Regulation of Transporters and Channels by Membrane-Trafficking Complexes in Epithelial Cells

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The vectorial secretion and absorption of fluid and solutes by epithelial cells is dependent on the polarized expression of membrane solute transporters and channels at the apical and basolateral membranes. The establishment and maintenance of this polarized expression of transporters and channels are affected by diverse protein-trafficking complexes. Moreover, regulation of the magnitude of transport is often under control of physiological stimuli, again through the interaction of transporters and channels with protein-trafficking complexes. This review highlights the value in utilizing transporters and channels as cargo to characterize core trafficking machinery by which epithelial cells establish and maintain their polarized expression, and how this machinery regulates fluid and solute transport in response to physiological stimuli.

A hallmark physiological function of epithelial cells is to effect secretion and absorption by many organs through vectorial transport of fluid and solutes. Vectorial transport is established by the polarized delivery of distinct cohorts of membrane solute transporters and channels from the post-Golgi biosynthetic secretory pathway to either the apical or basolateral membrane of epithelial cells. The transporters and channels then work in series to mediate vectorial transport of solutes across the apical and basolateral membranes. In addition, once transporters or channels are delivered to their respective plasma membrane domains, the $V_{\max}$ of secretion or absorption can be

acutely regulated by changing the number of transporters or channels at the apical or basolateral membrane by endocytotic and recycling pathways, in a physiologically responsive fashion to hormones or neurotransmitters.

Characterization of the trafficking machinery that regulates these transporters and channels, particularly those localized to the apical membrane, has progressed significantly in past years owing to multiple factors. First, epithelial cells in organs tend to behave stereotypically. Thus, in these systems, the activities of the transporters, channels, and their associated membrane-trafficking protein complexes tend to be circumscribed to fulfill a specific function,

Editor: Keith E. Mostov

Additional Perspectives on Cell Polarity available at www.cshperspectives.org

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Cite this article as *Cold Spring Harb Perspect Biol* 2017;9:a027839

C.T. Okamoto

which in turn facilitates the characterization of the interactions between transporters or channels and membrane-trafficking machinery and the functional consequences thereof. In addition, many of these cells endogenously elaborate specific subsets of often relatively common trafficking machinery to fulfill their function within their physiological niche. Thus, the expectation is that these proteins will be coexpressed and colocalized in the same relevant epithelial cells, and that their interactions will be functionally relevant. Second, the greater availability of tools, such as instrumentation and reagents, has facilitated the characterization of these sets of proteins and their functions. A related advantage is that transporters and channels often offer a straightforward functional readout of their activity. Third, the independent findings from a cell biological approach can inform inquiry into these physiologically based systems, and findings from a physiologically based system could as well inform the cell biology of these trafficking protein complexes. Indeed, an important criterion to fulfill is whether any findings in heterologous expression systems are recapitulated in vivo or in primary cultures of relevant epithelial cells to help mitigate concerns over artifactual interactions observed because of heterologous overexpression. Finally, dysregulation of the trafficking of transporters and channels are clearly linked to disease. A greater mechanistic understanding at the molecular level of these systems will potentially provide more precision to translational opportunities in therapeutic approaches.

The progress made in the characterization of trafficking machinery that regulates the recycling or localization of select apical membrane transporters or channels, with a couple of exceptions, will be reviewed, focusing on protein–protein interactions and functional consequences thereof, as well as their intersection with key intracellular signaling pathways in their regulation. This review complements recent general reviews on apical membrane recycling pathways (Swiatecka-Urban et al. 2007; Golachowska et al. 2010; Stoops and Caplan 2014; Goldenring 2015; Vergés 2016).

H⁺-K⁺-TRANSPORTING ATPase (H,K-ATPase) IN THE GASTRIC PARIETAL CELL

One of the first systems in which regulated recycling was first proposed nearly 40 years ago as a mechanism for regulating the $V_{\max}$ of a membrane transporter was that of the gastric H,K-ATPase in the regulation of gastric HCl secretion by parietal cells (Forte et al. 1977). The H,K-ATPase is a heterodimeric P-type ATPase, a close relative of the ubiquitous Na,K-ATPase, and is comprised of a 100-kDa polytopic 10-transmembrane (TM)-spanning domain catalytic α -subunit and a type II single transmembrane-spanning glycosylated β -subunit; it is distinct from, and unrelated to, the V-type H⁺-ATPase of endosomes, lysosomes, and H⁺-transporting cells of the kidney.

In nonsecreting parietal cells, the H,K-ATPase is stored in an extensive array of tubular, vesicular, and cisternal intracellular membrane compartments, collectively known as “tubulovesicles” (Forte et al. 1977; Aoyama and Sawaguchi 2011). When the parietal cell is stimulated by gastrointestinal neurotransmitters and hormones (“secretagogues”) to secrete HCl, intracellular cAMP and/or Ca²⁺ increase, and tubulovesicles fuse with the apical membrane, thereby delivering the H,K-ATPase to the apical membrane (N.B., a parietal cell’s apical membrane is highly invaginated and anastomosing and is also known as the canalicular membrane). At the apical membrane, the H,K-ATPase couples the hydrolysis of ATP to the efflux of H⁺ in exchange for extracellular K⁺, thus driving HCl secretion. When the stimulus is withdrawn, there is a massive re-uptake of apical membrane and H,K-ATPase, and typically within an hour, the tubulovesicular compartment is reestablished. There, the H,K-ATPase resides until the parietal cell is again stimulated to secrete HCl. Thus, functionally, the tubulovesicular compartment behaves as a recycling pathway regulated by secretagogues. Early ultrastructural studies of parietal cells showed a reciprocal relationship between the abundance of tubulovesicles and apical membrane surface area during the secretory cycle (Forte et al. 1981). Later, subcellular fractionation and bio-

chemical studies showed that the membrane environment of the H,K-ATPase changed on stimulation of the parietal cell (Wolosin and Forte 1981, 1984). Although such morphological and biochemical studies supported the regulated recycling hypothesis, the discovery that Rab11a was highly expressed in parietal cells and was localized predominantly to tubulovesicles began to solidify its identity as a regulated apical recycling compartment (Calhoun and Goldenring 1997; Calhoun et al. 1998). Conversely, the presence of Rab11a on tubulovesicles helped define Rab11's role as a major regulator of recycling pathways in other cells (Casanova et al. 1999; Hsu and Prekeris 2010; Goldenring 2015).

