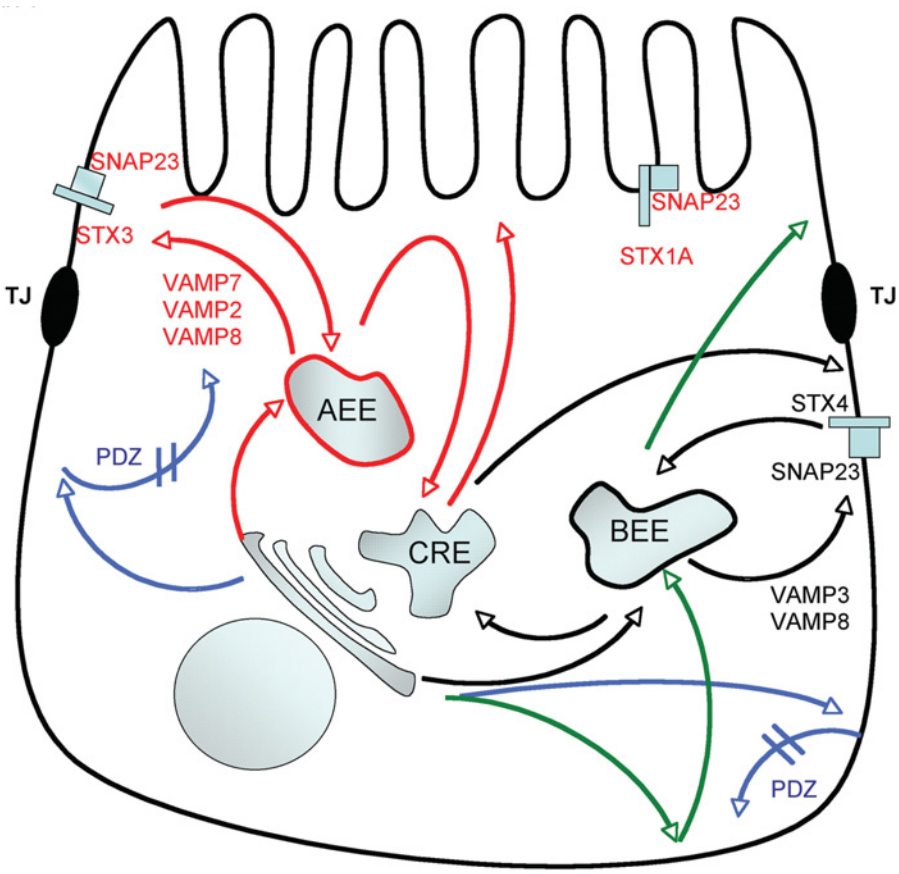
Sorting information

According to our present understanding, polarized trafficking is determined by signals contained within a membrane protein's structure. They are read, interpreted and acted on by the intracellular sorting machinery, which in turn shuffles, retains, or retrieves molecules to the appropriate membrane domain.

Direct demonstration of a sorting sequence requires its inactivation by mutagenesis/deletion. However, the type of mis-sorting phenotype produced depends on the presence or absence of other sorting signals that may be simultaneously contained within the polarized membrane protein. When the protein is devoid

Figure 1 | Trafficking routes in epithelial cells and the SNAREs involved in the membrane-specific fusion events

To date three different routing pathways have been identified in epithelial cells. Newly synthesized proteins can follow a direct route (red pathways for apical proteins and black pathways for basolateral proteins). In some polarized systems, apical proteins may also be sorted in a transcytotic route (green pathways). Alternatively, proteins can be randomly targeted to both membrane domains and achieve their asymmetric distribution by selective stabilization or retention at one cell surface following the so-called random route (blue pathways). Membrane fusion is the final and irreversible step of each trafficking route and is mediated by SNARE proteins. Under normal homoeostatic conditions, only the appropriate organelles fuse with the cognate membrane. SNARE proteins mediating fusion events at the apical (red) and basolateral (black) membrane are depicted. For clarity, this Figure does not include many other factors that have important roles in trafficking, but whose description is reported in the text and in Table 1.



of any other sorting information, ablation of the sorting signal will generally result in a non-polarized expression pattern. In contrast, removal of a basolateral sorting signal can unmask a subordinated apical sorting signal that drives the apical localization of the resulting protein. Once a particular sequence is identified as necessary for the polarized sorting of a protein, it is instructive to determine whether it is sufficient to act as an autonomous sorting signal. The experimental approach to address this issue usually involves transplanting the sorting sequence of interest on to a reporter protein that is otherwise expressed in a non-polarized fashion and examining whether it confers a polarized sorting phenotype.

