Complex polarity: building multicellular tissues through apical membrane traffic.

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Abstract
The formation of distinct subdomains of the cell surface is crucial for multicellular organism development. The most striking example of this is apical-basal polarization. What is much less appreciated is that underpinning an asymmetric cell surface is an equally dramatic intracellular endosome rearrangement. Here, we review the interplay between classical cell polarity proteins and membrane trafficking pathways, and discuss how this marriage gives rise to cell polarization. We focus on those mechanisms that regulate apical polarization, as this is providing a number of insights into how membrane traffic and polarity are regulated at the tissue level.

Key words
Apical transport, cell polarity, morphogenesis, endocytosis, exocytosis, lumen formation, Rab GTPases, Crumbs, Podocalyxin, epithelia, 3D culture, trafficking

Introduction
The evolution from uni- to multi-cellularity provided such organisms with unprecedented adaptive possibilities. This allowed cell surface subdomains to be shielded from the outside world, and thereby, to adopt unique and novel functions. This simple principle can be used iteratively, in different combinations, to give rise to tissue, organ, and organismal patterning. One of the most versatile cell types during organogenesis is epithelium, which adopts different shapes and functions to compartmentalize the body into physiological systems (1-3). How are the characteristic distinct cell surface subdomains of epithelia created when cell surface polarity does not yet exist? Recent advances in understanding protein transport during cell polarization reveal a key interplay between classical cell polarity proteins and the membrane trafficking pathways underpinning morphogenesis. We provide an update to this interplay, focusing on epithelia, as these have been instrumental in our evolving understanding.

Epithelia: the jack of all trades
Epithelial cells have discrete membrane domains with specific functions. One of these is an apical domain that lines the lumen of biological tubes (Figure 1A). This functions in exchange of materials into and out of tissues (4). Asymmetric from the apical surface is the basal and lateral domains, which are contiguous and are as such collectively referred to as the basolateral domain (3). These domains function in contact with the *E*xtra*c*ellular *m*atrix (ECM) and adjacent cells, respectively. The

apical and basolateral domains are physically separated by adherens junctions (AJs), which establish cell-cell contacts. The most apical region of the lateral domain of vertebrates possesses Tight Junctions (TJs), which form a paracellular diffusion barrier that also restricts diffusion between apical and basolateral membrane components (5, 6). In invertebrates this function is performed by Septate Junctions (SJs), which are located basally to the adherens junctions on the lateral domain (7) (Figure 1A). Although decades of work exist on mechanisms that regulate traffic to these distinct surfaces, much of this involves analysis of traffic in already polarized monolayers, such as in cell culture. Less is known about how trafficking pathways are utilized to generate distinct cell surface asymmetry *de novo* during polarity establishment. How junctions are regulated by trafficking pathways is the subject of other excellent reviews in this issue. We focus upon the interplay between polarity proteins and membrane traffic to generate apical-basal polarity, and highlight the emerging role of endocytic recycling in this process.

Core, conserved polarity proteins

The formation of cell surface asymmetry involves sets of evolutionarily conserved proteins, of which three dynamic modules have been described: the Par, Crumbs and Scribble complexes (Figure 1A). The main polarity determinants were first identified in genetic screens in *C. elegans* to examine protein *par*titioning *d*efects in the daughters of dividing cells, giving rise to the 'Par' protein namesake (e.g. Par6, gene name PARD6) (8). The requirement of these polarity proteins for morphogenesis was corroborated in other model organisms and mammal systems, leading to the identification of Par-interacting proteins involved in polarity such as the kinase aPKC and the GTPase Cdc42, a known yeast polarity determinant (9-12). Although not exclusively, many polarity proteins are scaffolds, which possess varying protein-protein interaction domains and act to nucleate multi-protein signalling complexes. By assembling different complexes, polarity proteins can be used iteratively to tailor different configurations to the specific needs of a cell. Such modular characteristics may explain the remarkable polarity determinant conservation across the Metazoan kingdom (1, 3, 13-15).

Traditionally, two main apical polarity modules have been described: the Crumbs and Par complexes (16) (Figure 1). The most apical is comprised of the transmembrane protein Crumbs (Crb) and its associated cytoplasmic scaffold proteins Pals1 and Patj. The 'apical' Par module consists of Par3, Par6, the GTPase Cdc42 and an atypical protein kinase C, aPKC. These complexes have extensive and direct intra- and inter-module interactions, and it appears that they form a number of spatiotemporal subcomplexes during polarity establishment (3) (Figure 1B). The basolateral polarity module is often referred to as the Scribble complex, but whether this represents a physical or functional complex remains unclear. This module consists of the scaffolds Lethal Giant Larvae (Lgl), Scribble, Discs Large (Dlg). The serine-threonine kinases Par1 (reviewed in (1, 15) and Par4 (LKB1) do not form part of either module, but nonetheless localize to the basolateral membrane (17). Par5 is a 14-3-3 family protein, which localizes to the cytosol (16). Par2 stands as a peculiarity amongst the polarity proteins in that in *C. elegans* it encodes an ubiquitin

ligase (18), but its orthologue through evolution is unclear. Though there are single genes for these proteins in *C. elegans* and *D. melanogaster*, the complexity of potential complexes is greatly increased in mammals where in some cases multiple paralogues, and/or multiple splice variants, exist. For more detailed explanation of polarity complexes, the reader is directed to other excellent reviews (1, 3, 13-15, 19). We focus on their interplay with trafficking machinery and pathways, to illuminate how these cooperate to build and maintain polarity. Examples of the influence of polarity regulators on membrane traffic during morphogenesis is presented in Table 1.