The functional role of Rab11a in the regulated recycling of the H,K-ATPase was shown when Rab11a was shown to translocate together with the H,K-ATPase to the apical membrane on stimulation (Calhoun et al. 1998), and the expression of dominant-negative Rab11a blocked the secretagogue-stimulated delivery of H,K-ATPase from tubulovesicles to the apical membrane in primary cultures of parietal cells (Duman et al. 1999). In addition, through colocalization and/or functional inhibition studies with SNARE-cleaving toxins, the membrane fusion SNARE proteins syntaxin-1 (Stx1), -3 (Stx3), SNAP-25, and VAMP2 have been shown to regulate tubulovesicle and H,K-ATPase delivery to the apical membrane (Calhoun and Goldenring 1997; Peng et al. 1997; Ammar et al. 2002; Karvar et al. 2002a,b; Lapierre et al. 2007), analogous to their regulation of the delivery of membrane proteins to the apical membrane in other epithelial cells.

The other potential regulators of regulated recycling identified on tubulovesicles or the apical membrane by either imaging or biochemical studies is a potentially exciting list of trafficking-related proteins, including membrane-trafficking proteins, such as clathrin and the AP family of clathrin adaptors (Okamoto et al. 1998, 2000), the tetraspanin CD63 (Duffield et al. 2003), epsin 3 (Ko et al. 2010), rab11 family of interacting proteins (Hales et al. 2001; Lapierre et al. 2007), arf6 (Matsukawa et al. 2003), rab25 (Calhoun and Goldenring 1997; Calhoun et al. 1998; Hales et al. 2001; Lapierre et al. 2007), and

rab27b (Suda et al. 2011b); cytoskeletal proteins, such as ezrin (Hanzel et al. 1991; Yao et al. 1996; Zhou et al. 2003a, 2005; Tamura et al. 2005; Yoshida et al. 2016), LIM- and SH3-domain-containing protein-1 (lasp-1) (Chew et al. 1998, 2000, 2002, 2008), cdc42 (Zhou et al. 2003b), IQGAP1 and/or IQGAP2 (Zhou et al. 2003b; Chew et al. 2005), and myosin IIB (Natarajan et al. 2014); and proteins that bridge membrane-trafficking pathways with the cytoskeleton, such as myosin Vb (Myo5b) (Hales et al. 2001; Lapierre et al. 2001) and Hip1R (Jain et al. 2008). Evidence for the functional significance of many of these proteins in the regulated recycling of the H,K-ATPase has been shown to varying degrees. There is indirect evidence that the β -subunit possesses a functional cytoplasmic YXX ϕ internalization motif (Courtois-Coutry et al. 1997; Nguyen et al. 2004); however, direct interaction of the H,K-ATPase with clathrin adaptors has not been shown, nor is there convincing evidence that the internalization phase of regulated recycling is clathrin mediated. Thus, the role of clathrin in H,K-ATPase trafficking remains to be characterized.

Apical targeting motifs have been characterized in both subunits (Gottardi and Caplan 1993; Dunbar et al. 2000). In addition, the expression of either H,K-ATPase subunit (Scarff et al. 1999; Spicer et al. 2000; Miller et al. 2002), putative tubulovesicular Cl⁻ channels (Xu et al. 2008; Nighot et al. 2015), or Hip1R (Jain et al. 2008) appears to be critical for the biogenesis of the tubulovesicular compartment, as parietal cells from knockout mice for these proteins are devoid of tubulovesicles, suggesting that the expression of cargo or specific trafficking proteins are critical for the biogenesis of tubulovesicles.

Significant progress has been made with respect to the role of the actin- and membrane-binding protein ezrin in H,K-ATPase recycling. Ezrin, a founding member of the ezrin-radixin-moesin (ERM) family of scaffolding proteins, was first characterized in parietal cells as a substrate of protein kinase A (PKA) (Hanzel et al. 1991) and expressed at the apical membrane (Yao et al. 1996; Sawaguchi et al. 2004). Two major PKA phosphorylation sites on ezrin

C.T. Okamoto

in parietal cells have been characterized, S66 and T567 (Zhou et al. 2003a, 2005; Zhu et al. 2008). Overexpression in primary cultures of parietal cells of a nonphosphorylatable S66A ezrin inhibits H,K-ATPase recruitment (recycling/exocytosis) to the apical membrane, whereas the overexpression of the phosphomimetic S66D ezrin results in a stimulated morphology owing to recruitment of tubulovesicular membranes to and fusion with canalicular membranes (Zhou et al. 2003a). On the other hand, overexpression of the T567A ezrin localized to the canalicular membrane and did not inhibit H,K-ATPase recruitment, whereas T567D ezrin was predominantly localized to the basolateral membrane and effected the relocation of the H,K-ATPase to or near the basolateral membrane (Zhu et al. 2010). Knockdown of ezrin in mice resulted in achlorhydria, an inability to secrete gastric acid, and in ultrastructural changes in tubulovesicles and canalicular membranes (Tamura et al. 2005; Yoshida et al. 2016). Ezrin has been shown to bind to the adaptor protein PALS1, which mediates ezrin localization, and knockdown of PALS1 blocks membrane recruitment in stimulated cells (Cao et al. 2005). Myosin IIB also appears to be critical for ezrin localization (Natarajan et al. 2014). Ezrin binds to and recruits ACAP4, the GTPase-activating protein (GAP) for ARF6, to canalicular membranes in a stimulation- and ezrin-S66-phosphorylation-dependent manner (Ding et al. 2010). Overexpression of a GAP-deficient ACAP4 inhibits membrane recruitment and HCl secretion, but not ezrin localization. Finally, ezrin interacts with Stx3 as well as the cell polarity kinase MST4 (Yu et al. 2014; Jiang et al. 2015). Thus, ezrin appears to reside within a key nexus for protein–protein interactions in the recruitment of the H,K-ATPase to the canalicular membrane on stimulation of the parietal cell, although ezrin itself has not been reported to interact directly with the H,K-ATPase. Other ERM proteins and their regulation of apical expression of transporters and channels are reviewed below.

