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Molecular Regulation of Lumen Morphogenesis Review

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Abstract

The asymmetric polarization of cells allows specialized functions to be performed at discrete subcellular locales. Spatiotemporal coordination of polarization between groups of cells allowed the evolution of metazoa. For instance, coordinated apical-basal polarization of epithelial and endothelial cells allows transport of nutrients and metabolites across cell barriers and tissue microenvironments. The defining feature of such tissues is the presence of a central, interconnected luminal network. Although tubular networks are present in seemingly different organ systems, such as the kidney, lung, and blood vessels, common underlying principles govern their formation. Recent studies using *in vivo* and *in vitro* models of lumen formation have shed new light on the molecular networks regulating this fundamental process. We here discuss progress in understanding common design principles underpinning *de novo* lumen formation and expansion.

Introduction

The essence of metazoa is the organization of cells into tissues. The most fundamental type of tissue is epithelia, which consist of a layer of polarized cells that line a surface and thus serve to divide the organism into compartments. Some epithelia cover the outside of the organism, but almost all metazoa contain internal hollow spaces or lumens, which are lined by a layer of epithelial cells. Such lumens may serve to isolate specific functions, such as digestion, or to allow the movement of fluids, gases or cells between different parts of larger animals. Some very small lumens are surrounded by a single cell, such as the terminal branches of the *Drosophila* trachea, but most lumens are encompassed by multiple cells [1]. The simplest overall structure of lumen-containing organs is a sphere, such as the thyroid follicle. Most typically, though, these organs are elongated into tubules, which can be unbranched (e.g. sweat gland) or branched, often ending in spherical caps, termed acini or alveoli (e.g. mammary gland or lung) [2]. Some tubules form anastomosing networks, such as the vasculature, which is lined by specialized epithelia known as endothelia. All of these networks have in common a central lumen.

Lumens form during development by remarkably diverse mechanisms, including the wrapping, folding, invagination or evagination of polarized cell sheets to generate a hollow lumen [2]. Loosely adherent mesenchymal cells can also convert into polarized epithelia, termed the mesenchymal–epithelialtransition [2], and create lumens between the cells.

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Several reviews of tubule formation have described the molecular control of these processes in different organs [1–7].

Certain common design principles underpin the seemingly enormous diversity of lumen and tubule formation mechanisms. In nearly all cases, lumens are lined by the apical surfaces of the limiting epithelial cells [3]. (A fascinating variation is the circulatory system of certain invertebrates, which lacks endothelial cells and in which the basal surfaces of cells line the lumen, which is initially filled with extracellular matrix (ECM) [8].) Formation of the apical surface involves the coordination of membrane trafficking machinery with the polarity complexes that define polarized plasma membrane domains [9]. Moreover, in the case of multicellular lumens, cells must coordinate the orientation of their apical surfaces to face the lumen, which requires interaction of the cell with other cells and the ECM [10].

What basic design principles are required for cells to form a lumen *de novo*? The first principle must involve cell–matrix and cell–cell recognition — sensing one's environment and neighbors. This is a pre-requisite for determining where to form the lumen. The second principle must involve apical-basal polarization, spatiotemporally coordinated with neighboring cells. This can happen by one of at least three principal ways: hollowing, i.e. vectorial apical membrane transport to a common point between apposing cells, generating luminal space *de novo*; cavitation, i.e. clearing of non-ECM-contacting inner cells from a cell cluster, such as by apoptosis, resulting in a polarized layer surrounding luminal space;or focalized contact, where adjacent cells adhere only at their lateral-most apposing edges, generating luminal space between contacts (Figure 1). A third design principle involves the expansion of the luminal space, such as by fluid and ion efflux. Here, we consider recent advances in our understanding of common design principles, across different species, tissues, and cells, of *de novo* lumen generation and expansion.

Cell–Cell and Cell–Matrix Recognition

When non-epithelial cells coalesce to form tubular epithelia *de novo*, polarization must begin with a cell determining the directionality of lumenogenesis. Typically, lumens form at a shared position between neighboring cells, often perpendicular to the ECM-contacting surface, such as in kidney tubular epithelium, although other luminal positions (e.g. laterally between hepatocytes) can occur. Signals from the ECM provide one axis from which to orient lumen positioning; neighboring cells, through cell–cell contacts, provide a second axis. These combinatorial inputs provide molecular cues, and thus spatial coordinates, for generation and positioning of apical membranes.

Role of Cell–Matrix and Cell–Cell Recognition

How are signals transduced from the ECM into cells to effect polarization? Heterodimeric integrin molecules, consisting of an α- and β-integrin pair, play crucial roles in sensing ECM in a variety of cell types, with β1-integrin-containing complexes having key roles in tissue polarization [11,12]. In *Drosophila* tracheal terminal branches, deficiency of certain β- or α-integrins, or talin, which connects integrins to the cytoskeleton, leads to multiple lumens [13]. In mice, global loss of β1 integrin leads to embryonic lethality [14]. Tissue-specific knockouts have varying severity, although lumens are often perturbed. While the loss of β1-integrin from kidney collecting ducts does not abolish polarization or luminal network formation, kidneys were hypoplastic, and lumens were often cystic or dilated [15]. Endothelial-specific β1-integrin knockout mice are embryonic lethal, associated with luminal and branching defects [16,17]. Analysis of later blood vessel development using hypomorphic alleles revealed perturbed vessel polarity, filled lumens, and mistargeting of cell–cell junction proteins [18]. In these knockouts, the polarity protein Par3 was downregulated, and Par3 re-expression partially rescued lumen occlusion, suggesting that

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polarity proteins are key β1-integrin signaling targets. Indeed, in 3D cultures (Figure 2B) a signaling module involving α2β1 integrin, the adhesion proteins Jam-B/C, and the polarity proteins Par-3–Par6β–Cdc42 controls ECM remodeling by the matrix metalloprotease MT1-MMP to form endothelial tubes [19].

Similarly, in MDCK 3D cultures, a laminin–β1-integrin– Rac1 module controls apical-basal polarization [10,20]. β1-integrin deficiency perturbs the normal orientation of the apical surface to a central region between cells, through inappropriate activation of a RhoA–ROCKI–myosin II pathway [21], suggesting that ECM-derived signals can influence lumenogenesis via regulating cytoskeletal tension [22]. Lumen positioning (apical versus lateral) in MDCK is also controlled by regulation of the cytoskeleton by the Par protein Par1b [23–25]. In *Drosophila* laminin mutants, development of most organs, including the gut, airway and nervous system, is defective [26]. Thus, the ECM, β1 integrins, and polarity proteins are key regulators of apical surface and lumen orientation.