Competition and cooperation between polarity proteins

An important consideration in understanding how polarity proteins generate asymmetry is that they are able to form distinct spatiotemporally regulated subcomplexes (3). Multiple layers of positive and negative feedback between complexes ultimately results in zones of cell surface polarization, the most dramatic of which is the asymmetry between the apical and basolateral surfaces. The apical Par module provides examples of both positive and negative feedback between polarity modules. The scaffold, Par6, is normally autoinhibited. Upon GEF-mediated activation of Cdc42, such as by Ect2 (20), Dbl3 (21), and probably others, GTP-loaded Cdc42 binds Par6 to relieve the latter's autoinhibition. In turn this facilitates association of Par6 with aPKC (12). In this way, the apical Par complex acts as an interdependent module, the function of which is to stimulate aPKC-mediated phosphorylation of target substrates, by displacing aPKC's interaction with its own N-terminal pseudosubstrate (22).

Phosphorylation of targets by aPKC often acts to exclude the substrate from the surface domain at which the Cdc42-Par6-aPKC module resides, allowing polarity to remodel (Figure 1B). For example, during cellularization in *D. melanogaster* embryo, Par3 is one of the first polarity proteins recruited to a developing apical surface, helping to recruit E-Cadherin for the formation of AJ (23, 24), Par6-aPKC for the stabilization of these junctions (23, 25-27) and the Crumbs complex component Pals1 to the cortex (28). As polarity matures, aPKC-mediated phosphorylation of Par3 leads to its dissociation from aPKC/Par6 and Pals1, instead directing Par3 to the adherens junctions (29), and probably allowing stabilization of Crumbs at the apical membrane (28, 30).

aPKC can also act on basolateral determinants (Figure 1C), phosphorylating Par1 (31-33), Lgl (34-36) and Yurt (37) to promote their association with the 14-3-3 protein Par5 in the cytosol. This prevents the anchoring of these proteins at the (often apical) membrane. Conversely, the kinase Par1 can phosphorylate Par3, disrupting precocious assembly of any Par3-Par6-aPKC complex components at the basolateral domain (38). Several additional layers of antagonism exist (3, 16). All of this supports the notion that these polarity proteins form mutually antagonistic, and spatiotemporally distinct complexes.

How is it that these complexes become distinct if the same protein can associate with multiple modules? Positive feedback events can be sufficient to promote

spontaneous cell polarity generation (39, 40), and also promote stabilization and maintenance of asymmetry (41, 42). If two such domains are mutually antagonistic, this can explain the formation of stable asymmetry between the apical and basolateral domain. However, remodelling of cell surface domains *de novo* requires input from additional pathways to 'tip the balance' between mutually antagonistic domains. For example, Rho kinase (ROCK) can phosphorylate Par3 at a site very close to, and thereby sterically hindering, the major aPKC phosphorylation residue. This functions to modulate association of Par3 with Par6-aPKC (43). Thus, signalling inputs allow for remodelling of polarity complex composition either focally and/or temporally. However, and as an example, there are at least 11 spliceforms of Par3, and dozens of unique phosphorylation sites, suggesting that we have only scratched the surface of understanding how polarity proteins, let alone complexes, are regulated.

Polarity proteins and membrane traffic: a *pas de deux*.

Despite a significant body of work examining how polarity proteins regulate cellular asymmetry, this has focused mainly on cytoplasmic proteins (16). Similarly, there are now decades of research into the machinery that regulates vesicle formation, sorting and transport in polarized cells (3). Yet, for what now seems a *fait accompli*, our understanding of how polarity modules interface with plasma membrane organization and polarized membrane trafficking is still in its infancy.

The identification of Cdc42 as part of the Par6-aPKC module was an early indicator that Par proteins may regulate membrane trafficking, as Cdc42 was a known regulator of polarized transport through actin cytoskeleton regulation (12, 44, 45). This concept was solidified by seminal work from Bath Grant's laboratory, who identified roles for the 'apical' Par complex (Par3-Par6-aPKC-Cdc42) in endocytic recycling in *C. elegans* and mammalian cells (46). This was followed by identification of roles for Cdc42 and the 'apical' Par complex in regulating the integrity of the adherens junctions by controlling endocytosis of AJ components (26, 47, 48). It is important to point out that such defects may be secondary to effects on apical Par and Crumbs complex localization (26, 49-51).

A possible clue to the underlying mechanism for this came from Marino Zerial's laboratory, who demonstrated that the Par complex controls membrane traffic by influencing endosomal network positioning, possibly by controlling the cytoskeleton (52)(Figure 2). Apical Par (Par3, Par6), or dynein-dynactin, complex perturbation caused scattering of endosomal compartment organization in the *C. elegans* intestine. In contrast, yet in keeping with the notion of mutually exclusive apical-basal zones, basolateral Par1 or Par5 depletion resulted in mispositioning of (the normally sub-apical) Rab11-positive *a*pical *r*ecycling *e*ndosome (ARE) at the basolateral domain. This was associated with ectopic basal F-actin clusters, and mistargeting of a slew of normally apical molecules to these clusters. Importantly, the Par5 RNAi phenotype could be reversed by co-depletion of RhoA or Rac1 homologues. Thus, the antagonism between the apical and basal Par complexes extends to intracellular organelle asymmetry by controlling cytoskeleton

organization. This concept sits nicely with the demonstrated roles of Par proteins in controlling the cytoskeleton, during morphogenesis (47, 48, 53, 54).