One of the more interesting recent findings is that patients with loss-of-function mutations in mucolipin-1 (MCOLN1), resulting in the ly-

sosomal storage disease mucopolipidosis type IV (MLIV), present with achlorhydria, which is unique among lysosomal storage diseases (Schiffmann et al. 1998; Lubensky et al. 1999; Bargal et al. 2000; Sun et al. 2000b; Venugopal et al. 2007; Chandra et al. 2011). MCOLN1 is a member of the transient receptor potential (TRP) channel family and is localized to late endosomes and lysosomes (Puertollano and Kiselyov 2009). Its function in the endolysosomal pathway has not been clearly defined, but it has been shown to play multiple roles in regulating endolysosomal function, such as lysosomal pH (Soyombo et al. 2006), autophagy (Vergarajaregui and Puertollano 2008), lysosomal exocytosis (Park et al. 2015), transport of lipids to lysosomes (Pryor et al. 2006), or clearance of apoptotic cells (Venkatachalam et al. 2008).

MLIV is a rare inherited disorder and, in addition to achlorhydria, patients also show the more severe symptoms of delayed psychomotor development and progressively impaired vision (Schiffmann et al. 1998; Lubensky et al. 1999; Bargal et al. 2000; Sun et al. 2000b; Venugopal et al. 2007; Chandra et al. 2011; Wakabayashi et al. 2011). It is not clear how the loss-of-function mutations in MCOLN1 result in any of the symptoms of MLIV. However, ultrastructural analysis of parietal cells from these patients, as well as from knockout mice for MCOLN1, reveals an accumulation of lysosomal inclusion bodies (Lubensky et al. 1999; Chandra et al. 2011). It suggests that normal turnover by the endolysosomal system of membrane protein, and perhaps of the H,K-ATPase in particular, may be important for maintaining HCl secretion by the parietal cell. Because the parietal cell is rather selectively sensitive to mutation of MCOLN1, analysis of its role in parietal cell function could lead to insights into its role in normal endolysosomal function and how mutations in it lead to the multiple symptoms of MLIV.

AQUAPORIN-2 (AQP2) IN RENAL EPITHELIAL CELLS

Another well-characterized regulated apical recycling system is that of the vasopressin (also

known as antidiuretic hormone)-sensitive water channel AQP2 in renal epithelial cells (Brown 2003; Moeller and Fenton 2012; Fenton et al. 2013; Wilson et al. 2013; Verkman et al. 2014). AQP2 is a polytopic 6-TM domain membrane protein that assembles homotypically to form water channels. AQP2 resides in an intracellular membrane storage compartment when water does not need to be reabsorbed back into the body from nascent urine. When dehydrated, the body needs to reabsorb water from nascent urine, and the posterior pituitary gland secretes vasopressin. Vasopressin stimulates renal epithelial cells, and these AQP2-containing intracellular membranes fuse with the apical membrane to mediate water reabsorption. When vasopressin is no longer present, AQP2 is internalized from the apical membrane, and the recycling storage compartment is reestablished. AQP2 undergoes regulated recycling through a Rab11-positive storage compartment (Nedvetsky et al. 2007; Rice et al. 2012), analogous to the regulated recycling of the H,K-ATPase. Recent novel data indicate, however, that the delivery of a significant fraction of newly synthesized AQP2 may be targeted first to the basolateral membrane and then transcytosed to the apical membrane *in vitro* and *in vivo* (Yui et al. 2013). The remainder may traffic directly to the apical membrane from the *trans*-Golgi network (TGN) in a pathway that is dependent on the phosphatidylinositol-4-phosphate-binding adaptor protein FAPP2 (Yui et al. 2009).

Phosphorylation of AQP2 regulates the rates of exocytosis at, and endocytosis from, the apical membrane (Moeller et al. 2010; Rice et al. 2012). Exocytosis is mediated by the v-SNAREs VAMP2, VAMP3, and VAMP8 (endobrevin) on AQP2 vesicles, and t-SNAREs Stx3, Stx4, and SNAP23 at the apical membrane (Procino et al. 2008; Mistry et al. 2009; Wang et al. 2010). The Sec1/Munc18-like (SM) protein Munc18-2, which helps prime SNAREs for membrane fusion, is also localized at the apical membrane (Procino et al. 2008). Thus, the membrane fusion machinery for AQP2 exocytosis is also relatively commonplace.

Endocytosis occurs through a clathrin- and dynamin-dependent pathway (Sun et al. 2002;

Moeller et al. 2010; Rice et al. 2012) and possibly a clathrin-independent, lipid-raft-dependent pathway (Yu et al. 2008; Fenton et al. 2013), and ubiquitylation enhances AQP2 endocytosis (Fenton et al. 2013; Moeller et al. 2014), whereas association of AQP2 with the apical membrane detergent-resistant protein (MAL, also known as vesicle integral protein of 17 kDa [VIP17]) attenuates internalization (Kamsteeg et al. 2007). Recent data suggest that AQP2 interacts with members of the 14-3-3 scaffolding protein family in a phosphorylation-dependent fashion, which in turn regulates AQP2 ubiquitylation, trafficking, and degradation (Moeller et al. 2016). It is not currently clear what fraction of AQP2 internalized from the apical membrane is sorted to the recycling pathway versus the degradative pathway.

Of note is that loss-of-function inherited mutations in AQP2 result in its misfolding and retention in the endoplasmic reticulum and thus its inability to be delivered from the biosynthetic pathway to the recycling compartment or apical membrane (Tamarappoo et al. 1999; Frick et al. 2014; Dollerup et al. 2015). These mutations are a subset of the causes of nephrogenic diabetes insipidus, presenting as a chronic production of a dilute urine and consequent imbalance in body water content, and is one example of diseases caused by inherited defects in the proper folding of a membrane transporter or channel.

