Polarized transport of membrane and secreted proteins during lumen morphogenesis

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Abstract

A ubiquitous feature of animal development is the formation of fluid-filled cavities or lumina, which transport gases and fluids across tissues and organs. Among different species, lumina vary drastically in size, scale, and complexity. However, all lumen formation processes share key morphogenetic principles that underly their development. Fundamentally, a lumen simply consists of epithelial cells that encapsulate a continuous internal space, and a common way of building a lumen is via opening and enlarging by filling it with fluid and/or macromolecules. Here, we discuss how polarized targeting of membrane and secreted proteins regulates lumen formation, mainly focusing on ion transporters in vertebrate model systems. We also discuss mechanistic differences observed among invertebrates and vertebrates and describe how the unique properties of the Na^+/K^+-ATPase and junctional proteins can promote polarization of immature epithelia to build lumina *de novo* in developing organs.

Keywords

Lumen; epithelial; polarity; fluid secretion; morphogenesis; zebrafish

1. Introduction

During animal development, tube formation allows the transport of gases, fluid, and macromolecules across organs [1]. Tubes range from simple structures made from just a few cells, as in the *Caenorhabditis elegans* excretory system, to complex branched ductal networks, as in the mammalian lung [2]. Tubes consist of epithelial cells that encapsulate an interior space or lumen. A variety of mechanisms are known to generate lumina from epithelial sheets or unpolarized mesenchyme [2–4]. *De novo* lumen formation, in which a fluid-filled cavity is generated within clusters of cells or through individual cells, is a common and conserved process, as examples have been described in nearly all invertebrate

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and vertebrate model systems [5–12]. While distinct cellular mechanisms can facilitate *de novo* lumen formation, some fundamental principles are prevalent among different systems. For example, luminal contents (fluid or solid) drive the opening and enlargement of lumina and tubes [11–18]. The roles of cytosolic trafficking machinery in lumen formation has been recently reviewed [3], and lumen formation *in vitro* (e.g. 3D cell culture models) has also been thoroughly characterized and reviewed [19–24]. Here, we focus on how polarized sorting and trafficking of key proteins facilitates lumen opening, expansion, and coalescence during organ development mostly in *in vivo* models, and we describe some *in vitro* work that has offered molecular insights. Throughout this review, we consider how epithelial polarization relates to lumen formation.

2. The problem of polarity and trafficking in immature, developing epithelial organs

Cell polarization is a general feature of mature lumina, with distinct domains of the plasma membrane in contact with the lumen and surrounding tissue. The apical membrane generally faces the lumen, and the basolateral membrane is adhered to the basement membrane by cell-extracellular matrix (ECM) interactions. Some tissues, such as the mammalian blastocyst, have a less common organization, with the basolateral membrane facing the lumen [25]. Epithelial polarization is critical for cell and organ physiology, which depend on secretion and absorption of ions and macromolecules to and from the lumen [1]. Epithelial cells accomplish these tasks by expressing membrane proteins such as channels and transporters asymmetrically, at either the apical or basolateral surface. Membrane proteins are generally sorted, or segregated, into different vesicular carriers during biosynthetic transport. Segregation of apical and basolateral proteins can occur in the trans-Golgi network (TGN) and sorting endosomes, or in recycling endosomes after internalization [26–28]. While mature, highly polarized epithelia have robust sorting and trafficking mechanisms delivering cargoes to the cell surface in a highly polarized fashion, in developing organs, lumen morphogenesis can coincide with epithelial polarization [29]. The lack of polarization in immature epithelia presents a problem because channels and transporters must precisely localize to their target membrane to facilitate lumen formation. Randomized sorting of polarized channels and transporters to apical and basolateral membranes would abolish ion gradients that are needed for secondary, coupled transport and fluid secretion. Recent studies have revealed how critical polarized membrane proteins can be correctly targeted in newly polarizing epithelial cells, and how lumina can rapidly form *de novo* within immature epithelia. In the following sections, we highlight the key roles of the Na^+/K^+-ATPase in this process and describe how its basolateral sorting can be tightly regulated prior to mature epithelial polarization.