Apical sorting signals

The first signal for polarized sorting to be rigorously established was the GPI, which when added to certain proteins resulted in the apical localization of these chimaeric plasma-membrane proteins (Brown et al., 1989; Lisanti et al., 1989; Powell et al., 1991). However, in Fisher rat thyroid cells, endogenous

GPI-anchored proteins are delivered to the basolateral plasma membrane (Zurzolo et al., 1993) and the typical GPI-anchored protein, PLAP (placental alkaline phosphatase), is expressed on the apical membrane by a GPI-independent mechanism (Lipardi et al., 2000). Moreover, the addition of a GPI-anchoring motif to rGH (rat growth hormone), a soluble protein that is secreted in a non-polarized manner, is not sufficient to ensure its apical targeting (Benting et al., 1999), confirming that GPI anchoring is not a universal mechanism for protein targeting to the apical membrane.

A second group of apical sorting signals comprises N-glycans and O-glycans. Early indications for an involvement of *N*-glycosyl chains in apical protein targeting came from studies using *N*-glycosylation inhibitors. Treatment of MDCK cells with tunicamycin, a GlcNAc analogue that inhibits the first steps of *N*-glycosylation, mis-sorts apical gp80 (glycoprotein 80; clusterin) to both membrane domains of MDCK cells (Urban et al., 1987).

Moreover, mutagenic removal of N-glycosylation sites in the gastric H^+/K^+-ATPase β-subunit (Vagin et al., 2004) or bile salt export pump (Mochizuki et al., 2007) significantly decreases their apical content and increases their intracellular accumulation. Conversely, sequential addition of one to five N-glycans to the basolaterally located Na^+/K^+-ATPase β1-subunit caused an increasing fraction of this subunit to be redirected to the apical membrane in HGT-1 cells (Vagin et al., 2005). Recent work also suggests that terminal glycosylation, rather than core glycosylation, of N-glycans seems to be important for apical sorting (Potter et al., 2004; Vagin et al., 2004).

O-linked glycosyl chains may also act as apical targeting signals. The p75NTR (neurotrophin receptor p75) and the hydrolase SI (sucrase isomaltase) are characterized by the presence of heavily *O*-glycosylated stalk domains in close proximity to the membrane. Deletion of these domains results in a shift in the sorting of these proteins from the apical plasma membrane to both surface domains (Yeaman et al., 1997; Jacob et al., 2000).

Despite several lines of evidence suggesting that N-glycans can act as apical sorting signals, this hypothesis remains controversial primarily because N-glycans are important for the processes that precede or follow the actual sorting event such as protein folding, quality control, ER (endoplasmic reticulum)-associated degradation, ER trafficking and retention of glycoproteins in the apical membrane. Moreover, there is a large population of basolateral plasma-membrane proteins that are glycosylated, demonstrating that the glycosylation is not, in itself, sufficient to specify selective apical targeting.

A third group of apical sorting signals includes proteinaceous motifs in transmembrane or cytoplasmic domains that can range from a few amino acids to stretches of up to 30 residues. Indeed, apical sorting signals of a variety of different compositions have been located in the cytosolic tails of rhodopsin (Chuang and Sung, 1998), megalin (Marzolo et al., 2003; Takeda et al., 2003), receptor guanylate cyclases (Hodson et al., 2006), M_2 muscarinic receptors (Chmelar and Nathanson, 2006) and the ATP7B copper-ATPase (Braiterman et al., 2009).

Conformational determinants are essential components of these apical sorting signals. For instance, for the gastric H,K-ATPase it has been suggested that TM4 (the fourth transmembrane spanning domain) and its flanking regions induce conformational sorting motifs that direct the ion pump exclusively to the epithelial apical membrane (Dunbar et al., 2000). Similarly, Grati et al. found that the apical targeting of the PMCA2 (plasma-membrane Ca^{2+}-ATPase 2) depends on the size but not on the sequence of an amino acidic stretch in the first intracellular loop, suggesting that the conformation of this cytoplasmic loop plays a role in apical sorting (Grati et al., 2006).

More recently, we identified two regions within the C-terminus of the renal NKCC2 (Na-K-2Cl cotransporter type 2), which specifically co-operate in generating a functional apical sorting signal. Our results suggest that the actual functional apical sorting signal might arise from a conformational cross-talk between these sorting motifs and their neighbouring sequences in the protein's structure (Carmosino et al., 2008) (Table 1).

Apical sorting machinery

The most likely hypothesis as to the nature of the apical sorting machinery 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.