A crucial concept in understanding the dance between membrane traffic and polarity proteins is that polarity proteins are both regulators and cargoes of endocytic recycling pathways. Examples of membrane traffic machinery that regulate polarity during morphogenesis are presented in Table 2. Targeting polarity determinants to their proper localization is essential for correct polarity establishment and maintenance. This extends to both transmembrane cargo such as Crumbs, but also to the cytoplasmic Par proteins. Blockade of endocytosis and early endosome formation (Dynamin, Rab5 and early endosome fusion machinery) results in varying disruption to apical-basal polarization, particularly to the Crumbs and Par complex components, ultimately leading to tissue integrity defects (53, 55-59). Similarly, perturbation of exocytosis via dynamin, clathrin and the clathrin adaptor AP-1 complex is required in *C. elegans* to maintain the correct asymmetry of Par6 and Cdc42 (59-61). There thus appears to be a well-choreographed tango between polarity proteins, the cytoskeleton, and the membrane compartments that transit cargo (which includes polarity proteins) to their correct locale. The interplay between these is required to generate and maintain an apical-basal polarized cell.

The *entr'acte*: cell surface polarity regulation at REs.

The transit of membrane proteins through Rab11-positive endosomes is emerging as key to maintaining cell surface asymmetry. Such 'REs' are intracellular sorting stations for both endocytic proteins, and biosynthetic cargo, *en route* to the cell surface from the Golgi (62-65). That Par protein function ensures the correct subapical positioning of Rab11-positive REs underscores such a notion (52). In polarized MDCK cells, Rab11a switches from a general recycling regulator to exclusively regulating apical transport (66), with the prominent exception of the otherwise basolateral E-cadherin (62, 67). Disruption of either entry into, general organization, or egress from Rab11 endosomes leads to a progressive loss of apical, then adherens junction and overall tissue organization (68-71). Studies into three apical transmembrane proteins, which each control morphogenesis of their respective cell type, have provided key insights: the polarity protein Crumbs (Crb), the major light-regulated element of the photoreceptor Rhodopsin (Rh1), and the lumen formation-regulating sialomucin Podocalyxin (Podxl). As we discuss Podocalyxin specifically in a later section, we focus on Rhodopsin and Crumbs here.

Unlike most polarity determinants, Crumbs is a transmembrane protein with a short intracellular domain that contains both FERM binding (FBM) and PDZ binding (PBM) motifs in its C-terminus (72, 73) (Figure 3A). Correct apical localization, and levels, of Crumbs ensures tissue homeostasis by maintaining apical-basal cell polarity, with both loss and gain-of-function of Crumbs affecting tissue integrity (42, 55, 72-77). Moreover, extensive and direct interaction between the Crumbs complex and Hippo pathway regulators ensure that nuclear translocation of the proliferation regulator YAP/TAZ is dampened in apical-basal polarized cells (78-81). Despite the clear importance of Crumbs in apical-basal polarity, it is still not clear what the function of

the extracellular domain of Crumbs is, though in some Crumbs paralogues, this is to provide cell-cell interactions with neighbouring cells (42, 82). Regardless of this hole in our understanding, the stereotypical and penetrant phenotypes that result from Crumbs perturbation have allowed mapping of the trafficking pathways that regulate apical transport to control cell polarity. Analogously, the *Drosophila* opsin Rhodopsin 1 (Rh1) is essential for rhabdomere development, the light-sensing organ in the fly eye (83). A unique feature of Rh1, at least compared to Crumbs, is that it also undergoes light-induced internalization, after which it can be degraded in lysosomes or recycled to the membrane (84). Defects in Rh1 traffic lead to photoreceptor degeneration both in mammals and *Drosophila* reviewed in (84). This feature, combined with studies into Crumbs, has allowed dissection of the pathways regulating both constitutive and stimulated trafficking underpinning polarization.

The earliest known regulator of transport of these key apical proteins occurs via interaction of an R(X)(K/R)R di-basic motif present in Crumbs-intracellular domain, which ensures its ER export by interaction with the Sar1 GTPase (85)(Figure 3). This overlaps with the FERM-binding region, suggesting that Sar1 associates before FERM partners can bind Crumbs (Figure 3A). Crumbs and Rh1 are both glycosylated and pass through the Golgi with the help of chaperone proteins, such as ninaA for Rh1 (86). After Golgi exit, these both transit via the Rab11 RE *en route* to the apical surface (68-70, 87). The Rab11 endosome is organized in concert with a slew of effectors, including *Rab11 family interacting proteins* (Rab11FIPs), the exocyst complex, and the actin-binding motor Myosin-5b (68, 69, 88). Perturbation of any of these effectors disrupts transit of Rh1 or Crumbs from REs to the surface, leading to Rh1 and Crb loss-of-function phenotypes.