Sensing neighboring cells occurs via a multitude of adhesion receptors, including cadherins and nectins [27]. Defining individual roles of these molecules during lumenogenesis has been complicated by the partial redundancy of multiple family members. For instance, N-cadherin- and VE-cadherin-null mice display varying developmental defects, including aortic and vascular luminal perturbations [28,29]. While global E-cadherin knockout mice are not viable, tissue-specific knockouts, such as in thyroid follicles, reveal that lumens are present, but often smaller [30]. Similarly, epithelial polarization occurs in DE-cadherin-null *Drosophila* [31]. Thus, although fundamental roles for adhesion molecules in generating tissue polarization have been postulated from decades of studies in 2D culture, if and how these molecules actually regulate lumenogenesis *in vivo* still remains largely unclear.

Establishment of Apical-Basal Polarity

Once newly polarizing cells recognize the ECM and their neighbors, luminal space can be generated, either by hollowing, cavitation, or focalized contact and repulsion (Figure 1). Each mechanism requires the spatial and temporal coordination of cellular processes such as directional vesicle trafficking (hollowing), luminal cell death (cavitation) and localized formation of cell–cell contact and repulsion (focalized contact). Recent data suggest that these processes may not be mutually exclusive; if one process is perturbed, compensatory induction of the other may ensue [32].

Creating an Apical Surface *de Novo*

To generate a luminal domain *de novo*, neighboring cells must coordinate delivery of apical membrane components to a common site (Figure 1B). One possible mechanism may be to utilize a molecular landmark on the cell surface, common between neighboring cells. Such a landmark may form at the midbody during mitosis, as occurs in budding yeast [33]. During vegetative growth of *Saccharomyces cerevisiae*, the ‘bud scar’, retained from a previous cytokinetic event, provides a landmark for anchorage of the cytoskeleton and for localized membrane growth leading to the new bud formation. Recent studies [34,35] indicate that in mammalian cells, vesicles containing apical proteins are delivered to a discrete, common landmark between neighboring cells to initiate the lumen, a region termed the apical membrane initiation site (AMIS [35]).

A cohesive picture of the regulatory networks that control lumen initiation has begun to emerge (Figure 3). Prior to lumen initiation (in MDCK cysts), the polarity protein Crumbs3a and the apical glycoprotein podocalyxin/gp135 accumulate in Rab11a-positive vesicles [34–36]. Similar subapical vesicles, containing the apical glycoprotein Muc1, are observed *in vivo* at the onset of murine pancreas lumenogenesis [37]. Rab11a initiates a GTPase

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cascade, recruiting the Rab guanine nucleotide exchange factor (GEF) Rabin8 to sub-apical vesicles, in turn activating Rab8a/b at this locale [35]. This Rab cascade drives vesicle surface delivery, possibly by activating motor proteins such as myosin-5B [38–40]. Transport and docking of these vesicles with the AMIS is promoted by the hetero-octameric exocyst complex [35]. Fusion of apical vesicles with the plasma membrane to create an apical surface *de novo* is likely to occur via SNARE proteins, with syntaxin-3 acting as one likely key regulatory SNARE [41].

The AMIS is demarcated by a polarity complex comprising Par3 and atypical protein kinase C (aPKC) and the exocyst subunit Sec8 (though other polarity proteins and exocyst members also overlap with both the AMIS and non-AMIS regions of the cell–cell contact) [35]. Notably, an AMIS-like structure has been observed in mouse aorta lumenogenesis [34,42], zebrafish neuroepithelial lumen formation [43], *Drosophila* pupal photoreceptor and tracheal tube intercellular lumenogenesis [7,44], and during the formation of the intracellular lumen of *Drosophila* terminal tracheal cells [45], suggesting it as a common *de novo* lumen-initiating structure both in 3D culture and *in vivo*. Targeting of apical vesicles, the exocyst and Par3–aPKC complexes to the AMIS is mutually interdependent and, moreover, requires the upstream Rab8–Rab11 cascade [35], and in *Drosophila*, the Arf-like3 (Arl3) GTPase (which localizes to Rab11 endosomes) [46,47]. Studies in *Drosophila* trachea also suggest that cadherin-mediated adhesions may be prerequisite for AMIS formation [7,44]. All of these components are likely required to form a single lumen. This suggests that the apical exocytosis and polarity machinery operate in a positive feedback loop to establish and expand an apical domain during lumen initiation.

In yeast, Cdc42-directed networks play a critical role in targeting of vesicles to the new bud site [48]. Unlike unicellular yeast polarization, however, metazoa usually require the contribution of multiple cells to form a lumen. Thus, the orientation of cell division must be coupled to apical surface generation. In mammalian cells, Cdc42 plays a critical role in both processes. Cdc42 is activated at the apical pole of cells by Rab8–Rab11, and the Cdc42–Par3–aPKC polarity complex, in conjunction with the phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$)-binding protein annexin 2 (Anx2), controls exocytosis to the AMIS [35,49].

The AMIS matures into a ‘pre-apical patch’ (PAP), an early apical domain between cells where the luminal space has not yet expanded [34,35]. Here, the Par3–aPKC polarity complex, and the plasma-membrane-localized exocyst subunits (Sec8–Sec10) relocalize to tight junction regions. How is this lumen maturation step controlled? In MDCK cysts, Par3 and aPKC kinase activity are required for apical trafficking to the AMIS to expand to a PAP [34,35]. In *Drosophila*, aPKC-mediated Par3 phosphorylation disrupts Par3–Pals1 association [50], presumably to allow formation of the apical Crumbs–Pals1–PatJ complex, which is then involved in dissociation of Par3 from Par6 and aPKC [51,52]. Crumbs3a delivery to the nascent lumen may similarly exclude Par3 and other junction proteins from this region, helping to establish and expand the nascent apical domain. To this end, during *Drosophila* photoreceptor development, Cdc42, in conjunction with Par6, recruits Crumbs to the apical membrane, facilitating restriction of Par3 to the boundary between the apical membrane and the nascent adherens junction [52]. Thus, different combinations of polarity complexes form during the development of apical polarity, regulating maturation and expansion of the apical domain itself.

A critical design principle for making a single lumen is that, once the AMIS lumen is formed, subsequent divisions should reinforce, not disrupt, this polarized architecture (Figures 2C and 3C). Accordingly, Cdc42, in conjunction with Par3, also controls the correct orientation of the mitotic spindle [43,53–55] via apical recruitment of aPKC [56].