3. Na^+/K^+-ATPase and fluid secretion in lumen formation

Ion transport, which is mediated both by active transport and by secondary transport along electrochemical gradients, can promote lumen formation by controlling fluid flux [30]. The resulting hydrostatic pressure from accumulating fluid drives lumen inflation and can also promote coalescence of many smaller lumina [11]. Active Na^+ and K^+ transport by

the Na^+/K^+-ATPase provides the driving force for fluid transport in most mature epithelia [1], and it is ultimately responsible for lumen inflation in most developing organs. This was first shown in the *Drosophila* tracheal system [31] and the zebrafish brain ventricle [15]. Subsequently, work in the zebrafish gut, which develops from an endodermal rod of unpolarized mesenchyme that undergoes cord hollowing [32], established a role for basolateral transporters and junctional pores in lumen morphogenesis [11]. Lumen formation in the zebrafish gut begins as small cavities arise adjacent to newly forming junctional domains marked by ZO1 [11]. Coalescence into a single lumen then proceeds as the cavities enlarge, expand, and remodel membrane contacts [33]. The process is genetically controlled by transcriptional regulation via Tcf2, which is required for expression of the Na^+/K^+-ATPase and the paracellular pore forming protein Cldn15la in the gut [11]. Perturbation of the pathway results in many small lumina that fail to resolve due to impaired fluid secretion that is needed for lumen expansion. Apical trafficking, on the other hand, is required for later stages of single lumen formation, where Rab11a promotes membrane contact remodeling during the final step of coalescence. Contact remodeling is facilitated by recycling of basolateral membrane proteins, including E-cadherin [33]. A similar sequence of events, but controlled by parasympathetic innervation, was shown for lumen formation in the mouse salivary gland [34], although fluid secretion in that organ relies on the CFTR channel (see section 5).

Similar to the zebrafish gut [11], mammalian salivary glands [34], and avian lung [35], the mouse blastocoel forms as several pressurized microlumina arise throughout the embryo in a process that has been compared to hydraulic fracturing [36]. Formation of microlumina depend both on fluid transport via the activity of the basolateral Na^+/K^+-ATPase [36] and on secretion of cytoplasmic vesicles [37]. Microlumina begin to coalesce as they are guided to a centralized cavity according to differences in cell adhesion and tension [36]. Cells lining the cavity polarize and rapidly inflate the lumen by additional fluid transport that is driven largely by the basolateral Na^+/K^+-ATPase [38] and tight junction (TJ)-localized paracellular pores of the Claudin family [39]. Basolateral adhesion molecules are also crucial for morphogenesis during blastocoel inflation. Growth of the embryo occurs cyclically as hydrostatic pressure exceeds the tensile strength of adherens junctions, leading to their collapse and repair, allowing them to withstand yet increased pressure during subsequent growth cycles [5, 38]. Lumen formation within the proamniotic cavity, which forms shortly after the blastocyst stages, follows similar principles [29]. Cells of the epiblast are initially unpolarized, and restriction of the adherens junction protein E-Cadherin to the nascent basolateral domain decreases adhesion at presumptive apical membrane domains. Basolateral membrane protein segregation coincides with the activity of ion transporters that drive fluid secretion, allowing for hydraulic fracturing to initiate lumen formation at polarized membrane domains. Apical polarization commences concurrently and facilitates the delivery of proteins that further inhibit adhesion at apical membrane domains, while the continued activity of transporters enhances fluid secretion to inflate the proamniotic cavity. Much of the fluid used for inflating the lumen of the proamniotic cavity appears to derive from the blastocoel [29], suggesting that one of its functions is to act as a fluid reservoir for other tissues in the embryo. Formation of the blastocoel and proamniotic cavity, while generally similar, also show important mechanistic differences. For example, as described

above, disruption of the epithelial barrier of the blastocyst results in fluid leakage and lumen size reduction [36]. By contrast, when epiblast cells surrounding the proamniotic cavity undergo division and disrupt the epithelial barrier, this allows additional fluid flow into the lumen rather than leakage [29]. This finding suggests that, unlike the blastocoel, osmotic gradients are not necessarily limiting for lumen expansion of the proamniotic cavity, and that much of the fluid that enters the lumen is coupled to cell division, which may help to relax tension that builds with rapid lumen growth.