EPITHELIAL SODIUM CHANNEL (ENaC)

Although expressed in the apical membrane of a number of epithelial cells, in renal epithelial cells ENaC plays a critical role in sodium balance and is the target of the diuretic amiloride (Rossier 2014; Hadchouel et al. 2016). There are three similar but distinct subunits, α , β , and γ , all with two membrane-spanning domains and two short cytoplasmic tails. To form a functional channel, these subunits may be assembled in homomeric fashion, but maximal channel activity requires assembly with one of each subunit isoform.

ENaC was one of the first demonstrations of a gain-of-function mutation in an epithelial ion

C.T. Okamoto

channel linked to disease. In Liddle's syndrome, a mutation results in the loss of the PY motif in one of the cytoplasmic tails of ENaC, the consequence of which is constitutively active Na^+ reabsorption by the kidney and chronic hypertension, caused by the inability to internalize ENaC efficiently from the apical membrane (Schild et al. 1996). ENaC activation is also controlled by proteolysis of a segment of its extracellular domain (Svenningsen et al. 2011); however, this mode of activation will not be reviewed here.

The molecular mechanism for the defect in Liddle's syndrome is an inability to ubiquitylate ENaC, and that ubiquitylation is required for the rapid internalization of ENaC (Staub et al. 1997). The molecular machinery for ubiquitylation was identified as the ubiquitin ligase Nedd4-2 that binds to the PY motif. Ubiquitylated ENaC then binds to the alternate clathrin adaptor epsin and is endocytosed via clathrin-coated vesicles (Shimkets et al. 1997; Staruschenko et al. 2005; Wang et al. 2006; Weixel et al. 2007). In addition, the YXX ϕ motif downstream from the PY motif ([S/T]PPPYXX[S/T] ϕ) can bind independently to the μ -2 subunit of the AP-2 clathrin adaptor and may also mediate its endocytosis (Staruschenko et al. 2005).

Postendocytotic trafficking of ubiquitylated ENaC may transit through either a recycling or a degradative pathway. Transport to a lysosome-dependent degradative pathway appears to be mediated by hepatocyte growth-factor-regulated tyrosine kinase substrate Hrs, a component of the endosomal sorting complexes required for transport (ESCRT)-0 complex (Zhou et al. 2010). Hrs can bind to ENaC also through the PY motif. Thus, mutation of this motif in Liddle's syndrome affects both Nedd4-2 and Hrs binding. Diversion to a recycling pathway may be mediated by the ubiquitin-specific peptidase 8 (USP8), which can also bind to ENaC and deubiquitinate it (Zhou et al. 2013). When coexpressed with ENaC in various cell systems, USP8 increased ENaC current and abundance at the cell surface. Interestingly, another USP, USP2-45, increased surface expression of ENaC by reducing its endocytosis rather than reducing its degradation (Oberfeld et al. 2011). The recycling

compartment for ENaC is likely to be a Rab11-positive compartment (Karpushev et al. 2008; Butterworth et al. 2012; Frindt et al. 2016).

Recent data have shown that inhibition of ubiquitylation occurs by acetylation of key lysines targeted for ubiquitylation (Butler et al. 2015). Acetylation was also shown to increase cell-surface abundance of ENaC, and acetylated ENaC was shown to be a substrate of histone deacetylase 7, which is expressed abundantly in the kidney, particularly in the cells enriched in ENaC. Acetylation of ENaC may represent a novel pathway for regulation of ubiquitylation of ENaC and therefore its function.

In characterizing the exocytosis of ENaC, a fusion-independent role for the SNARE Stx1 was observed. When coexpressed with ENaC, Stx1 (but not Stx3) was shown to inhibit directly the activity of ENaC by reducing both the abundance and activation of gating of ENaC at the cell surface, and were mediated by the cytoplasmic domain of Stx1 interacting with the carboxy-terminal cytoplasmic tail of ENaC (Qi et al. 1999; Peters et al. 2001; Condliffe et al. 2003; Berdiev et al. 2004; Condliffe et al. 2004), suggesting a system of coordinate regulation of delivery or ENaC and its activity.

Aldosterone is a steroid hormone that is thought to regulate Na^+ balance by transcriptional up-regulation of transporters and channels, and their protein activators, for Na^+ reabsorption (Rossier 2014). However, recent data suggest that the relatively short time course of the onset of some of aldosterone's effects is too rapid to be mediated by changes in gene expression. These rapid aldosterone-dependent changes in ENaC activity involve the activation of protein kinase D1 resulting in the stimulation of the delivery of a pool of ENaC from the TGN to the apical membrane (Dooley et al. 2013; Quinn et al. 2014).

In addition, a number of kinases affect Nedd4-2 activity, such as serum glucocorticoid-regulated kinase (SGK), PKA, and 5' AMP-activated kinase (AMPK). Those kinases that activate Nedd4-2 (e.g., AMPK) ultimately inhibit ENaC activity (Carattino et al. 2005; Bhalla et al. 2006), and those that inhibit Nedd4-2 (e.g., SGK, PKA) stimulate ENaC

activity via interaction of phospho-Nedd4-2 with 14-3-3 proteins instead of ENaC (Snyder et al. 2004; Bhalla et al. 2006; Nagaki et al. 2006). Recent data have also suggested that With-No-Lysine kinase 4 (WNK4), a kinase mutated in pseudohypoaldosteronism type II, inhibits ENaC activity by enhancing internalization and also reducing the recycling pool of the channel (Ring et al. 2007; Yu et al. 2013; Hadchouel et al. 2016). These WNK4 effects are independent of Nedd4-2.