Once at the apical surface, the total levels of Crb and Rh1 must be tightly controlled. Both proteins undergo endocytosis via a Dynamin, clathrin and Rab5, and possibly AP-2-dependent pathway, leading to the early endosome (55-58). Notably, the AP2 interaction motif in Crumbs overlaps with that of the PBM (Figure 3A), the latter of which is critical for stabilization at the membrane (30). This suggests that stable association of Crb with Pals1 might prevent Crb endocytosis via blocking association with AP2. aPKC is also thought to stabilize Crb at the cell surface, potentially by regulating Moesin association with the Crumbs complex (89, 90), though the exact mechanisms by which this occurs is unclear. Blockade of endocytosis mirrors Crumbs overexpression phenotypes, causing polarity defects (58), and suggesting that tightly regulated turnover is essential for balancing the antagonism between apical-basal domains. Conversely, blockade of endocytosis mirrors loss-of-function Rh1 phenotypes (57), indicating that Rh1 internalization is obligate for Rh1 signal transduction.

Once at the early endosome, both Crb and Rh1 have the similar option of alternate fates (Figure 3). Transport to late endosomes and lysosomes leads to the degradation of both proteins (1, 84). However, for maintenance of protein levels, transport back to the RE facilitates subsequent recycling to the apical surface. Interfering with early endosome fusion machinery (Rab5, rabenosyn, Vps45, syntaxin-7) or late endosomal/lysosomal function (ESCRT complex) blocks turnover

of these proteins (55, 91-94). Similarly, interference with the Retromer complex, which acts to retrieve proteins away from a degradative route, blocks recycling of Crb and Rh1, leading to their turnover. Notably, Crumbs interacts directly with the Vps35 subunit of the Retromer, ensuring that there is a tight regulation of Crumbs levels in cells (50, 51, 75). The Retromer can function in retrograde transport of endocytic cargo back to the Golgi, and/or transport into recycling compartments (95). It is not clear whether the retromer regulates sorting from early endosomes to the Golgi, or directly to the ARE for these cargos. Given that post-Golgi, Rh1 and Crb transit via the RE, either option may result in an eventual similar outcome. Intriguingly, the Scribble complex regulates the localization and transport of apical polarity determinants, seemingly through the Retromer complex (75). Exactly how Scribble achieves this remains to be demonstrated. Thus, transit via the RE is a critical step in the development and maintenance of epithelial polarity, which is underscored by defects in apical transport and polarity upon perturbation of the Rab11 GEF protein, Crag (71).

Crumbs is also required for preventing light-exposed adult eye degeneration (96). Surprisingly, Crumbs, through it's intracellular domain, stabilizes Myosin-5 in photoreceptors, protecting it from proteasomal degradation, and thus allowing the transport of Rh1 to the membrane (88). Myosin-5 and its homologues are essential for exocytosis in diverse morphogenetic contexts (97). Given such a crucial function of Rab11 vesicles in polarity, it will be important to determine whether this is a universal function of Crumbs in stabilizing Myosin-5-dependent apical transport. Collectively, these data tell us that polarity proteins are both cargoes and regulators of membrane trafficking pathways, which all appear to converge on the Rab11-positive RE. The consequences of disruption to apical AREs on human disease are presented in Box 1.

De novo apical polarity generation: building a lumen.

How is membrane traffic organized during development to build a domain, such as the apical membrane, when it does not yet exist? Studies in MDCK 3D spheroids, where single cells are embedded inside gels of ECM, provide an isotropic environment where cells must generate their own apical and basal surfaced *de novo* (4). This means that the trafficking pathways that can tell apical from basal must also be organized *de novo*. Studies in this system also highlight a central role of the Rab11a ARE in apical polarization. Notably, a similar morphogenetic cascade is observed in intestinal Caco-2 cyst cultures (98), and in mouse embryonic stem cells (99, 100), suggesting that this is an evolutionarily conserved process. We focus mostly on MDCK only for ease of explanation.

A schema of the morphogenesis of MDCK cysts is presented in Figure 4. Upon embedding in ECM, single MDCK epithelial cells undergo proliferation and morphogenesis to generate an apical-basal polarized monolayer organized radially around a single, central lumen. The process involves a series of stereotyped steps (101). Initially, single cells lack an appreciable apical-basal polarity. A cell division event occurs to generate a doublet. At this stage polarity is 'inverted', with the apical

membrane protein Podxl at the ECM-abutting surface, and the cadherin complex at the lateral contact between the doublet. The orientation of polarity is corrected by detection of the ECM via β1-integrins, leading to destabilization of Podxl at the periphery, and Podxl endocytosis (102). Once internalized, Podxl transits into Rab11a-positive REs and is transcytosed across the cell to a common site at the cell-cell contact between the cell doublet called the *A*pical *M*embrane *I*nitiation *S*ite (AMIS). This process involves realignment of the centrosomes of doublets from the periphery to face the AMIS in a mirror image configuration (17). Notably, Par4 (LKB1) drives peripheral actin contractility via the RhoA-ROCK-Myosin-II pathway, and this must be temporarily dampened to allow the centrosomes to reorient. Given the tight association of the Rab11 compartment with the centrosome (103-105), this is further example of how Par protein-dependent regulation of the cytoskeleton controls membrane traffic via regulating endosome positioning.