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aPKC phosphorylates and excludes the spindle-orientating LGN– NuMA complex from the apical surface, consequently preventing division in the apical-basal axis and maintaining growth only in the plane of the monolayer [56,57]. Recent reports identify Tuba and Intersectin-2 as the sole Cdc42 GEFs that control localized Cdc42 activation during lumenogenesis [54,55]. Notably, while depletion of either GEF disrupts spindle orientation, only Tuba regulates apical exocytosis [35]. Whether Tuba directly regulates spindle orientation or acts indirectly through modulating exocytosis remains to be elucidated. Nonetheless, the Cdc42–Par6– aPKC–Par3 module co-operates with a Rab11-directed network to integrate apical exocytosis, apical polarity complex maturation, and cell division orientation — all processes fundamental for *de novo* lumenogenesis.

Generating Apical-Basal Identity

The breaking of cortical symmetry in cells during AMIS formation results in the differential apical-basal polarization not only of proteins, but also of lipids, particularly phosphoinositides (Figure 3B). Moreover, certain phospholipids themselves specify membrane identity; $PI(4,5)P_2$ is enriched at, and specifies, apical/luminal membrane identity, whereas phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) localizes solely to, and specifies, basolateral membrane identity [58]. Addition of exogenous PIP_3 to the apical surface of cells induces basal-membrane-like protrusions from the apical surface [59], while conversely $PI(4,5)P_2$ addition to the basal surface relocalizes apical proteins to this domain [32]. Exclusion of PIP_3 from the apical surface during lumen initiation is controlled by the lipid phosphatase PTEN, a pool of which localizes to the apical membrane and metabolizes PIP_3 into $PI(4,5)P_2$, a process crucial for lumenogenesis [32] (Figure 3B). What regulates PTEN localization during *de novo* lumen formation is not yet clear, but direct binding to Par3 may contribute at early stages [60,61] (although Par3 localizes to tight junctions, whereas PTEN is apical once lumens form). Notably, direct binding of Par3 to phosphoinositides contributes to its plasma membrane localization (though exactly how this occurs is controversial) [62,63]. As Par3 is both a target for phosphoinositides and a regulator of their metabolism (through PTEN), the Par complex might be a master regulator of both protein and phosphoinositide asymmetry. Accordingly, aPKC controls $PI(4,5)P_2$/PIP_3 asymmetry during development of MDCK monolayers [64].

Whether $PI(4,5)P_2$ at the lumen is solely generated by PTEN is unknown. For instance, $PI(4,5)P_2$ can also be generated from either PI(4)P or PI(5)P by a combination of type-I, -II, and -III phosphatidylinositol kinases (PIKs) [65]. Notably, PI4K-IIIβ generates PI(4)P at, and recruits Rab11a to, exocytic vesicles [66,67], with PI(4)P required for subsequent recruitment of the Rabin8 homologue, Sec2p, in yeast [68]. Rab8a/11a vesicles, which control delivery of apical proteins to initiate the lumen (see above), are enriched for both PI(4)P and $PI(4,5)P_2$ [69]. Notably, the $PI(4,5)P_2$-binding protein Anx2 is present on, and regulates exocytic traffic of, Rab8a–Rab11a vesicles to the lumen [35,49]. Furthermore, Anx2 binds Cdc42 and regulates its localization. This suggests that $PI(4,5)P_2$ may have a key role in regulating the apical exocytic machinery [70]. However, whether $PI(4,5)P_2$ is actually delivered to the AMIS via Rab11 vesicles remains to be demonstrated. Furthermore, the identity of the key targets of $PI(4,5)P_2$/PIP_3 in effecting protein asymmetry are unknown, although the exocyst complex, some subunits of which directly bind phosphoinositides, is one likely candidate [71–73].

Hollowing and Lumen Initiation

A design principle underlying hollowing lumenogenesis is that once apical membrane is delivered to the cell surface, extracellular space must be generated between neighboring adherent cells. How is space initiated? Along with mucin 1 (Muc1) and Crumbs, the CD34 family of anti-adhesins, including podocalyxin, are some of the earliest known proteins to

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localize at nascent lumens, both in 3D cysts and *in vivo* [34,42]. Notably, these proteins are extensively glycosylated and/or sialylated, resulting in highly negatively charged extracellular domains [74,75] that can act as anti-adhesive molecules [76,77]. In developing mouse aorta, electrostatic repulsion of podocalyxin from apposing endothelial plasma membranes provides the key initiating step necessary for subsequent endothelial lumen expansion [78]. Whether the Crumbs3 extracellular domain plays a similar role is not yet clear, but loss of podocalyxin or Crumbs3 impairs generation of this intercellular space [42,78–80]. The intracellular domains of these proteins also play key roles in lumen initiation, with the cytoplasmic tail of Crumbs recruiting ezrin–radixin–moesin (ERM) family members, and the polarity proteins Par6 and aPKC [81]. Podocalyxin also controls subapical recruitment of an F-actin–ERM–RhoA–myosin-II network, which may generate force for lumen expansion and maintenance [75]. Accordingly, ezrin knockout mice have defects in the formation of mouse intestinal lumens [82]. This apical actin network, in cooperation with the Diaphanous family of formins, may also be required for subsequent secretion during lumen maturation [38]. Thus, Crumbs and CD34 family molecules may participate in extracellular and intracellular remodeling events required for *de novo* lumen formation; however, whether a core requirement for such molecules exists in all lumen-forming tissues is yet to be elucidated.

Tubular Polarity by Cavitation

An alternative design principle for lumenogenesis is that there must be a mechanism to form luminal space when large clusters of cells, many of which may not be in contact, are present. This must also begin with sensing ECM and neighboring cells. Cells in the cluster periphery receive ECM-derived polarization and survival cues; those in the interior die by anoikis (loss of ECM contact) — a process termed cavitation [83] (Figure 1C).

Cavitation is the predominant lumenogenesis mode during mammary branching and salivary gland development, where highly proliferative ductal outgrowths form multilayered terminal end buds [5,84]. Bim and Bmf, pro-apoptotic members of the Bcl-2 family, regulate luminal apoptosis *in vitro* [85,86]. Bim-null mammary glands show transient lumen filling, but the lumens eventually clear through caspase-independent mechanisms, indicating the presence of alternative cavitation pathways [87]. The role of Bmf in mammary morphogenesis *in vivo* is unclear [87]. Cells undergoing anoikis during mammary morphogenesis also strongly upregulate autophagy (self-eating) pathways [5]. Surprisingly, autophagy suppresses, rather than promotes, apoptosis [88], suggesting that our understanding of luminal clearance is incomplete. Notably, in MDCK cysts, which normally undergo lumenogenesis via hollowing, these cells switch to cavitation as an alternative lumenogenesis mechanism when rapid polarization is disrupted [32]. In contrast, in 3D prostate cultures lumenogenesis is driven by hydrostatic pressure rather than cell death [89]. While the exact molecular details are only recently coming to light, it is important to note that, although multiple lumenogenesis mechanisms occur, built-in redundancy between these alternative mechanisms ensures a lumen eventually occurs.