NA⁺-H⁺ EXCHANGER 3 (NHE3)

NHE3 is a member of a family of polytopic 11-TM domain antiporters that catalyze the electroneutral efflux of H⁺ to the influx of Na⁺ (Donowitz et al. 2009). NHE3 plays an important role in Na⁺ and pH homeostasis. NHE3 in its basal state resides predominantly in the microvilli of the brush border membranes of intestinal and renal epithelial cells. NHE3 tethering to microvilli is predominantly attributable to its direct binding to ezrin (Cha et al. 2006) and other scaffolding proteins, namely, the NHE regulatory factor (NHERF) family of proteins, identified in seminal work beginning in 1997 with ERM-binding phosphoprotein of 50 kDa (EBP50, also known as NHERF1), NHERF2, and NHE3 kinase A regulatory protein (E3KARP) (Yun et al. 1997, 1998; Lamprecht et al. 1998). The NHERF proteins possess two PSD-95/discs large/zonula occludens-1 (PDZ) domains, one of which binds to a PDZ-binding motif in the carboxy-terminal cytoplasmic tail of NHE3. The NHERF proteins also possess a domain to bind ERM proteins. Thus, the NHERF proteins also recruit ezrin to NHE3, where ezrin may serve as an actin cytoskeletal linker and/or PKA anchor (Lamprecht et al. 1998; Yun et al. 1998; Kurashima et al. 1999). The NHERF family of proteins has since been shown to regulate the intracellular trafficking of other membrane proteins, such as G-protein-coupled receptors (Cao et al. 1999; Wheeler et al. 2007).

Signaling pathways that raise intracellular Ca²⁺, intracellular cAMP, or intracellular cGMP activate the trafficking of NHE3 down microvilli,

in a clathrin-associated myosin VI-dependent fashion (Hegan et al. 2012; Chen et al. 2014), to the intermicrovillar cleft (region) where it may be endocytosed into endosomal compartments (Donowitz and Li 2007). Thus, its surface expression is reduced, lowering its $V_{\max}$ at the cell surface. EBP50/NHERF1 also regulates cAMP sensitivity of NHE3 (i.e., the cAMP-dependent inhibition of activity), whereas NHERF2 regulates cAMP, cGMP, and Ca²⁺ sensitivity (Yun et al. 1997; Lamprecht et al. 1998; Murtazina et al. 2011; Sarker et al. 2011). Endocytosis, however, has been reported to be both clathrin-dependent (Hu et al. 2001; Musch et al. 2007) and clathrin-independent, but lipid-raft-dependent (Zachos et al. 2014).

The exocytosis of NHE3 from an endosomal compartment, and therefore an increase in $V_{\max}$ of NHE3 transport, can also occur (Donowitz and Li 2007). A number of other signaling pathways (epidermal growth factor, glucocorticoid signaling through SGK, phosphatidylinositol-3 kinase, apically applied lysophosphatidic acid or UTP) have been shown to regulate the stimulation of NHE3 in an NHERF-dependent fashion (Li et al. 2001; He et al. 2011; Murtazina et al. 2011; Sarker et al. 2011). Angiotensin II, a peptide hormone that stimulates Na⁺ reabsorption by renal epithelial cells, also stimulates the trafficking of NHE3 up the microvilli of renal epithelial cells to stimulate the $V_{\max}$ of NHE3 transport and, therefore, Na⁺ reabsorption in the kidney, and NHE3 is trafficked along with myosin VI, EBP50/NHERF1, ezrin, and clathrin (Riquier-Brison et al. 2009). Thus, the NHERF proteins play key roles in integrating signaling, trafficking, and localization of NHE3, as well as of other transporters, channels, and membrane receptors.

CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR (CFTR)

The CFTR is an ATP-binding cassette (ABC) transporter encoded by the cystic fibrosis (CF) gene. It is a 12-TM domain polytopic membrane protein that is localized at the apical membrane of epithelial cells where it functions as a cAMP-activated Cl[−] channel. Through a

C.T. Okamoto

variety of interactions with adaptor proteins, CFTR serves as a putative hub of macromolecular complexes of transporters and channels, such as ENaC and NHE3, and actin cytoskeletal elements in reciprocal regulation of their activities (Farinha and Matos 2016).

CF is the most common genetic disorder among Caucasians (Elborn 2016). It is well known that mutations in CFTR cause defects in Cl^- secretion, leading to the most overt symptoms of CF. The most prevalent mutation, ΔF504 , results in improper trafficking of the CFTR, owing to its misfolding, leading to a defect in Cl^- secretion. As might be expected, CFTR interacts with many members of the trafficking protein complexes mentioned above, such as PDZ domain-containing proteins (Short et al. 1998; Moyer et al. 1999, 2000; Sun et al. 2000a; Karthikeyan et al. 2001; Naren et al. 2003; Cheng et al. 2004; Li et al. 2005; Bossard et al. 2007; Gee et al. 2011; Arora et al. 2014; Loureiro et al. 2015; Lobo et al. 2016), ezrin (Sun et al. 2000a; Naren et al. 2003; Li et al. 2005; Loureiro et al. 2015; Abbattiscianni et al. 2016), clathrin adaptors (Bradbury et al. 1994; Weixel and Bradbury 2000, 2001a,b; Peter et al. 2002; Collaco et al. 2010; Cihil et al. 2012), v- and t-SNARES (Peters et al. 2001; Bilan et al. 2013), Rab11 (Swiatecka-Urban et al. 2007; Silvis et al. 2009; Bilan et al. 2013; Holleran et al. 2013), and Myo5B (Swiatecka-Urban et al. 2007) and -VI (Ameen and Apodaca 2007; Collaco et al. 2010); some of these interactions may be cell-type-specific, but these will not be reviewed here. Rather the focus will be on amelioration of CF by drugs that may work at novel trafficking loci for CFTR (Zhang et al. 2011; Hanrahan et al. 2013; Holleran et al. 2013; Loo et al. 2013; Abbattiscianni et al. 2016; Farinha and Matos 2016).