This hypothesis is supported by strong experimental evidence. First, many apical proteins, such

Table 1 | Sorting signals and the elements of the sorting machinery

Signals	Examples	Elements of the sorting machinery
Apical		
Lipid rafts	HA, PLAP, GPI-anchored proteins	VIP17/MAL, galectin-4, FAPP2, annexin-13b, annexin-2, kinesin, KIFC3
Glycosylation	Clusterin (gp80), H,K-ATPase β-subunit, P75NTR, LPH, SI, glycoprotein g114	Lipid rafts, galectin-3, kinesin, KIF5B
Cytoplasmic sequences	Rhodopsin, megalin, receptor guanylate cyclase, M_2 muscarinic receptors, ATP/B, copper-ATPase, NKCC2, PMCA2	Dynein light-chain Tctex
Transmembrane sequence	H,K-ATPase α-subunit	
PDZ motifs	CFTR, Na^+/H^+ exchanger, NaPiII	NHERF, NaPi-Cap2
Basolateral		
Cytoplasmic YXXΦ (Φ-bulky hydrophobic) motifs	LDL receptor, VSV-G, pIgR, hTfnR (human transferrin receptor)), TGN38, AQP4	AP-1, AP-4, exocyst
Cytoplasmic [DE]XXXL[LI] motifs	E-cadherin, sulfate/bicarbonate/oxalate anion exchanger sat-1, MCAM, NKCC1 CD147, MHCII, furin	AP-1, exocyst
PDZ motif	Kir 2.3, GABA transporter, BGT-1, GABA transporter, GAT-2, Syndecan-1, α5β-1 integrin	Syntrophin, Lin-7/CASK, PSD-93, SAP97, Cdc42
Non-canonical sequences	Transferrin, NCAM, TGF-β receptor	

as GPI-anchored apical proteins and HA, become insoluble in non-ionic detergent at 4°C as they reach the Golgi complex. Detergent insolubility reflects lipid raft association since raft lipid components are insoluble in non-ionic detergent at 4°C (Brown and Rose, 1992). Moreover, depletion of glycosphingolipids or cholesterol results in the mis-sorting of GPI-anchored apical proteins and influenza HA on the way to the apical plasma membrane in MDCK cells (Keller and Simons, 1998).

This simple hypothesis was challenged by subsequent studies. For instance, cholesterol depletion did not affect the apical sorting of some GPI-anchored proteins (Lipardi et al., 2000) and lipid raft association did not correlate necessarily with apical membrane targeting, since some basolateral proteins can partition into raft-like compartments (Fujii et al., 2008; Lebreton et al., 2008). These observations suggest that lipid rafts seem to be necessary but not sufficient for apical membrane targeting.

Interestingly, Paladino et al. showed that GPI-anchored GFP (green fluorescent protein) is targeted to the apical membrane in MDCK cells, but is mis-sorted to the basolateral membrane when mutations are introduced to GFP that prevent its natural tendency to oligomerize (Paladino et al., 2004). Parallel experiments imply that clustering is important in the general sorting of GPI-associated proteins, suggesting that lipid rafts are converted into functional apical sorting platforms by a 'clustering event'.

Until now, a variety of clustering factors or lectin-raft-associated proteins have been identified. The VIP36 (vesicular integral protein 36) has been isolated by Simons and co-workers from detergent-insoluble fractions (Fiedler et al., 1994). This lectin is present in the Golgi apparatus, at the apical plasma membrane and in endosomal or vesicular structures, and has been assigned a putative role in glycoprotein trafficking from the TGN to the plasma membrane. Nevertheless, further studies have demonstrated that VIP36 is in fact involved in early trafficking steps, e.g. in trafficking from the ER to the Golgi apparatus, and may not represent an essential sorting factor for apical glycoproteins (Fullekrug et al., 1999; Hara-Kuge et al., 2002). Another candidate, the VIP17/MAL (myelin and lymphocyte protein) protein is present in lipid microdomains, interacts with GPI-apical proteins and may either escort its associated proteins to the apical membrane or retain associated proteins within the apical membrane (Cheong et al., 1999; Puertollano et al., 1999; Kamsteeg et al., 2007; Ramnarayanan et al., 2007). Interestingly, overexpression of VIP17/MAL in the kidney of transgenic mice results in dramatic amplification of the apical surface and ultimately to cyst formation in distal nephron structures, consistent

with the hypothesis that it plays an important role in apical sorting or in maintaining the stability of the apical membrane (Frank et al., 2000).

Moreover, recent reports demonstrated that VIP17/MAL clusters, formed either by spontaneous clustering of VIP17/MAL or by antibody-mediated cross-linking of FLAG-tagged VIP17/MAL, laterally concentrate markers of sphingolipid rafts in COS cells, placing MAL as a key component in the organization of membrane rafts domains (Magal et al., 2009).

Recent results have highlighted the involvement of a new lectin family, the galectins, in apical protein trafficking. Depletion of galectin-4 by RNA interference impairs raft formation and affects apical trafficking in polarized intestinal HT-29 cells (Delacour et al., 2005; Stechly et al., 2009). However, it should also be noted that galectin-3, a lectin of 29 kDa, has been identified in raft-independent apical carrier vesicles from MDCK cells (Delacour et al., 2006). Galectin-3 interacts directly with the apical proteins LPH (lactase-phlorizin hydrolase), the p75NTR and the sialoglycoprotein gp114 in a glycan-dependent manner, suggesting that the clustering events are necessary for the apical sorting regardless of the association with lipid rafts (Delacour et al., 2007).