Focalized Contact and Repulsion

A variant on the hollowing method is to combine focalized cell–cell contacts with active membrane repulsion, such as is employed in the developing *Drosophila* cardiac tube [90,91] (Figure 1A). Here, non-contacting myoendothelial cell rows line up along the midline, forming cadherin-mediated adhesions only at the ventral-most, then dorsal-most regions. That the luminal membranes do not form contacts is ensured by a gradient of secreted Slit protein in the intercellular space, acting on the Robo receptor to induce active membrane repulsion. The combined actions of focalized adhesive and repulsive cues, which must be facilitated by differential polarized membrane trafficking of cadherins and Slit/Robo to these

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sites, allows generation of the intercellular luminal space *de novo*. It remains to be demonstrated whether the molecules that break symmetry of the plasma membrane to allow such differential polarized exocytosis to adhesive/repulsive sites are the same as those in epithelial/endothelial cells (i.e. Par complexes).

Lumen Expansion

Once lumens are formed they must expand to their mature, functional size. Hydrostatic pressure, regulated by apical delivery and activation of pumps and channels, is thought to account for part of luminal expansion in most tissues [3]. In addition, roles for an apical 'matrix' are becoming clear. Expansion of the luminal network may also involve division of cells in the wall of the epithelium.

Role of Pumps and Channel Proteins

During the development of several organs in a number of species, multiple smaller 'micro lumens' normally coalesce to form a single lumen [2,37,82,92,93]. Key roles for the Na-K-ATPase and the claudin family of tight-junction proteins have emerged in this lumenogenesis step. Loss of the Tcf2 transcription factor in the gut strongly inhibits Na-K-ATPase and claudin-15 expression, resulting in multiple lumens. Parallel MDCK cyst analysis revealed that chloride-channel-mediated ion transport and Na-K-ATPase-mediated fluid transport are essential for lumen expansion, with claudin-15 forming a paracellular pore regulating these channel activities [92]. Similarly, in zebrafish ventricle lumen expansion claudin-5a regulates paracellular permeability across the neuroepithelial barrier and is crucial for ventricle lumen expansion [94]. Claudin-4 and −6 play similar roles in mouse blastocyst lumen expansion, while the *Drosophila* claudin Kune-Kune controls tracheal tube size [95,96]. Na-K-ATPase (Atp1α1) expression is also required for brain ventricular lumen expansion, but whether its pump activity is required is controversial [97,98]. Nonetheless, lumen expansion seems to occur in multiple organs via a conserved interplay between claudin-regulated paracellular permeability, and Na-K-ATPase-modulated luminal hydrostatic pressure.

Control of chloride transport through the cystic fibrosis transmembrane conductance regulator (CFTR) also appears to be essential for regulating fluid transport into epithelial lumens [99]. Chloride currents are controlled by protein kinase A (PKA)-dependent CFTR phosphorylation events in response to signals promoting local increases in cyclic AMP levels [100]. Pharmacological hyper-activation of CFTR-dependent fluid transport results in overexpansion of the gut lumen in developing zebrafish [101], and also in MDCK cysts [102]. A recent genetic screen identified Cse1l as an inhibitor of CFTR function during zebrafish gut development [101]. Notably, Cse1l loss-of-function leads to lumen overexpansion in both zebrafish gut and MDCK cysts through CFTR hyperactivation. Whether cyclic AMP/CFTR-dependent signaling affects lumen formation solely through chloride secretion, or through as yet unappreciated mechanisms, remains unclear.

Luminal Matrix in Lumen Expansion

Luminal matrices are increasingly being appreciated as key regulators of lumen expansion. For example, the *Drosophila* trachea contains a remarkable set of interconnected tubes formed by both intercellular and intracellular lumens [103]. Maturation of this lumen is a multistep process involving secretion of a luminal matrix, followed by rapid clearing and the initiation of gas exchange [104]. The matrix transiently secreted into the lumen is composed of fibrillar chitin and is required for lumen expansion [105]. Interestingly, the two putative chitin deacetylases, Vermiform and Serpentine, which are secreted into the lumen and assumed to regulate clearance of chitin to allow mature lumen function, appear to regulate

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tube length but not diameter [106,107], suggesting that our understanding of the role of the chitin matrix in lumen morphogenesis is far from complete. Instead, tracheal-tube expansion appears to be a cell-autonomous process whereby endoplasmic reticulum (ER)–Golgi transport pathways contribute to apical membrane expansion, cell flattening and luminal cuticle formation [108], although the mechanisms behind this process remain largely unclear. In contrast, lumen expansion in the *Drosophila* photoreceptor requires apical secretion of the proteoglycan Eyes Shut (Eys) [109], perhaps acting to induce membrane repulsion, similar to podocalyxin or Muc1. Notably, mutations in Eys in humans are associated with autosomal recessive retinitis pigmentosa [110,111], suggesting conservation in the mechanisms of retinal lumen morphogenesis between insects and humans. We suggest that, while a chitin-based extracellular matrix appears not to play a role in vertebrate lumen development, an analogous luminal matrix consisting of the 'glycocalyx' [112] provided by luminal proteins such as podocalyxin, Muc1 or Eys may instead be involved. Our understanding of these events, however, is still in its infancy.

ER–Golgi Transport and Lumen Expansion

As mentioned above, bidirectional ER-Golgi transport has somewhat surprisingly emerged as a key regulator of apical transport and lumen morphogenesis, rather than being a generalized membrane transport step. The coat protein complex II (COPII), comprising Sar1 (regulatory GTPase), Sec23–Sec24 (cargo-binding subunits), and Sec13–Sec31 (coat components), regulates anterograde ER–Golgi transport, while the COPI complex regulates retrograde transport (Golgi-to-ER) [113]. Most subunits exist as multiple isoforms, allowing different combinatorial complexes [114], such as for general versus apical secretion. Perturbation of Sar1 or Sec24 (COPII complex) in *Drosophila* attenuates apical secretion, luminal matrix deposition, and luminal expansion without affecting apical-basal polarization [104,108]. Sec24B mutant mice fail to complete neural tube closure (and thus lumen formation), due to defective transport of the planar cell polarity regulator Vangl2 [115]. Loss of function of γCOP (COPI complex) also disrupts luminal secretion during *Drosophila* tracheal tube [116] and salivary gland maturation [117]. Notably, Sec23A mutant mice are defective in proteoglycan and collagen secretion [118]. This suggests that isoform-specific COP-complex-regulated secretion of matrix proteins (structural and proteoglycan) may be a fundamental step in lumen morphogenesis in diverse tissues and organisms. Whether these pathways only control matrix secretion or also control apical transport of expansion-regulating pumps and channels remains to be demonstrated [119].