Apically destined Podxl- and Crb-containing vesicles are exocytosed to the AMIS, creating a nascent lumen and the beginnings of correctly orientated apical-basal polarity (101, 106, 107). Morphogenesis continues by maturing the AMIS into a *P*re-*a*pical *p*atch (PAP), a temporary stage with two closely interdigitated apical membranes, before the lumen is ultimately expanded through luminal secretion. Proliferation in the plane of the monolayer continues to expand the total cyst cell number. This transcytosis cascade is regulated by an intricate and complex rearrangement of intracellular organelle polarity to generate correctly oriented surface asymmetry (101). At the ARE, Rab11a recruits the Rab8 GEF, Rabin8 to activate Rab8a on REs. This Rab11-Rab8 cascade is essential for recruitment of Myosin-5b to REs (108), as well as for RE-localized activation of Cdc42 via the GEF Tuba (101). Notably, whilst Cdc42 localized to the RE, Par3-aPKC localized to the AMIS, supporting a model whereby Cdc42 also functions outside the Par complex on endosomes, but joins up with Par3-aPKC once it is delivered to the apical surface. Mirroring that seen for Crumbs and Rh1, Podxl-positive Rab11a vesicles also recruit Rab11FIP5 (109, 110), and the exocyst component Sec15 (101). Other exocyst components, such as Sec8 and Sec10 localize to the AMIS. A theme thus arises that the full assembly of the apical Par complex with Cdc42, and the exocyst holocomplex may only occur once Rab11 vesicles are delivered to the AMIS. This provides a neat mechanism to ensure that apical vesicles are delivered, and fuse, solely in the correct place for apical-basal polarization.

Less well understood are the basolateral membrane rearrangements that facilitate AMIS formation, which is essentially the conversion of a previously basolateral membrane into the apical surface and lumen. Despite doublets already having TJs, Par3-aPKC and several other TJ markers, initially form a contiguous line at the AMIS before apical vesicles are delivered (101), representing the unusual scenario of formation of a third TJ per cell. As the AMIS matures, Par3 and TJ proteins now split to become two new TJs on either side of the PAP, and the previous TJs are disassembled. This extensive rearrangement of polarity, particularly TJs facilitates development of apical-basal polarity in a new locale. This is consistent with the initial characterisation of the Par3-Par6-aPKC complex as regulators of TJ formation in mammalian epithelia (12, 111-113).

Delivery of apical vesicles is also regulated by Rab11FIP5-dependent interplay between SNX18 and the motor KIF3A (109, 110, 114). SNX18 appear to regulate the budding of vesicles from the Rab11a endosome, whilst KIF3A is part of the Kinesin-II microtubule motor. Phosphorylation of Rab11FIP5 by GSKβ3 controls the former's mutually exclusive binding to either SNX18 or KIF3A (109, 114). This suggests that a cascade of Rab11FIP5 interactors may regulate progressive stages of apical transport. Given that Rab11a leads to activation of so many effectors, it is unclear of whether this reflects the sequential action, or multiple pools, of differential Rab11-effector complexes to generate apical polarity.

Several other Rab proteins are involved in ensuring the fidelity of apical transport solely to a single AMIS. A comprehensive screen for Rab-dependent trafficking of Podxl revealed several Rabs regulate likely processive steps in transit to the lumen (115). Rab3b, Rab27a/b act in concert with the apical SNARE protein, Syntaxin-3, and the Synaptotagmin-like proteins, Slp-2a and Slp-4a (116). The transit of Podxl-containing vesicles to the AMIS is essential for interaction of many vesicle-localized trafficking machinery, as blockade of Myosin-5b function attenuates association with the Rab11-Rab8 cascade with the AMIS localized Syntaxin-3 and munc18-2 SNARE machinery (98). Defects in any of these proteins cause the formation of multiple lumens and in some cases, the formation of multiple ectopic lumens per cell. Rab35 is a crucial player in ensuring fidelity of apical vesicle delivery, as it binds directly to the cytoplasmic tail of Podxl and acts as a tethering factor at the AMIS (117). Notably, mistargeting of Rab35 to mitochondria is sufficient to target not only Podxl to mitochondria, but also aPKC, Crumbs and Cdc42. This suggests that correct targeting of Podxl is essential for subsequent establishement of apical transport. As such, Podxl depletion or blockade of its interaction with Rab35 results in the subapical accumulation of these apical polarity proteins (101, 117). The particularly severe phenotype of Rab35 depletion may be compounded by the requirement for Rab35, and its effector ACAP2, in β1-integrin traffic (118), the lattermost of which regulates the orientation of apical traffic in cyst cultures (115). Rab11 endosomes also carry a number of kinases that act on ERM proteins (119), the latter of which is required to stabilize some proteins, including Podxl and Crumbs (120), at the apical surface, potentially explaining such defects.

The interdependence of polarity proteins and membrane traffic is also highlighted by these studies. Inhibition of aPKC or Par3 results in a failure to align the AMIS of each doublet cell, thereby disrupting co-ordinated vesicle delivery to form a single lumen (101). Par3 regulates centrosome migration in other systems (121), and whether this is a consequence of misaligned centrosomes is unknown. Conversely, inhibiting exocyst-dependent vesicle transport results in a lack of Par3 enrichment at the AMIS (101). This suggests that membrane traffic to the AMIS is part of the positive feedback loop that stabilizes the apical polarity complex. Given that Crumbs and Cdc42 both transit via Rab11 vesicles to the lumen (101, 106), the recycling of Crb and Cdc42 back to aPKC-Par6 at the apical surface may be a major function of Rab11 endosomes during apical polarity establishment and maintenance. The pathophysiological consequences of ARE dysfunction is discussed in Box 1.