Interestingly, the transport of apical cargo from the Golgi was shown to be specifically decreased by RNA interference directed against FAPP2 [PI(4)P (phosphatidylinositol-4-phosphate) adaptor protein]. By binding to glucosylceramide, which is the only known glycolipid that is synthesized in the cytoplasmic leaflet of the Golgi, FAPP2 could, by oligomerization, contribute to stabilization of the raft cluster. Moreover, via its proline-rich motif, FAPP2 could bind to other components of the apical transport machinery (Vieira et al., 2005). The same authors went on to show that ciliogenesis, the final stage in the polarization process, is defective in FAPP2-knockdown cells. Furthermore, they demonstrated that FAPP2 depletion impairs the formation of condensed apical membrane domains, suggesting that FAPP2 is a crucial component of the apical transport machinery and in cilium formation in epithelial cells (Vieira et al., 2006).

Some polypeptides of the annexin family also participate in apical transport of raft-associated membrane proteins. Annexin-13b has been identified in post-Golgi vesicles from MDCK cells and associates with lipid rafts (Lafont et al., 1998). This annexin recruits the ubiquitin protein ligase Nedd4, which might modulate the dynamics of membrane microdomains (Plant et al., 2000). Antibodies directed against annexin-13b inhibited the transport of the influenza HA glycoprotein from the TGN to the apical plasma membrane (Fiedler et al., 1995). Annexin-2, another member of the annexin family, interacting in a heterotetrameric complex with lipid rafts, modulates the membrane trafficking of several channel proteins (Girard et al., 2002; van de Graaf et al., 2003). Moreover, inhibition of annexin II decreases the apical delivery of proteins that reside in annexin II-positive vesicles (Danielsen and Hansen, 2006).

Vectorial transport to the apical membrane involves transport along microtubules with both dynein and kinesin as microtubule motor proteins, whereas the basolateral surface transport depends on kinesin alone (for a review, see Musch, 2004). Evidence for a substantial role of kinesin motors comes from studies based on microinjection of antibodies against the KIF5B (kinesin family member 5B), which blocks the apical sorting of the apical marker p75NTR in MDCK cells (Jaulin et al., 2007). For lipid raft-dependent apical transport routes, an involvement of the minus-end-directed KIFC3 (kinesin family member C3) in surface delivery of influenza HA has been demonstrated. This motor interacts with annexin 13b in Triton X-100-insoluble membrane organelles, suggesting a mechanism that links membrane raft association with an apical membrane-directed delivery process (Noda et al., 2001).

In contrast, the dynein light-chain Tctex-1 binds to the cytosolic tail of rhodopsin and mediates translocation of this exogenously expressed photopigment to the apical membrane domain of MDCK cells (Tai et al., 2001).

PDZ proteins that are resident in the apical membrane may participate in the apical localization of their interacting partners. The apical expression of the CFTR protein as well as the Na^+/H^+ exchanger is maintained through the interaction with the NHERF (Na^+/H^+-exchanger regulatory factor) (Guggino and Stanton, 2006; Donowitz and Li, 2007; Singh et al., 2009). The apical localization of the NaPiII (sodium phosphate co-transporter type 2) requires an intact PDZ-binding motif for the interaction with the apical PDZ protein NaPi-Cap2 (sodium phosphate C-terminal-associated protein 2) (Biber et al., 2004) as well as NHERF-1 (Capuano et al., 2007) (Table 1).

Basolateral sorting signals

Basolateral protein delivery is often specified by minimal amino acid motifs in the cytoplasmic region of a wide range of membrane proteins. Although extensive heterogeneity exists, certain features are commonly found in these amino acid sequences. Specifically, the targeting of many basolateral proteins, including the LDL (low-density lipoprotein) receptor and the VSV-G protein, have been demonstrated to be regulated by tyrosine-based motifs YXXØ, where X is any amino acid and Ø represents a large hydrophobic residue (Matter et al., 1992; Thomas et al., 1993; Sun et al., 2001; Lopez et al., 2005).

Alternatively, dileucine/hydrophobic signals have been identified in the C-terminus of the epithelial adhesion molecule E-cadherin (Miranda et al., 2001), sulfate/bicarbonate/oxalate anion exchange sat-1 (Regeer and Markovich, 2004), Fc receptors (Newton et al., 2005), MCAM-1 (melanoma cell adhesion molecule-1) (Guezguez et al., 2006) and NKCC1 (Carmosino et al., 2008) which direct each of these protein's basolateral sorting behaviours.

The frequent finding that both Y and LL-dependent basolateral motifs are collinear with endocytic determinants has led to the long-standing suggestion that the basolateral and the endocytic-sorting machineries share some common elements (Rodriguez-Boulan and Musch, 2005). Studies involving clathrin adaptors and most recently clathrin itself in basolateral sorting support this notion (Deborde et al., 2008).