Polarity Proteins in Lumen Expansion

Members of the conserved polarity complexes regulate apical-basal polarization and lumen size, but how these regulate the latter is poorly understood. Generally, apical and basolateral polarity complexes are thought to act in mutual opposition, negatively regulating the overexpansion of one domain into the other [120]. The basolateral complex comprising Scribble, Discs large (Dlg) and Lethal giant larvae (Lgl) negatively controls lumen expansion in the *Drosophila* trachea [121]. However, of these, only Lgl loss abrogates luminal matrix deposition, while Scribble and Dlg control lumen size in a matrix-independent pathway (the precise mechanism of which remains to be elucidated) [121]. The loss of another basolateral polarity protein Yurt leads to tracheal tube enlargement. In contrast, Crumbs overexpression results in apical membrane overexpansion [81]. As Yurt negatively regulates apical Crumbs, lumen enlargement in Yurt mutants may be due to an imbalance of the mutual inhibition between basolateral and apical polarity complexes [121,122]. The apical polarity proteins Crumbs, Par6γb, and aPKC also regulate zebrafish brain ventricle size [123–125], with embryos lacking these proteins having brain ventricle expansion defects.

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As aforementioned, fusion of multiple rudimentary lumens into a single lumen occurs normally during development of several vertebrate organs [2,82,92]. During zebrafish gut lumen formation, multiple clusters of actin-enriched foci form and eventually fuse, in an aPKC-dependent manner, at a single focal point prior to lumen expansion [123]. A similar phenomenon occurs during mouse pancreatic tube formation, where multiple microlumens fuse to form a central lumen. This fusion is dependent on Cdc42 acting upstream of aPKC and loss of either Cdc42 or aPKC leads to multilumen phenotypes [37]. The apical ERM protein ezrin similarly controls progression from multiple to single lumens in the mouse gut [82]. Thus, Cdc42,as part of the apical polarity complexes, and probably together with ezrin-mediated effects on the apical cytoskeleton, regulates multiple steps in lumen formation and maturation. How changes in these polarity proteins and apical cytoskeleton influence cellular mechanisms that regulate lumen expansion remain poorly understood, but may involve modulation of membrane transport pathways [9,35]. Indeed, in *Drosophila*, Diaphanous formins modulate apical actin networks to facilitate myosin-5B-directed apical secretion during tracheal morphogenesis [38]. Thus, polarity proteins likely control lumen formation by acting as an interface node between ECM-derived signaling networks, cytoskeletal organization, and membrane transport.

Integrating Morphogens, Polarity, and Membrane Transport

A fundamental design principle for developmental morphogen systems specifying epithelial or endothelial cell fate is that these signals must induce polarization networks to form a lumen. How do traditional ‘fate-generating’ signals induce such cellular behaviors?

Recent studies reveal that transcription factors regulating epithelial differentiation modulate transcription of apical polarity and transport factors (Table 1). In particular, the Rab11 GTPase family (Rab11a/b and Rab25), required for lumen morphogenesis in diverse systems [35,36,40, 126,127], appears to be a key transcriptional target. For instance, Snail,a transcription factor that induces the epithelial–mesenchymal transition, directly represses Crumbs3, Rab25 and PTEN transcription [128–130], all of which are required for lumen formation [35,49]. In *Drosophila*, loss of the transcription factor Ribbon decreases Rab11a expression and consequently apical Crumbs levels, resulting in impaired expansion of tracheal and salivary gland lumens [131]. Rab11a similarly regulates apical Crumbs3a and polarity protein delivery in MDCK cysts [35,80]. Notably, the homeobox transcription factor Cdx2, which specifies intestinal fate, regulates lumenogenesis via the key transcriptional targets Rab11a and the kinesin-II subunit, Kif3b [132] (which is linked to Rab11 via its effector Rab11–FIP5 [133]). Similarly, during *Drosophila* airway branching morphogenesis, interplay between Wingless and Decapentaplegic morphogen gradients controls the expression of Rab11a and Rip11, affecting the type of epithelial lumen that forms [127]. These studies reveal that apical transport and polarity proteins are key targets of morphogen systems regulating epithelial differentiation.

Conclusions and Future Directions

Despite the seeming multitudes of morphogenetic processes governing *de novo* lumen formation, several key design principles have emerged in the last few years, which we have described here. More recently, key common molecular regulators of these processes have been elucidated: β1 integrin, which transduces ECM-derived signals [10,18,20,21]; Rab11a, which directs apical transport [35,36]; Cdc42, which functions in apical polarity, membrane transport, and cell division [35,49,53–55]; and the Par3– aPKC complex, which integrates all of these signals together into polarity- and lumen-generating modules [18,35,49]. Several questions remain. How are these key polarization molecules regulated, both transcriptionally and via upstream regulators? How is such regulation differentially controlled to give rise to

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different luminal structures during diverse morphogenetic events? Parallel analysis of such questions using *in vivo*, *in vitro* and *in silico* models (Figure 2) should allow us to uncover further regulators of this complex morphogenetic event, a key requirement for improving human health (Box 1).

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Box 1

Lumen formation and human health

Lumen formation was a crucial step in metazoan evolution, enabling essential functions such as nutrient uptake, gas exchange, and circulation. The dysfunction of luminal networks is often fatal. Hyperdilated tubules associated with reduced renal function occur in polycystic kidney diseases, which is caused by mutation in numerous genes, and can also be induced by long-term renal dialysis [138]. Such dilation may therefore be a final common pathway resulting from perturbation of the finely balanced control of lumen diameter. Stenosis, or reduction of lumen size, is associated with vascular diseases such as hypertension [4]. Defective brain ventricle closure or expansion leads to anencephaly, schizencephaly and hydrocephalus [139]. Early stages of many epithelial cancers display luminal filling, such as in ductal carcinomas *in situ* [83]. Understanding the molecular mechanisms controlling formation and maintenance of lumens is therefore key to effectively treating such common human diseases.