Concluding Remarks and Future Considerations

One of the most important aspects in considering how polarity proteins and trafficking pathways cooperate to regulate morphogenesis is the model system and tissue used. Different organisms, and the milieu of different cell types in the same organism, may have vastly different expression of both polarity proteins and trafficking proteins. This may result in stark contrasts in the requirement for certain polarity and trafficking pathways in different tissues. The focus on select systems and model cargo is not intended to under-illuminate the importance of other cargo, or their contribution to our understanding of morphogenesis. Rather, these are meant to highlight the common themes that have emerged. It is also important to recognize that differences in trafficking of the same molecule, such as Podxl, occur between regular '2D' versus 3D culture methods (115, 116). Some trafficking pathways are specifically upregulated to handle the additional requirement of building an apical surface *de novo*, such as in 3D (116). Such differences are likely compounded further as one moves from 3D into *in vivo* systems. A crucial question for the future is thus how the described pathways are rewired to suit different epithelia with different physiological functions. This is intrinsically linked with understanding how membrane trafficking and cell polarity proteins are transcriptionally controlled, of which we know very little. For instance, do classical cell fate-inducing morphogens do so by controlling expression of proteins that regulate specialized trafficking pathways? Or just as intriguingly, does differential expression of trafficking and polarity pathways only allow certain cell types to respond to a particular morphogen? This later scenario has been elegantly demonstrated by the Martin-Belmonte lab, which showed that expression of proteins that promote recycling of apical SNARE proteins is required to control how the Notch signalling pathway is handled, thereby controlling where Notch-induced developmental fate occurs (122). Now that we know some of the rules of how membrane traffic and polarity proteins interplay, perhaps it is time to consider the cause and consequence of their differential expression in model systems apart from our powerful, but limited, epithelial 'work-horse' systems.

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Box 1. Apical polarity, membrane traffic, and Microvillus Inclusion Disease

The importance of the interplay between the ARE and apical polarity is exemplified by Microvillus Inclusion Disease (MVID), a rare but severe congenital neonatal disease presenting with intractable secretory diarrhoea. Mutations in Myosin-5b (MYO5B) are the major genetic cause of MVID (123). MYO5B mutations occur throughout the coding sequence and are inactivating, either through truncation, uncoupling of association with Rab8 or Rab11, and slowing of the processivity of the myosin motor. Atypical variant MVID also occurs from mutations in the apical SNARE protein Syntaxin-3 (STX3) (124). MVID-like symptoms in familial hemophagocytic lymphohistiocytosis type 5 patients can also occur from mutations in the Syntaxin-3-binding protein munc18-2 (STXBP2) (125). MVID is typified in the presence of the appearance of vacuolar intracellular 'inclusions' which contain microvilli. In addition, numerous normally apically localized transporter proteins are lost from the apical surface, and are varingly mislocalized to these inclusions (123). Although the majority of defects involve apical transport insufficiencies, some basolateral cargoes can be mislocalized, though this varies between model systems and patients. What is remarkable is that all of the identified human mutations associated with MVID encode for defective proteins that are normally crucial for transport from the ARE to the apical surface. All of these are identified to regulate either Crumbs or Podxl apical traffic (101, 106, 116). Moreover, knockout (KO) mice for Rab11a, Rab8a and Cdc42 all display MVID-like intestinal phenotypes (126-129), underscoring the importance of the ARE. This further emphasizes the notion that Rab11, Rab8, Cdc42, Myosin-5b, Syntaxin-3, and Munc-18-2 are acting together as a functional unit to ensure apical protein transport. Indeed, recent work has shown that these molecules, and many additional molecules identified in MDCK studies on lumen formation, form a complex that is dependent on Myosin-5b for proper assembly (98). Accordingly, what is invariant in these different *in vivo* models is that the localization of each of the other complex members is disrupted when one is knocked out. Indeed, the apical Par3-aPKC-Par6 module is diminished at the apical surface in MVID patients (130). Defects in the interplay between the ARE and apical polarity proteins thus results in severe defects in physiology, soon after birth.

Table 1. Select examples of polarity determinants and GTPases controlling trafficking pathways

Polarity determinant	Model System	Function / Defect	Reference
Apical Par Complex (Cdc42-aPKC-Par6-Par3)	*Drosophila* (various epithelia)	Apical (Crumbs) and AJs component endocytosis	(26, 47, 48)
	C. elegans intestine	Control of endocytic recycling	(46)
	C. elegans intestine	Retrograde transport from recycling endosomes to the Golgi	(131)
	MDCK cyst	Apical exocytosis during lumen formation	(101)
Par5/Par1	*C. elegans* intestine	Control of endosome positioning, particularly Rab11	(52)
Scribble complex (Scrib-Lgl-Dlg)	*Drosophila* (various epithelia)	Regulate retromer-dependent endosomal trafficking	(75)
aPKC	*M. musculus* retinal endothelia	Phosphorylate Dab2 endocytic adaptor to inhibit VEGF receptor endocytosis	(132)
Crb	*Drosophila* photoreceptor	Protect MyoV from proteasomal degradation	(88)
Rac1	*Drosophila* trachea	Control tracheal elongation, and promote Crb endocytosis	(133)
Lgl	*Drosophila* eye	Regulation of Notch endocytosis	(134)
	Budding Yeast	Co-operate with SNARE-mediated exocytosis	(135-137)

Table 2. Select example machinery and model systems illuminating trafficking pathways controlling apical polarity.