Bipartite basolateral sorting motifs are not unusual. For example, the LDL receptor and the AQP4 (aquaporin 4) water channel each have a tyrosine-containing element as well as one encompassing a cluster of acidic residues (Matter et al., 1992; Madrid et al., 2001). Moreover, basolateral sorting of furin relies on a cluster of four acidic residues (EEDE) followed by a separate FI (phenylalanine–isoleucine) pair (Simmen et al., 1999). Similarly, the basolateral sorting signal of the cell surface transmembrane glycoprotein CD147 requires the presence of both a mono-leucine and an upstream cluster of acidic residues (Deora et al., 2004).

Basolateral motifs lacking any canonical consensus sequence have been also described in pIgRs (Casanova et al., 1991), transferrin (Odorizzi and Trowbridge, 1997), the NCAM (neural cell adhesion molecule) (Le Gall et al., 1997) and TGF-β (transforming growth factor-β) receptor (Donoso et al., 2009).

Other basolateral sorting signals, like those in the Kir 2.3 (inwardly rectifying potassium channels 2.3) (Le Maout et al., 2001), GAT-2 [GABA (γ-aminobutyric acid) transporter 2] (Perego et al., 1999; Brown et al., 2004) and Syndecan-1 (Maday et al., 2008), are found at the extreme C-terminus and appear to involve juxtaposed PDZ-binding motifs.

Interestingly, the α-subunit of the Na^+/K^+-ATPase contains binding sites for ankyrin; ankyrin is a component of the ankyrin–spectrin subcortical cytoskeleton that is linked to the basolateral actin cytoskeleton, which mediates the selective retention of Na^+/K^+-ATPase at the basolateral membrane (Morrow et al., 1989). Moreover, it has been observed that the expression and basolateral localization of Na^+/K^+-ATPase are also related to plasma membrane contact or the interaction between sodium pump molecules in neighbouring cells (Shoshani et al., 2005), suggesting a novel and more complex mechanism for this protein's basolateral sorting.

Little is known about the three-dimensional structure of basolateral targeting signals. The atypical basolateral sorting signal of the polyimmunoglobulin receptor exists as a β-turn and a nascent helix, with a critical valine residue in the nascent helix (Aroeti et al., 1993). The two dihydrophobic sorting motifs of the MHC-associated invariant chain reside in a nascent helix and a turn with leucine–isoleucine in the nascent helix and methionine–leucine as part of a turn (Motta et al., 1997). Most recently, a basolateral sorting signal consisting of a single glutamic acid residue upstream of a novel FV (phenylalanine–valine) dihydrophobic motif has been identified in muscarinic receptor M_3. The single glutamic acid resides in a type IV β-turn, and the dihydrophobic motif exists in either a type I or IV β-turn (Iverson et al., 2005), suggesting that type 1 β-turns may be the conserved feature of at least some basolateral sorting signals (Table 1).

Basolateral sorting machinery

The resemblance of Y and LL-basolateral sorting signals to well-established, clathrin-dependent endosomal targeting signals has long suggested the possibility that the basolateral membrane sorting machinery might employ a related mechanism. Y and LL-endosomal sorting signals have been well known

to be recognized by heterotetrameric clathrin AP (adaptor protein) complexes called adaptins that link clathrin to membrane proteins. Four adaptins have been so far identified: AP-1, AP-2, AP-3 and AP-4. The adaptor proteins AP-1 and AP-2 are major components of clathrin-coated vesicles originating at the TGN and plasma membrane respectively. Each of these adaptors is composed of two large subunits (γ and β1 for AP-1 and α and β2 for AP-2), a medium subunit (μ1 and μ2) and a small subunit (δ1 and δ2). The interactions of AP-1 and AP-2 with transmembrane proteins occur mainly via two types of sorting signals: tyrosine-based YXXΦ (Φ-bulky hydrophobic) and dileucine-based [DE]XXXL[LI] motifs. Biochemical and structural studies have unequivocally determined that YXX motifs engage the μ subunits of adaptor proteins (Ohno et al., 1995; Owen and Evans, 1998). However, the binding site for dileucine-based motifs has been the subject of debate. It has been reported by various groups that this motif binds to the β-subunits of AP-1 and AP-2 (Bonifacino and Dell'Angelica, 1999; Geyer et al., 2002; Schmidt et al., 2006), to the μ subunits of AP-1 and AP-2 (Rodionov and Bakke, 1998; Craig et al., 2000; Hinners et al., 2003) and, more recently, to the γ/δ1 hemicomplex of AP-1 (Coleman et al., 2006) and α/δ2 of AP-2 (Doray et al., 2007; Kelly et al., 2008). There are two different subtypes of AP-1: AP-1A and AP-1B, containing a μ-1A subunit or μ-1B subunit respectively. The μ1A subunit is ubiquitous, whereas μ1B is specifically expressed in a subset of epithelial cell types (Ohno et al., 1999).