Via its carboxy-terminal PDZ-binding motif, wild-type CFTR interacts with EBP50/NHERF1 and resides in a functional macromolecular complex with the β_2 adrenergic receptor ($\beta_2\text{AR}$) at the apical membrane of airway cells (Naren et al. 2003). Ezrin, through binding to EBP50/NHERF1, is also part of this complex. An increase in cAMP from stimulation of $\beta_2\text{AR}$ blocks internalization of CFTR, thus increasing

CFTR V_{max} at the apical membrane; the cAMP sensitivity and increase in V_{max} is dependent on EBP50/NHERF1. In addition, ezrin-EBP50/NHERF1 interaction enables misfolded CFTR to evade peripheral protein quality control (Loureiro et al. 2015), which for mutated, misfolded CFTR likely involves evasion of rapid internalization from the cell surface and subsequent trafficking to a ubiquitin-dependent degradative pathway (Okiyonedo et al. 2010).

A class of small molecules, known as “correctors” or “rescuers,” are thought to act as small molecule chaperones to facilitate the folding of mutated CFTR, allowing it to be exported from the endoplasmic reticulum and ultimately be delivered to the apical membrane (Hanrahan et al. 2013; Holleran et al. 2013; Loo et al. 2013; Arora et al. 2014; Abbattiscianni et al. 2016; Farinha and Matos 2016). However, their efficacy is countered by the peripheral protein quality-control pathway mentioned above. Recent exciting work has shown that the activity of some of these correctors can be potentiated by enhancing the interactions among the proteins in the macromolecular complex at the apical membrane (Loureiro et al. 2015; Abbattiscianni et al. 2016), such as by stimulating a conformational change in EBP50/NHERF1 via activation of the F-actin-regulating small GTPase Rac1 (Loureiro et al. 2015). Exchange protein directly activated by cAMP 1 (EPAC1), a cAMP-dependent guanine nucleotide exchange factor for the small GTPases Rap1 and Rap2, also enhances the activity of correctors (Lobo et al. 2016). EPAC interacts directly with EBP50/NHERF1 to stabilize CFTR at the cell surface.

Interestingly, other routes of delivery to the cell surface may be exploited in the treatment of CF. ΔF504 CFTR trafficking to the cell surface can be rescued in vitro and in vivo by directing ΔF504 to a Golgi-independent pathway that relies on the PDZ-binding domain of CFTR and the PDZ domain of Golgi reassembly stacking protein 55 (GRASP55) (Gee et al. 2011). Blocking the conventional Golgi-dependent secretory pathway by pharmacological agents or molecular constructs can redirect ΔF504 to this unconventional Golgi-independent pathway. Further characterization of these CFTR protein-pro-

tein interactions that regulate its trafficking may provide novel opportunities to treat CE.

RENAL OUTER MEDULLARY K⁺ CHANNEL (ROMK) AND AUTOSOMAL RECESSIVE HYPERCHOLESTEROLEMIA (ARH) PROTEIN

The apical membrane ROMK channel (or KCNJ1 or Kir1.1) regulates K⁺ excretion (efflux from cells) into urine by the kidney. Endocytosis of ROMK from the apical membrane is a key mechanism to reduce K⁺ excretion under conditions of K⁺ deficiency. ROMK has been shown to interact with the alternate clathrin adaptor autosomal recessive hypercholesterolemia protein (ARH) (Zeng et al. 2002; Fang et al. 2009). ARH binds to the NPXY motif in the cytoplasmic domains of transmembrane proteins (Bonifacino 2014), although in ROMK, the motif appears to be YxNPxV. ARH regulates the constitutive endocytosis of ROMK by clathrin-coated pits, and ARH is expressed predominantly in regions of the kidney that also express ROMK (Fang et al. 2009).

The endocytosis of ROMK via ARH is also stimulated by the kinase WNK1 (Fang et al. 2009) and WNK4 (Kahle et al. 2003). Familial gain-of-expression mutations in WNK1 have been characterized in which the observed hyperkalemia may be caused by an increase in ARH abundance (Hadchouel et al. 2016). However, other studies have shown that the kinase activity of WNK1 is not required for its stimulatory effect on endocytosis, and that the effect of WNK1 may be mediated through its interaction with intersectin, a scaffolding protein of the clathrin-dependent endocytotic pathway (He et al. 2007). It is possible that two independent pathways exist for the regulation of ROMK via endocytosis, which would not be surprising given that redundant pathways often exist for the regulation of important physiological processes.

MULTIDRUG RESISTANCE PROTEINS (MRPs) AND THE TUMOR SUPPRESSOR LIVER KINASE B1 (LKB1)

LKB1 (also known as serine/threonine kinase 11 [STK 11] or in *Caenorhabditis elegans* parti-

tioning-defective 4 [PAR4]) (Baas et al. 2004) is a multidimensional kinase (Korsse et al. 2013). It regulates cellular metabolism, cell proliferation, cell polarity, and tumorigenesis. LKB1 is known to regulate many of these various activities mainly through regulation of AMP-dependent protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signaling. In addition, germ-line inactivating mutations in LKB1 in humans cause Peutz–Jeghers syndrome, in which patients have a predisposition to cancers of the digestive tract, breast, and reproductive organs.

Transport of bile salts and bile pigments into and out of hepatocytes for their ultimate excretion into bile relies on the proper expression of transporters for these solutes at the basolateral (sinusoidal) and apical (canalicular) membranes, respectively (Chang et al. 2013). Liver-specific knockouts of LKB1 in mice result in mice with abnormal bile canaliculi, abnormal bile duct (a structure dependent on the formation of patent bile canaliculi), and a loss of localization of a bile salt transporter ABCB11 (also known as bile salt export pump [BSEP]) from the canalicular membrane (Woods et al. 2011; Homolya et al. 2014). These mice are also underweight, hyperbilirubinemic, and jaundiced, and they do not survive beyond 4 weeks. However, the loss of ABCB11 expression from the canalicular membrane does not account for the inability to excrete bilirubin because knockout mice for ABCB11 do not develop jaundice (Wang et al. 2013a). On the other hand, all of these defects could be attributed to a general loss of polarity in the hepatocytes, although, interestingly, histological examination of livers from these LKB1-deleted mice does not show any frank aberrant polarized phenotype, other than the irregular bile canaliculi.