Group	Protein	Model system	Function / Defect	Reference
Endocytosis and early endosome	Avalanche/Syntaxin-7; Rab5, Rabenosyn-5, Vps45, Dynamin, clathrin AP-2 complex	*D. melanogaster*	Regulate cortical levels of apical proteins by controlling endocytosis Regulate tubular lumen shape Allow light-induced rhodopsin activation	(55-58)
	Rab5, Dynamin	*C. elegans* embryo	Maintain cortical levels of Par/anterior polarity determinants	(53, 59)
Exocyst	Sec5, Sec6, Sec15, Exo84	*D. melanogaster*	Delivery of Crumbs to apical membrane Recycling of internalized E-cadherin	(67, 68, 138)
	Sec15A, Sec8, Sec10	MDCK cyst	Traffic of apical determinants to the AMIS for *de novo* lumen formation	(101)
Apical Recycling Endosomes	Rab11a, Rab8a, Rabin8, Rab27a/b, Rab3b, Rab25, Rab11FIP5, Myo5B, SNX18, GSK3β	MDCK cyst	Traffic of apical determinants to the AMIS for *de novo* lumen formation	(101, 106, 108-110)
	Myo5B Rab8	Human enterocytes Mouse intestine	Trafficking of apical and basolateral proteins. Defects in Myo5B causes MVID	(126, 139)
	Plasmolipin, EpsinR, Syntaxin-7	*D. rerio* intestine	Recruitment of apical SNAREs to ARE Controls Crumbs and Notch endocytosis	(122)
	Rab11, dRip11, MyoV	*Drosophila* epithelia	Delivery of apical determinants to apical cortex and maintenance of E-Cad membrane levels	(69, 70)
	Crag	*Drosophila* photoreceptor	Control Rab11-dependent apical trafficking of Rhodopsin from the TGN Controls basement membrane deposition.	(71, 140, 141)
	KIF3A, Rab11FIP5	MDCK cyst	Transport of apical proteins to lumen	(114, 142)
Retromer	Vps35, Vps29, Vps26	*Drosophila* epithelia	Retrieval of apical proteins (Crumbs, Rh1) away from the degradative pathway	(49-51)
Apical domain	Rab35	MDCK cyst	Tethers Podxl-containing vesicles to the AMIS via direct binding to Podxl	(115, 117)
	Slp-2a, Slp-4a, Munc18-2, Stx3, VAMP7	Caco-2 cyst	Apical SNARE fusion complex controlling lumen formation	(98, 116)
Other	Clathrin AP-1 adaptor complex, SOAP-1, Clathrin	*C. elegans* intestine	Apical sorting of E-Cad, Cdc42 and Par determinants	(60, 61, 143)
	FMNL3, RhoJ	HUVEC 3D culture	Polarized trafficking of Podxl during lumen formation	(144)
	NinaA	*Drosophila* photoreceptor	Rh1 trafficking through the secretory pathway	(145)
	COPII machinery (Sar1, Sec24CD)	*Drosophila* embryo	Crumbs ER export	(85)
	ESCRT	*Drosophila* embryo	Altered Notch trafficking and overproliferation of tissue	(93, 94, 146)
	Rab3a, Rab3d, Rab4a/b, Rab5a, Rab5b, Rab12, Rab15, Rab17, Rab19, Rab32	MDCK cyst	Traffic of apical determinants to the AMIS for *de novo* lumen formation through unknown mechanism.	(115)

Figure Legends

Figure 1. Polarity complexes regulating epithelial polarity.
A. Common and distinct regulators of epithelial apical-basal polarity. Schematic representation of an epithelial tubule, with the apical domain lining the internal lumen. Presented is a prototypical epithelial cell comparing the common and distinct polarity determinants between both mammals and *Drosophila*. Proteins belonging to the same complex are represented by the same colour. Three major polarity complexes are present in all epithelial, including the apically localized Crumbs (green; Crumbs, PATJ, Pals1) and Par (blue; Cdc42-Par6-aPKC) complexes, and the basolateral Scribble (pale red; Scrib, Lgl, Dlg) complex. Each module partner acts to stabilize its own module. In addition, Par3 localizes to adherens junctions (AJs), while Par1 and Par3 can associate with Par5 when phosphorylated, leading to their cytoplasmic sequestration. In Mammals, the tight junction localizes above the AJ, in concert with the cytoplasmic scaffolding zona occludens proteins (ZO1-3). In *D. melanogaster*, an alternate junctional complex, the septate junction, forms basally to the AJ, whereat another type of polarity complex localizes with the Na+/K-ATPase (along with Yurt, Coracle, Neurexin). The single Drosophila ZO-1 protein Polychaetoid (Pyd) localizes to adherens junctions. Yellow circles represent phosphorylation.

B. Sequential formation of polarity protein subcomplexes in the *Drosophila* embryo. ***1.*** During cellularization, Par3 helps recruit DE-Cadherin to establish AJs. ***2.*** Activated Cdc42 and Par3 contribute the recruitment of Par6 and aPKC to the cortex. Par3 also participates in Pals1 recruitmet. ***3.*** As polarity matures, aPKC then acts to phosphorylate Par3, thereby excluding Par3 from the apical membrane, restricting Par3 to a more basal location, contributing to the definition of the apical-lateral border. ***4.*** aPKC-mediated phosphorylation of Par3 also disrupts Par3-Pals1 interaction, allowing Pals1 to now stabilize Crb in the apical membrane. Par6-aPKC and Scrib complex proteins further contribute to polarity maintenance through reciprocal inhibition.