The first experimental evidence that the μ1B adaptin is involved in the basolateral trafficking in epithelial cells comes from studies that utilized the cell line LLCPK-1 (Lilly Laboratories cell porcine kidney cells) lacking the endogenous expression of μ-1B. Roush et al. demonstrated that proteins that depend on Y-based sorting signals for their basolateral localization in MDCK cells were delivered to the apical membrane when expressed in LLCPK-1 cells (Roush et al., 1998). Following these observations, Folsch et al. developed an LLCPK-1 cell line stably expressing μ1B and tested whether the expression of this adaptin was able to rescue the basolateral sorting defect. Surprisingly, both the LDL and transferrin receptors, which were mislocalized in LLCPK-1 cells, were appropriately expressed on the basolateral membrane in μ1B-expressing LLCPK-1 cells (Folsch et al., 1999). Although these studies clearly demonstrate the involvement of AP-1B in basolateral sorting, this protein's expression is not, however, required for the basolateral sorting of all proteins that contain a Y-based motif (Duffield et al., 2004).

The AP-2 complex is ubiquitously expressed and links cargo proteins with the clathrin coat mediating the endocytosis of its cargo, whereas AP-3 has been implicated in an alternative pathway to endosomes or lysosomes (Robinson and Bonifacino, 2001).

AP-4 is a recently identified member of the clathrin adaptor family. Simmens et al. have proposed a role for AP-4 in the basolateral sorting of the LDL, transferrin and mannose-6-phosphate receptors (Simmen et al., 2002). Tyrosine-based motifs interact with the μ-subunit of the AP-4 clathrin adaptor complexes (Simmen et al., 2002).

As previously described, there is a growing body of evidence suggesting the role of PDZ-binding proteins in polarized epithelial sorting processes. There are examples of PDZ proteins that predominately reside at the basolateral membrane of certain intestinal and renal epithelia. These include syntrophyn, Lin-7, ERBIN (Erbb2 interacting protein), the CASK (calcium/calmodulin-dependent serine protein kinase), the PSD-93 (postsynaptic density protein 93) and the SAP97 (synapse-associated protein 97) (for reviews, see Brone and Eggermont, 2005). CASK and Lin-7 co-immunoprecipitate and co-localize on the basolateral membrane of MDCK and native kidney epithelial cells (Straight et al., 2000; Olsen et al., 2002). By linking extracellular matrix receptors and the cytoskeleton, the Lin-7–CASK complex is able to anchor stably epithelial BGT-1 (betaine/GABA transporter 1) (Perego et al., 1999) and Kir 2.3 on the basolateral membrane (Olsen et al., 2002).

Another protein that can interact with a PDZ domain and is associated with basolateral sorting is the small Rho GTPase Cdc42 (cell division cycle 42). Interfering with the function of Cdc42 using a constitutively inactive mutant, the basolateral VSV-G protein is mis-sorted to the apical membrane (Kroschewski et al., 1999). The removal of perinuclear actin and the inhibition of basolateral transport from the TGN by constitutively activated Cdc42 were mimicked by actin-disrupting drugs, indicating that the regulation of basolateral transport by Cdc42 is mediated by the actin cytoskeleton (Musch et al.,

2001). Members of the Rab family of small GTPases, particularly Rab8, have been implicated in basolateral membrane sorting events. Hubert et al. showed that a Rab8-inhibitory peptide could selectively inhibit biosynthetic membrane trafficking of the VSV-G protein from the TGN to the basolateral membrane without affecting the apical sorting of the influenza HA protein to the apical surface (Huber et al., 1993). Recent findings are consistent with Rab8 controlling the delivery of basolateral secretory traffic from the TGN to the REs, rather than functioning later in the process of delivery to the surface (Henry and Sheff, 2008). Moreover, Rab8-knockout mice showed a mis-sorting of apical peptidase and transporters in the small intestine, suggesting a role of Rab8 in the localization of apical proteins in intestinal epithelial cells (Sato et al., 2007).

The exocyst comprises eight subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) that govern the delivery to the basolateral surface of certain proteins as well as normal renal tubulogenesis (Lipschutz et al., 2000). It was first discovered in yeast and later shown to be important for basolateral targeting of LDL receptors. Addition of function-blocking antibodies to Sec8 inhibits delivery of newly synthesized LDL receptor from the TGN to the basolateral membrane. Blocking Sec8 does not interfere with the delivery of apical proteins in MDCK cells (Grindstaff et al., 1998). Overexpression of Sec10 stimulates the synthesis and delivery of basolateral, but not apical membrane proteins (Lipschutz et al., 2000), whereas mutations in Sec5 or Sec6 inhibit trafficking of DE-cadherin (*Drosophila* epithelial-cadherin) from REs to the basolateral domain of *Drosophila* epithelial cells (Langevin et al., 2005). Furthermore, AP-1B recruits the exocyst complex to REs (Folsch et al., 2003), implying that AP-1B vesicles utilize the exocyst for fusion with the basolateral membrane. As Sec6 and Sec8 are localized laterally below TJs, it has been suggested that the exocyst specifies the location at the plasma membrane to which basolateral vesicles are delivered (Yeaman et al., 2004) (Table 1).