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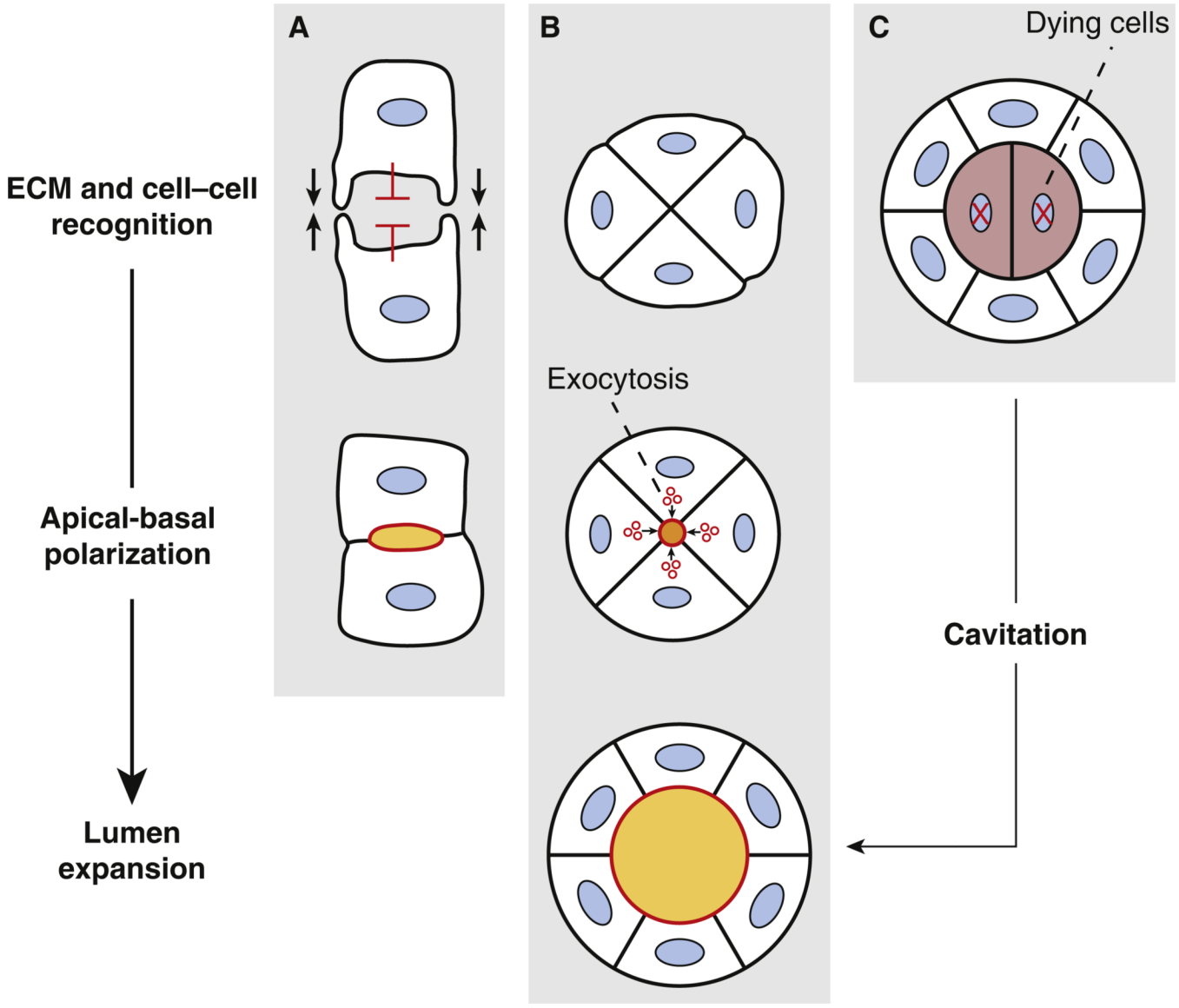

Figure 1. Design principles for *de novo* lumen formation

Lumen formation in various contexts relies on the co-ordination of three consecutive basic design principles: extracellular matrix (ECM) and cell–cell recognition, apical-basal polarization, and lumen expansion. Molecular instructions for whether, where and how lumens will be generated are provided from integrating signals from the ECM (depicted by gray shading in all panels) and cell–cell contacts. In (A), adhesive contacts occur between neighboring cells only at discrete foci (indicated by black arrows), with non-contacting regions undergoing active repulsion (indicated by red inhibitory arrows). This leads to the formation of a luminal space between adhesions (called focalized contact), which allows apical-basal polarization. This occurs, for example, in *Drosophila* heart tube formation [3]. In (B), clusters of cells contacting the ECM initially adhere without a luminal space, then vesicles containing luminal components are exocytosed in a coordinated fashion to a central luminal region, generating apical-basal polarization (called hollowing). This occurs, for example, in developing mouse aorta [42] and MDCK cysts [32]. In (C), clusters of cells initially adhere without a luminal space; however, unlike (B) some cells do not contact the ECM, and thus undergo apoptosis. This results in generation of luminal space as these inner

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cells die (a process called cavitation). In addition, apical-basal polarization must occur. This occurs, for example, in mammary terminal end buds [5]. Thus, although *de novo* lumen formation occurs through seemingly different morphogenetic events, all make use of the common principles of ECM and cell–cell recognition and apical-basal polarization. Similarly, once lumens have formed, such as through these different processes, lumen expansion will occur to generate the appropriate lumen diameter. Red lines indicate apical/luminal membrane; blue ovals, nuclei; grey, ECM; maroon, dying cells.