MRP2 (also known as ATP-binding cassette transporter C2 [ABCC2] or canalicular multi-specific organic anion transporter [cMOAT]) plays a key role in the efflux across the apical membrane of a number of epithelial cells of the conjugates of phase II biotransformation, namely, conjugates of glutathione, glucuronate, and sulfate (Erlinger et al. 2014). MRP2 is expressed at the apical membrane of hepato-

C.T. Okamoto

cytes, renal epithelial cells, enterocytes, and placental syncytiotrophoblasts. MRP2 is mutated in patients with Dubin–Johnson syndrome, resulting in an inability of hepatocytes to efflux conjugated bilirubin, leading to hyperbilirubinemia. Therefore, MRP2 is thought to play a key role in the excretion of conjugated bilirubin, and its localization and activity may be regulated by LKB1. LKB1 may thus be a general regulator of the localization and activity of ABC transporters in hepatocytes or may regulate these transporters indirectly by its regulation of cell polarity (Jansen et al. 2009; Fu et al. 2010, 2011; Treyer and Müsch 2013; Müsch 2014).

The interaction of MRP2 with scaffolding proteins and the regulation of these interactions by kinases affect MRP2 function. Radixin is the predominant ERM protein expressed in hepatocytes and is localized to the apical canalicular membrane (Suda et al. 2011a). Radixin binds to the carboxy-terminal tail of MRP2 (Kikuchi et al. 2002). Deletion of radixin in mice results in a loss of canalicular expression selectively of MRP2 and in hyperbilirubinemia (Kikuchi et al. 2002). The localization of radixin to the canalicular membrane, and therefore MRP2 function, is affected by its phosphorylation (Suda et al. 2011a, 2016), the intracellular redox or metabolic state of hepatocytes (Sekine et al. 2011; Suda et al. 2015), and cholestasis (Kojima et al. 2008). An inability to excrete conjugated bilirubin or other metabolites caused by loss of MRP2 function may exacerbate hepatocyte damage initiated by the accumulation of metabolites. In enterocytes, however, ezrin may regulate the apical membrane expression of MRP2 (Yang et al. 2007; Nakano et al. 2013).

Via its PDZ-binding motif, MRP2 also binds to EBP50/NHERF1, and the interaction of EBP50/NHERF1 with either MRP2 or radixin is important for the efflux function of MRP2 (Karvar et al. 2014). Thus, ERM proteins, either through a direct interaction with the membrane transporter and/or indirectly through interaction with EBP50/NHERF1, appear to play a key role in the expression and function of select transporters at the apical membrane.

OTHER CORE TRAFFICKING MACHINERY REGULATING POLARIZED DELIVERY OF OTHER MEMBRANE TRANSPORTERS

Endogenous membrane transporters and channels have been increasingly used as markers for cargo destined for apical and basolateral membranes, resulting in further characterization of the machinery that regulates polarized sorting in epithelial cells. Mutations in the motor protein Myo5B and Stx3 are causes of the rare autosomal-recessive microvillus inclusion disease (MVID), whose patients suffer from chronic diarrhea and life-threatening dehydration, as well as metabolic acidosis (Davidson et al. 1978; Golachowska et al. 2010; Knowles et al. 2014; Vogel et al. 2015). The intestinal villi of these patients are severely atrophic (Davidson et al. 1978). Expression in cultured intestinal epithelial cells of an Myo5B with a mutation found in patients with MVID resulted in an inability of these cells to deliver a subset of apically targeted membrane transporter cargo, namely, NHE3, CFTR, and the facilitative glucose transporter isoform 5 (GLUT5) (Vogel et al. 2015). Myo5B and Stx3 were found to be part of a core complex that includes Rab11a, the v-SNARE Slp4A, Munc18-2, which mediates the interaction between Stx3 and Slp4A, and the v-SNARE Vamp7 (Knowles et al. 2014; Vogel et al. 2015). Other rabs that were found to interact with Myo5B and Stx3 include Rab8a and Rab3b (Vogel et al. 2015). The interaction of Myo5B with Rab8a supports a model in which this core trafficking complex may regulate other apical membrane delivery pathways, as Rab8a (and Rab11a) has been shown to regulate primary ciliogenesis (Das and Guo 2011; Deretic 2013). On the other hand, Rab8 regulates the *trans*-Golgi network-associated clathrin adaptor AP1b to regulate the delivery of basolateral membrane proteins (Ang et al. 2003). It is not clear how the hierarchy of Rab8 utilization in apical versus basolateral protein delivery is established, and it may be cell-type specific. In addition, interestingly, the mutated Myo5B did not inhibit the apical delivery of the glycosylphosphatidylinositol (GPI)-linked apical membrane proteins, dipep-

tidylpeptidase IV (DPPIV) or the disaccharidase sucrase-isomaltase (SI) (Vogel et al. 2015).

On the other hand, knockout or mutation of Rab8A in enterocytes of mice resulted in the inhibition of delivery of not only brush-border GPI-linked membrane proteins DPPIV, SI, and alkaline phosphatase, but also the polytopic absorptive transporters, such as the H⁺-dependent dipeptide transporter PepT1, responsible for the absorption of di- and tripeptides from the diet, and the Na⁺-dependent glucose transporter 1 (SGLT1) (Sato et al. 2007). Thus, the apical delivery routes are not segregated and regulated simply on the basis of GPI-linked versus integral membrane or polytopic membrane proteins.

Morphologically, enterocytes in these Rab8a mutant mice resemble those observed in patients with MVID, with villus atrophy, microvillar shortening, and microvillar incursions. In addition, a patient with MVID symptoms has been shown to have reduced levels of Rab8A (Sato et al. 2007). Functionally, the symptoms of the mutant mice recapitulate those of MVID patients, particularly diarrhea. The inability to complete the digestion of proteins and complex carbohydrates to single amino acids and monosaccharides, respectively, would be attributable to the inability to deliver dipeptidases and disaccharidases to the apical membrane. Coupled with the inability to absorb di- and tripeptides, as well as Na⁺ and glucose, because of the decrease in apical membrane PepT1 and SGLT1, respectively, the diarrhea of MVID mice is consistent with that of an osmotic diarrhea in MVID patients. In addition, these mice die soon after birth, and on necropsy, their intestines were observed to contain undigested milk. Although Rab8a clearly is involved in regulating apical membrane protein delivery, there may be subsets of protein-sorting complexes for different membrane cargoes, based on a yet-to-be-determined selection process.