Membrane fusion machinery: SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptors)

Polarized protein trafficking to the apical and basolateral plasma membranes requires different sets of SNAREs, a family of proteins specifically involved in the fusion of vesicles with their target membranes. Functionally, SNAREs can be classified into v-SNAREs (vesicle SNAREs) that are associated with the vesicle/container and t-SNAREs (target SNAREs) that are associated with the plasma membrane. The formation of a SNARE complex between v-SNAREs and t-SNAREs mediates the specific recognition and subsequent fusion of vesicles with their appropriate target membranes. SNAREs are assisted by several partners and regulators including the small GTPase Rab proteins (for a review, see Jahn and Scheller, 2006).

It has been hypothesized that different classes of transport vesicles and different acceptor membranes possess distinct isoforms of v- and t-SNARE respectively and that only the pairing of a matching combination would lead to successful vesicle fusion (Rothman and Warren, 1994). This so-called 'SNARE hypothesis' therefore postulates a proofreading mechanism in which the SNAREs would contribute to the specificity of vesicular fusion.

It has been shown that the t-SNARE Stx3 (syntaxin 3) is primarily expressed at the apical plasma membrane in MDCK cells, whereas Stx4 is expressed predominantly at the basolateral membrane domain of MDCK cells (Low et al., 1996). Moreover, Stx4 is primarily expressed at the basolateral surface, while Stx2 and Stx3 are enriched at canicular membranes in hepatocytes (Fujita et al., 1998), suggesting that these particular t-SNAREs might underpin polarized vesicle fusion processes in a variety of epithelial cell types.

In contrast, SNAP23 (23 kDa synaptosome-associated protein) [ubiquitously expressed homologue of SNAP25 (25 kDa synaptosome-associated protein)] and Stx2 are present in both membrane domains in MDCK cells (Low et al., 1998). Stx3 and SNAP23 constitute the apical t-SNARE complex, and they interact with the v-SNARE TI-VAMP [TeNT (tetanus-neurotoxin)-insensitive VAMP (vesicle-associated membrane protein); also known as VAMP7] in Caco2 cells (epithelial colorectal adenocarcinoma cells) (Galli et al., 1998). The strong interaction between VAMP7, Stx3 and SNAP23 has been further demonstrated by a yeast two-hybrid analysis (Martinez-Arca et al., 2003). In MDCK cells, overexpression of Stx3 inhibits biosynthetic transport from the TGN to the apical membrane (Low et al., 1998).

Furthermore, inhibition of VAMP7 with specific antibodies affects apical delivery of influenza HA but has no effect on the basolateral route (Lafont et al., 1999). These results together demonstrate that Stx3 and VAMP7 are important for apical transport of transmembrane and secretory proteins both in MDCK and Caco2 cells.

In the renal cortical collecting duct principal cells, VAMP2 along with SNAP23 is expressed in subapical AQP2-containing vesicles, suggesting that these proteins play an important role in vasopressin-regulated trafficking at the apical membrane in principal cells (Nielsen et al., 1995; Inoue et al., 1998).

Both VAMP2 and VAMP3 are associated with immunoisolated AQP2 vesicles, whereas Stx3 and SNAP23 are associated with the apical plasma membrane in MCD4 renal collecting duct cells. Protein knockdown coupled with apical surface biotinylation demonstrated that reduced levels of the VAMP2, Stx3 and SNAP23 strongly inhibited AQP2 fusion at the apical membrane (Procino et al., 2008).

In contrast it has been shown that the ubiquitously expressed v-SNARE cellubrevin (VAMP3) localizes to the basolateral membrane and to REs, where it co-localizes with AP-1B in MDCK cells. Furthermore, cleavage of VAMP3 with TeNT results in scattering of AP-1B localization and mis-sorting of AP-1B-dependent cargos, such as transferrin receptor and a truncated LDL receptor, LDLR-CT27. These results suggest that VAMP3 and AP-1B co-operate in basolateral membrane trafficking (Fields et al., 2007).

In contrast, Syntaxin 1A and SNAP23 are the t-SNAREs that regulate exocytotic insertion of H^+-ATPase-containing vesicles (Schwartz et al., 2007).