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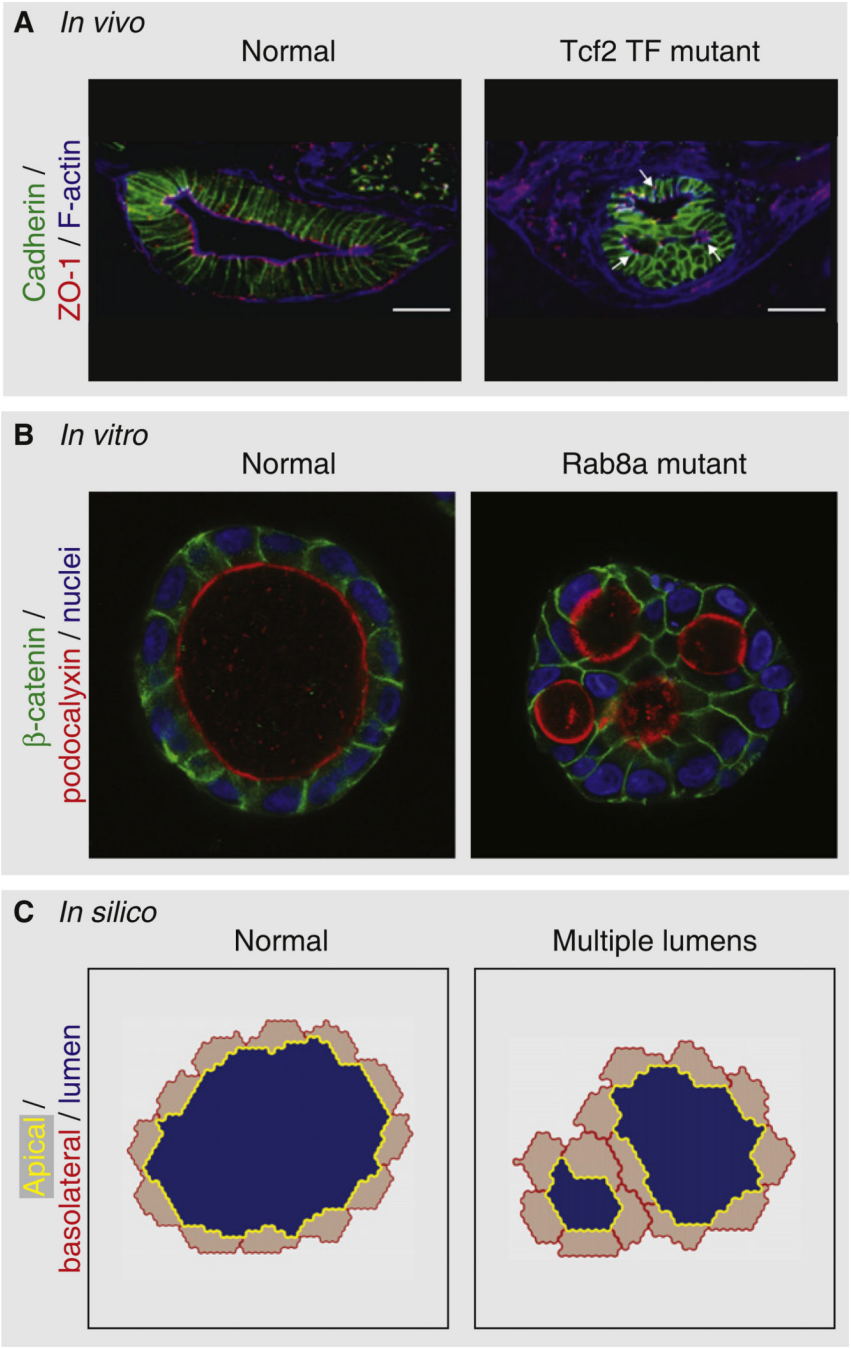

Figure 2. *In vivo, in vitro* and *in silico* models of lumen formation

Unraveling molecular mechanisms of lumen formation requires modeling the 3D organization of lumen-containing structures, precluding the use of traditional monolayer culture of cells on glass, plastic or Transwell filters. Thus, a combination of analyses from (A) *in vivo*, genetically engineered model organisms, (B) *in vitro*, 3D cysts of cultured cells, and (c) *in silico* models have begun to elucidate common design principles underpinning this process. (A) *In vivo* analysis of model organisms, such as during zebrafish gut development, presents as a powerful method, via both forward and reverse genetic analysis, to identify physiologically relevant regulators of tubular epithelium formation. Though genetically tractable, *in vivo* analysis is limited to a small number of molecular alterations due to the

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time-scale required to generate mutant organisms. (B) *In vitro*, 3D cyst analysis, which involves various techniques of growing cells in ECM-enriched conditions to allow self-organization into lumen-containing structures, complements *in vivo* analysis by allowing for rapid reverse genetic approaches. 3D cultures enable dissection of large molecular networks regulating lumenogenesis, using combinations of knockdown and protein overexpression technologies, such as the effect of compromised apical exocytosis (Rab8a knockdown) on lumenogene-sis. (C) *In silico* analysis has recently emerged as a further complementary approach to understanding lumenogenesis [134], facilitating derivation of common, and fundamental, design principles required to form polarized structures with a lumen. As such models develop, future aims should include creation of *in vitro* and *in silico* models of complex luminal networks, such as the hierarchical and modular lung branching program [135]. Presented are images from (A) *in vivo* analyses of a Tcf2 transcription factor (TF) mutant (the image is a cross-section through the developing intestine in zebrafish, and is reproduced from [92]), (B) *in vitro* analyses of an apical transport mutant (knockdown of Rab8a GTPase in MDCK cysts [35]), and (C) in silico analyses showing multilumen cysts under conditions in which cysts polarize later during cystogenesis [136,137], each of which disrupts single lumen formation, causing multiple lumens