The zebrafish inner ear is another organ highly dependent on basolateral transporters and fluid secretion for morphogenesis. The inner ear develops from the otic vesicle, a simple epithelium enclosing a lumen that rapidly inflates during development, increasing in volume nearly four-fold in just a day [40, 41]. Fluid secretion in the otic vesicle relies on the activity of at least two transporters, the Na^+/K^+-ATPase and cation-chloride co-transporter Nkcc1 [42]. *In vivo* studies demonstrated that lumen inflation in the otic vesicle generates hydrostatic pressure and because it is pressurized, puncturing the epithelium induces rapid volume loss [41]. Upon puncture, the otic epithelium quickly repairs and reinflates the lumen, but lumen growth during the repair process is abolished when Na^+/K^+-ATPase pump activity is inhibited [41]. Thus, the pump activity of Na^+/K^+-ATPase is a key driver of tubulogenesis across different vertebrate organs.

4. Polarization of the Na⁺/K⁺-ATPase in mature and immature epithelia

Given the important roles of fluid transport highlighted above, polarized sorting and trafficking of ion pumps and channels emerged as crucial for lumen formation *in vivo*. However, mechanisms underlying the asymmetric distribution of transporters during the onset of lumen formation in developing organs in animals are poorly described. In particular, how the Na^+/K^+-ATPase is sorted to the basolateral membrane in most epithelia during cell polarization is a major open question. In this section, we discuss mechanisms of Na^+/K^+-ATPase basolateral transport in mature versus immature epithelia, and we consider how *in vitro* studies of Na^+/K^+-ATPase trafficking may help us to understand the fundamental requirements of lumen opening in newly polarizing organs.

Basolateral sorting is generally mediated by receptor-like mechanisms in which cytoplasmic adaptor proteins bind to short peptide motifs on cargo proteins. An example is the di-leucine motif on the cytosolic tail of E-Cadherin, which partly relies on the AP (adaptor protein)-1A and AP-1B clathrin adaptors [43, 44]. Many basolateral membrane proteins are segregated in this way and become polarized by sorting and trafficking [45], whereas others are initially transported to the plasma membrane in a less polarized fashion and become asymmetrically enriched by selective stabilization through interactions with scaffolds and cytoskeletal adaptors [46, 47]. Basolateral targeting of the Na^+/K^+-ATPase in mammalian cells is complex and may be mediated by a combination of direct sorting and plasma membrane stabilization [48]. The minimal Na^+/K^+-ATPase holoenzyme consists of a catalytic α-subunit and an obligate ß-subunit, which is essential for biosynthetic trafficking of the pump and modulates its activity. In the absence of the ß-subunit, newly synthesized α-subunit is trapped in the endoplasmic reticulum (ER) lumen by the Coat Protein 1 (COP-1) factor ß-COP and subsequently degraded [49, 50]. ER export of the Na^+/K^+-ATPase requires

assembly of the α- and ß-subunits [50] and proper folding for release from chaperones, which occurs coincidently with binding of the α-subunit cytosolic tail to Ankyrin [51], an adaptor protein to the Spectrin cytoskeleton. These prerequisites seem to make ER export rate-limiting for biosynthetic transport of the Na^+/K^+-ATPase [52] and ensure that only functional pumps are trafficked to the plasma membrane [53]. After ER export, the Na^+/K^+-ATPase is transported to the plasma membrane rapidly [52, 54], segregating from other basolateral membrane proteins at the TGN and reaching the basolateral membrane directly without traversing intermediate endosomes [54]. Na^+/K^+-ATPase polarization has also been attributed to its stabilization at the basolateral membrane through its linkage to Spectrin [55, 56] by the adaptor Ankyrin [57], although this has not been shown directly and instead may relate to Ankyrin's role in the early biosynthetic pathway rather than at the plasma membrane [51].

The mechanism by which the Na^+/K^+-ATPase is segregated into basolateral carriers is unknown, and no specific sorting signals have been found on either the α- or ß-subunits. Both subunits lack common peptide sorting motifs, and their basolateral localization does not require AP-1A or AP-1B clathrin adaptors in mammalian cells [43, 45]. By contrast, in *Drosophila* photoreceptor cells, basolateral localization of the Na^+/K^+-ATPase does depend on Clathrin/AP-1 [58, 59]. Additionally, the GTPase Rab10, its GEF (Guanine Exchange Factor) Crag, its effector Ehbp1, and an unidentified GPI (Glycosylphosphatidylinositol)-anchored protein also regulate basolateral localization of the Na^+/K^+-ATPase [58, 59]. Because these factors localize to the TGN and their loss-of-function causes apical mistargeting [58], these results strongly suggest that this pathway regulates biosynthetic sorting of the Na^+/K^+-ATPase in *Drosophila* photoreceptors. Given the mechanistic differences observed for Na^+/K^+-ATPase targeting in *Drosophila* photoreceptor cells versus mammalian cells [43, 45, 58, 59], it is unclear whether Clathrin/AP-1/Rab10 is generally involved in Na^+/K^+-ATPase sorting in other epithelial organs.