VAMP8 is able to interact both with the basolateral t-SNARE Stx4 and with the apical t-SNARE Stx3. It has been shown that VAMP8 and Stx3 can form complexes with SNAP23 (Pombo et al., 2003). Furthermore, it has also been proposed that VAMP8, Stx4 and SNAP23 act together in regulating exocytosis in the endocrine system (Wang et al., 2007). Finally, VAMP8, Stx3 and Stx4 were co-immunoprecipitated in parotid acinar cells (Imai et al., 2003) and VAMP8 participates in endocytosis and apical recycling in MDCK cells (Steegmaier et al., 2000), but not in apical direct delivery (Lafont et al., 1999). All of these results suggest that VAMP8 could operate in the apical transcytotic pathway. This hypothesis has been recently confirmed by Pocard et al., who showed that VAMP8 knockdown had no effect on both the direct apical and basolateral delivery but deeply affected the transcytotic apical delivery in FRT (Fischer rat thyroid) cells (Pocard et al., 2007).

These results suggest that the direct basolateral delivery and the trancytotic pathway use different sets of v-SNAREs (Figure 1).

Conclusions and future perspectives

It is now clear that epithelial membrane polarity is achieved by a combination of intracellular sorting signals, vectorial movements towards the plasma membrane and membrane-specific retention processes.

Indeed, many proteins contain specific sorting signals in their cytoplasmatic region such as those based on Y and LL amino acid motifs, which are recognized by distinct molecular subunits of adaptor proteins, a pivotal mechanism in basolateral targeting.

A common requirement for apical sorting seems to be a clustering of the apical proteins into a specific membrane domain, perhaps with the help of lectins that recognize *N*- or *O*-linked glycans, or due to the ability of some apical directed proteins, such as GPI-anchored proteins, to oligomerize during their passage through the Golgi complex. Additionally, this apical clustering could be mediated by either lipid raft domains or by other non-raft carriers.

Newly synthesized proteins can travel directly from the TGN to either the apical or the basolateral surface. Alternatively, proteins can use an indirect pathway travelling first from the TGN to the basolateral membrane, followed by endocytosis and transcytosis to the apical surface. Interestingly, a novel class of sorting signal has been identified that drives the transcytosis from the basolateral to the apical surface (Luton et al., 2009).

Membrane fusion is the final and irreversible step of each trafficking route. Under normal homoeostatic conditions, only the appropriate organelles fuse with the cognate membrane. However, in many cases, the deletion of an individual SNARE does not prevent fusion, but rather impairs it by diverting the cargo on to an alternative pathway. These observations indicate that SNAREs can functionally replace each other to a certain extent and that further factors need to be invoked to ensure specificity.

Indeed, significant advances were made in defining sorting motifs as well as the sorting routes. However, only a small number of conserved sorting signals have

been definitively identified so far and even less is known about how most of these sorting signals are recognized and acted on.

Thus it would seem that the most pressing matter in the future research involves developing a complete inventory of polarized sorting signals and the machinery that interacts with them. For instance, the nature of the basolateral sorting signal machineries that recognize the clathrin-independent signals remains unknown and there is an even larger gap for apical proteins.

The scenario becomes more complicated with the observation that diverse epithelial cells have the ability to sort similar proteins to distinct surface distributions. For example, in MDCK cells, GPI-linked proteins are sorted to the apical membrane (Lisanti et al., 1989), whereas members of this family are directed to the basolateral domain in Fisher rat thyroid epithelial cells (Zurzolo et al., 1993). Additionally, both the Na^+/K^+-ATPase ion pump and NKCC1 reside in the basolateral membrane of most epithelial cells, but are localized to the apical membrane domain in cells of the choroid plexus (Masuzawa et al., 1984; Wu et al., 1998). The H^+/K^+-ATPase β-subunit, which contains a tyrosine-based motif in its cytoplasmic tail, was expressed at the basolateral membrane in MDCK cells, but was localized to the apical membrane in LLCPK-1 cells (Roush et al., 1998). It would appear, therefore, that principles of apical and basolateral sorting pathways are used to build up two different circuits in epithelial cells. As the signals directing proteins into different circuits function hierarchically, proteins can be then switched from one to the other pathway to meet the requirements imposed by their physiological functions.

Thus it will be equally important to elucidate how epithelial trafficking processes are regulated in concert with physiological demands. In this regard, it is important to underline that almost all of the present study has utilized epithelial cells cultured on permeable support where they constitute a simple monolayer. In real life, however, epithelial cells are found in more complex structures like cysts and tubules, which are the basic building blocks of most organs. During the development of such structures, epithelial cells must undergo alteration in shape, polarity and membrane trafficking. Related changes also occur in many diseases, such as cancer and polycystic kidney disease. Therefore studies should be extended to models in which proteins are ideally expressed in a physiological environment such as 3D cell cultures, isolated organs and animal models. In this respect, important technological advances in cellular imaging and transgenic organisms should be applied in this field.

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