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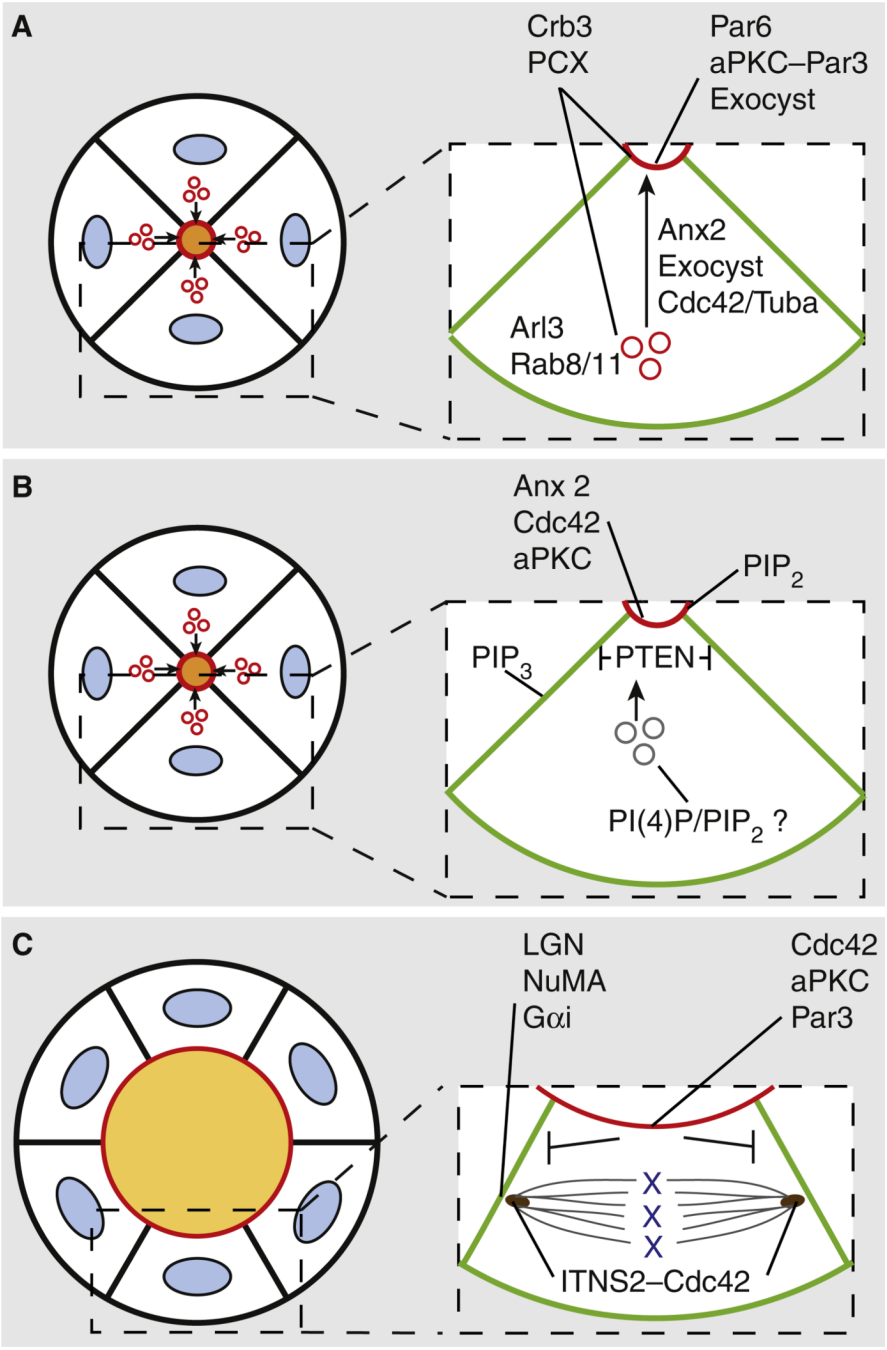

Figure 3. Molecular control of lumen generation and maintenance

(A) Exocytosis of apical membrane-initiating proteins (such as Crumbs3a (Crb3), podocalyxin (PCX) and Muc1) to the cell surface induces formation of the nascent lumen. These proteins are transported via Rab8/11-positive vesicles, in conjunction with the PI(4,5)P_2-binding protein annexin2 (Anx2), both of which are required for Cdc42 activation on these vesicles via the GEF protein Tuba. Delivery and docking of these vesicles with the cell surface at the apical membrane initiation site (AMIS) requires the concerted function of Arl3, the exocyst and Par3–aPKC complexes. The Cdc42– Par6 complex is required for efficient delivery of apical proteins, such as Crumbs. (B) As nascent lumens are formed, phosphoinositides also become asymmetrically distributed. PI(4,5)P2 (PIP_2) becomes

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enriched at the lumen, while PI(3,4,5)P_3 (PIP_3) is localized to the basolateral membrane. Apically localized PTEN excludes PIP_3 from this domain by dephosphorylating PIP_3, converting it to PIP_2. Anx2 associates with Cdc42, which in turn directs aPKC localization, with all three components acting to generate and maintain the PIP2-enriched apical membrane. The phospholipid content of exocytic vesicles destined for the lumen is not clear, although PI(4)P and PIP_2 are likely candidates. (C) Once a single lumen has been established, this polarized architecture is maintained during acini/tissue growth by orienting cell division events, in which Cdc42 plays a key role. Apically localized Cdc42, in conjunction with the Par3, recruits aPKC, which in turn phosphorylates LGN, excluding the spindle-orientating LGN–NuMA complex from the apical surface. This ensures mitosis occurs only in the plane of the monolayer. Cdc42, in concert with the GEF protein Intersectin-2 (ITSN2), also localizes to centrosomes during mitosis, and participates in spindle orientation. Discrete pools of active Cdc42, such as at apical versus centrosomal regions, are apparently controlled via the GEFs Tuba (A) and ITSN2, respectively

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Table 1

Selected transcription factors regulating development of tubular epithelial networks.

Transcription factor	Species	Tissue affected	Function/target genes	Lumen defect	Ref.
Caudal-type homeobox protein 2 (Cdx2)	Mouse	Intestine	Endocytic/exocytic proteins Pumps and channels Apical trafficking proteins (Rab11a, Kif3b)	Multiple lumens Disrupted apical polarity and surface morphology	[132]
Transcription factor CP2-like1 (Tcfcp2l1), related to Grainyhead	Mouse	Salivary gland, kidney	Channel, pumps and ion exchanger proteins	Dilated salivary gland lumen Defective renal tubule maturation	[140]
Grainyhead (Grhl1)	*Drosophila*	Trachea	Septate junction proteins (Fasciclin, Coracle, Sinuous)	Elongated/tortuous tracheal branches	[141,142]
Hairy (h)	*Drosophila*	Salivary gland	Represses Huckebein and Klarsicht	Branched and enlarged salivary gland lumen	[143]
Huckebein (hkb)	*Drosophila*	Salivary gland	Apical polarity (Crumbs) Microtubule transport (Klarsicht)	Dome-shaped glands, small lumens	[143]
Krüuppel-like factor 4 (Klf4)	Mouse	Sertoli cells	Exocytosis (sec 8l1) Lysosomal trafficking (Hps5) Apoptosis (Htatip2)	Delayed lumen formation (defect is rescued later in development)	[144]
No Tail-a (ntla)/Brachyury (T)	Zebrafish	Kupffer's vesicle	Mesenchymal to epithelial transition of dorsal forerunner cells to form Kupffer's vesicle	Uninflated Kupffer's vesicle lumen	[145]
Ribbon (rib)	*Drosophila*	Salivary gland	Apical polarity (Crumbs) Apical cytoskeleton (Moesin)	Short salivary gland tubes Decreased Rab11a expression Excessive microvilli	[131]
Spalt major/Spalt (salm)	*Drosophila*	Trachea	Inhibits tracheal dorsal trunk intercalation Membrane recycling (Rab11a, Rip11)	Excessive tracheal branch intercalation Disrupted Rab11a vesicle organization	[127]
HNF1 homeobox Ba (hnf1ba/tcf2)	Zebrafish	Gut	Ion pumps (Na/K-ATPase) Paracellular pores (Claudin 15)	Unexpanded, multiple lumens	[92]
Tramtrack (ttk)	*Drosophila*	Trachea	Septate junctions Cuticle formation	Increased tube size Impaired branching	[146]

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