C. Polarity proteins regulate zones of surface asymmetry. GEF-mediated activation promotes Cdc42 binding to Par6 to relieve the latter's autoinhibition, which in turn allows association with aPKC. aPKC-dependent phosphorylations are crucial for epithelial cell polarity. APKC can promote Crumbs complex stability. The apical Cdc42-Par6-aPKC module excludes Par3 and the basolateral determinants from the apical cortex. Further inhibition of Par3 by the basolateral determinants ensures Par3 is located to the AJ 'mid zone' between apical and basolateral. Positive feedback within the Scrib module ensures its formation. Inhibition of aPKC by the basolateral determinant ensures that the apical domain does not extend into the basolateral region. Green arrows represent positive feedback and co-operation. Red inhibitory arrows represent negative feedback and inhibition.

Figure 2. Control of endosome positioning by Par proteins.
In polarized epithelial cells, the ARE is localized in a subapical position, promoted by the apical Par (Cdc42-Par6-aPKC) complex. Upon loss of function of Par5 (L.O.F.), or Par1, the ARE become ectopically localized to a basal position, and misdelivery of

apical proteins to the basal domain occurs. Loss of function (L.O.F.) of the apical Par complex results in a generalized scattering of endosomal compartments. This suggests that the antagonism between basolateral (Par1/Par5) and apical Par determinants normally act in concert to maintain endosome positioning in a polarized cell. Red line, apical domain; black lines, basolateral domain.

Figure 3. Apical trafficking of cargoes in Drosophila.

A. Structure and motifs of the Crumbs cytoplasmic tail.

The cytoplasmic tail of *D. melanogaster* Crumbs (Crb) allows FERM interactions with Expanded, Yurt and Moesin through the FBM, which overlaps with the Sar1 interaction site, required for ER exit. The C-terminal PDZ motif interacts with Pals1 and Par6, which overlaps with the AP-2 interaction motif. Grey rectangle, extracellular (EC) region. Green oval, FERM-binding motif (FBM). Blue oval, PDZ-binding motif (PBM).

B. Crumbs intracellular trafficking.

Newly synthesized Crb exits the ER via association of Sar1. Crb is delivered to the apical surface via the Golgi, then *A*pical *R*ecycling *E*ndosome (ARE), where at it is assisted by Rab11 and the exocyst complex. At the apical cell surface, Crb is stabilized through interaction with Pals1, and an aPKC-dependent interplay with Moesin. Crb is removed from the apical surface by interaction with AP-2, Dynamin (Dyn) and Rab5, through an AP-2 interaction motif present next to the PDZ domain. In concert with early endosome formation machinery (Rab5 and its effectors, such as Stx7), Crb can be routed to two alternate fates: recycling or degradation. This choice is influenced by the Scribble complex, which regulates retromer-dependent retrieval of Crb away from the degradative late endosome (LE)/Multivesicular body (MVB) pathway, the latter of which is regulated by the ESCRT complex. Crb interacts directly with the retromer to ensure its recycling. Crb may either recycle through the Golgi or ARE.

C. Rhodopsin intracellular trafficking

Drosophila Rhodopsin (Rh1, red square) is a G-protein-coupled receptor whose trafficking follows a similar pattern to Crb. Rh1 is trafficked through the Golgi, assisted by the chaperone ninaA. Rh1 also passes through the Rab11-dRip11 ARE *en route* to the specialized apical light-sensing organelle, the rhabdomere. Light triggers the Dynamin-dependent endocytosis of Rh1, which is delivered to early endosomes also by Rab5 and Stx7. Mirroring Crb, Rh1 can either be degraded or recycled, depending on the retromer.

Acronyms: AEE: *A*pical *E*arly *E*ndosomes, ARE: *A*pical *R*ecycling *E*ndosome, LE: *L*ate *E*ndosomes, MVB: *M*ulti*v*esicular *B*ody, ER: *E*ndoplasmic *R*eticulum.

Figure 4. Membrane trafficking during *de novo* lumen formation in MDCK cysts.

MDCK cells embedded in extracellular matrix undergo a series of stereotyped, but not always synchronous, morphogenetic steps from a single cell to a cyst surrounding a single, central lumen. ***1.*** Single cells possess apical and basal proteins on the same surface, with some internalized apical proteins. ***2.*** After division, and at

the early doublet stage, Podxl is localized to the ECM-abutting periphery, and is then internalized and accumulates in Rab11-positive endosomes. ***3.*** Podxl vesicles are delivered to an *A*pical *M*embrane *I*nitiation *S*ite (AMIS), which develops transiently at the cell-cell contact and is composed of tight junction (TJ) proteins. ***4.*** Following delivery of apical proteins, TJs migrate to the two edges of the nascent apical domains, and a *P*re-*a*pical *P*atch (PAP) develops where apical-basal polarity has formed, but the lumen has not yet expanded. ***5.*** Expansion of the lumen and proliferation in the plane of the monolayer allows the cyst to develop. Examination of the machinery required for delivery of Podxl-containing vesicles reveals modules that ensure delivery of apical vesicles specifically to the AMIS. The most crucial concept is that full assembly of these modules requires motor protein-driven delivery of the Podxl vesicle to the AMIS. Note: Myo5b requires binding of both Rab11a and Rab8a for apical transport (arrows). Module members are similarly colour-coded. Red lines denote apical membrane; black denotes basolateral. Grey demarcates tight junctions.

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