Another hub for polarized membrane sorting may involve the sorting nexin protein 27 (SNX27). SNXs are a family of proteins that function as adaptor proteins for recycling of cargo between membrane compartments (Cullen and Korswagen 2011; Teasdale and Col-

lins 2012). SNXs all contain a phox homology (PX) domain, which binds to phosphoinositide 3-phosphate lipids; these lipids are enriched in endosomal membrane compartments, thus mediating the recruitment of SNXs to endosomes. SNXs work in recycling cargoes in conjunction with the protein complex known as the retromer (Bonifacino and Hurley 2008; Cullen and Korswagen 2011; Vergés 2016). Retromer is a highly conserved membrane coat complex that sorts cargo to be recycled from late endosomes to the TGN, and from recycling endosomes to the plasma membrane, via tubular membrane carriers.

SNX27 is a multidomain-containing protein, with a PDZ domain that interacts with a heterogeneous cohort of membrane receptors, transporters, or channels (Temkin et al. 2011; Ghai et al. 2013; McGough et al. 2014), and may also interact with the retromer subunit, VPS26 (Gallon et al. 2014). Moreover, SNX27 has an ERM domain that binds to another SNX, SNX1 (Steinberg et al. 2013), which contains a membrane-bending Bin-amphiphysin-rvs (BAR) domain. Thus, SNX27 is proposed to coordinate receptor recycling by binding directly to cargo via its PDZ domain and coupling it to membrane tubulation and recruitment of dynamic actin structures. SNX27 has recently been shown to regulate recycling of epithelial membrane transporters (Hayashi et al. 2012; Steinberg et al. 2013; Singh et al. 2015), neuronal membrane receptors (Cai et al. 2011; von Zastrow and Williams 2012; Wang et al. 2013b; Hussain et al. 2014; Loo et al. 2014), and G-protein-coupled receptors (Lauffer et al. 2010; Temkin et al. 2011; Chan et al. 2016; McGarvey et al. 2016). Interestingly, deletion of SNX27 in mice results in defective synaptic transmission and learning and memory deficits, presumably owing to the inhibition of neurotransmitter receptor recycling at postsynaptic neuronal membranes (Wang et al. 2013b). SNX27, therefore, may play a general role in recycling of membrane proteins, particularly in polarized cells.

In a global analysis of cells deleted in SNX27 or the VPS35 subunit of the retromer (Steinberg et al. 2013), there is a loss of cell-surface expres-

C.T. Okamoto

sion of a number of transporters that are expressed in epithelial cells, such as the Cu-transporting P-type ATPase, ATP7A. Mutations in ATP7A cause Menkes disease, a disease of copper deficiency (Polishchuk and Lutsenko 2013). The ATP7A also interacts with copper metabolism MURR1 domain-containing 1 (COMMD1) protein (Phillips-Krawczak et al. 2015), which, when mutated, also results in copper metabolic disorders. COMMD1 is the founding member of a family of proteins that are involved in proinflammatory signaling, hypoxia adaptation, and electrolyte transport (Maine and Burstein 2007). It is localized to endosomes and also interacts with FAM21, a component of the F-actin nucleating, endosome-associated, Wiskott–Aldrich syndrome protein and SCAR homolog (WASH) complex. The WASH complex also interacts with retromer. Thus, recycling of ATP7A is dependent on a SNX27-retromer-COMMD1-WASH axis. Interestingly, COMMD1 also interacts with CFTR (Drévillon et al. 2011) and ENaC (Ke et al. 2010) in cells that endogenously coexpress COMMD1 and either CFTR or ENaC. COMMD1 protects CFTR from ubiquitylation and sustains CFTR expression at the cell membrane; however, COMMD1 enhances ubiquitylation of ENaC, enhancing its internalization.

In cells deleted in SNX27 or VPS35, there is a loss of several other metal transporters, amino acid transporters, as well as the glucose transporter GLUT1, from the cell surface (Steinberg et al. 2013); however, not all of these transporters are localized to the apical membrane in epithelial cells. In fact, the multidrug resistance transporter MRP4 has been shown to interact with SNX27 (Hayashi et al. 2012), and this transporter is localized to the apical membrane in kidney epithelial cells, but basolaterally in hepatocytes. On the other hand, SNX27 has been shown to regulate NHE3 recycling at the apical membrane of epithelial cells (Singh et al. 2015). The overall role of SNX27 in the recycling of apical membrane transporters is, thus, not yet clear, although further characterization will likely support its role as an important hub in regulating polarized membrane recycling.

CONCLUSION

These examples of the regulation of membrane transporters and channels by membrane-trafficking pathways in epithelial cells hopefully have illustrated the value in focusing on and characterization of these proteins as cargoes, and there are numerous other examples that are unfortunately not included here. From these studies, it can be concluded that complexes that incorporate ERM proteins, NHERF family members, Rab8/11/Myo5B, and/or retromer are critical for the regulation of apical membrane localization and recycling. By applying cell biological approaches to transporter and channel function, a deeper mechanistic understanding of the physiology and pathophysiology of solute transport has been obtained, and it is clear that relatively commonplace trafficking machinery has been co-opted to effect a particular functional niche. In addition, the utilization of membrane transporters and channels as cargoes for membrane-trafficking pathways has resulted in novel findings regarding the function of select intracellular trafficking machinery complexes and the regulation thereof by intracellular signaling pathways in epithelial cell physiology.

ACKNOWLEDGMENTS

Apologies are extended in advance to those whose work has not been included here because of space constraints.

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C.T. Okamoto

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C.T. Okamoto

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Cold Spring Harb Perspect Biol 2017; doi: 10.1101/cshperspect.a027839 originally published online February 28, 2017

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