Importantly, the above-mentioned studies all described basolateral transport of the Na^+/K^+-ATPase in fully polarized cells. By contrast, *de novo* lumen formation in developing organs often proceeds before or together with epithelial polarization [11, 38]. This raises the question of how immature epithelial cells *in vivo* target the Na^+/K^+-ATPase to the basolateral membrane before membrane identity is specified. A possible answer may stem from the surprising discovery that the Na^+/K^+-ATPase, in addition to being an ion pump, also functions as a cell adhesion molecule [60, 61]. Co-culture studies of cells expressing ß-subunits from different species demonstrated that the Na^+/K^+-ATPase accumulates at the basolateral membrane only when homotypic interactions occur between neighboring cells, despite the fact that heterotypic cell borders still form adherens junctions [62]. Moreover, expression of the canine ß-subunit in Chinese Hamster Ovary (CHO) cells mediates localization of the Na^+/K^+-ATPase at heterotypic cell borders between canine MDCK and hamster CHO cells [62]. These experiments indicate that trans interaction of the ß-subunit across epithelial cells is both necessary and sufficient for basolateral localization of the Na^+/K^+-ATPase [31, 63].

Overall, these studies raise the intriguing possibility is that the sorting adaptor for the Na^+/K^+-ATPase catalytic α-subunit is simply its obligate ß-subunit. This mode of polarized

targeting would be unique from that of any other known membrane protein and could conceivably mediate proper localization of the Na^+/K^+-ATPase prior to establishment of epithelial polarization, provided that cells have established adherens junctions. This idea is supported by studies showing that expression of E-cadherin in unpolarized fibroblasts alone can mediate basolateral localization of the Na^+/K^+-ATPase [64], presumably by bringing cells close enough in proximity to facilitate trans interaction of ß-subunits [65]. In this system, basolateral targeting of the Na^+/K^+-ATPase occurs even before formation of TJ [64], suggesting that the Na^+/K^+-ATPase can be properly sorted in immature epithelial cells simply due to its intrinsic ability to adhere with pumps on neighboring cells. Further support for this model comes from studies showing that co-expression of E-cadherin and the Na^+/K^+-ATPase ß-subunit is sufficient to suppress a mesenchymal phenotype and induce epithelial polarization of a sarcoma-transformed MDCK cell line [58]. Moreover, the only know mechanism for apical targeting of the Na^+/K^+-ATPase, which supports the unique physiology of some organs [1], is expression of a separate, more highly glycosylated ß-subunit isoform [66–68], indicating that ß-subunit switching can mediate differential sorting of the Na^+/K^+-ATPase. Finally, of the many P-type ATPase subfamilies, a ß-subunit is unique to the Na^+/K^+-ATPase and H^+/K^+-ATPase pumps that share common ancestry [69]; some P4-ATPases also have an accessory subunit that is distinct from the Na^+/H^+K^+-ATPase ß-subunit [70]. These findings suggest that the ß-subunit may have evolved separately as an adhesion molecule before its integration with ion pumps [71].

Basolateral polarization of E-cadherin, although mechanistically distinct from that of the Na^+/K^+-ATPase, follows similar principles. In mature epithelia, the steady state localization of E-Cadherin relies on both biosynthetic sorting and recycling [43, 44]. E-Cadherin, like the Na^+/K^+-ATPase, is stabilized by homotypic interactions across neighboring cells to inhibit its turnover at the lateral membrane, and this is mediated in part by binding to Ankyrin [47]. Studies of newly polarizing cells showed that E-Cadherin localization to forming cell contacts immediately preceded delivery of subsequent basolateral membrane proteins, including the Na^+/K^+-ATPase and water channel forming protein Aquaporin-3 (AQP3). This finding led to the conclusion that Cadherin-mediated adhesion establishes a major positional domain to trigger epithelial polarization [72]. AQP3 also appears to reciprocally enhance cell adhesion complexes through expression of E-Cadherin [73] and β-catenin [74], suggesting that adhesion complexes, water channels, and ion pumps may exert a positive feedback effect to promote epithelial polarization and stabilize the basolateral machinery needed for lumen formation. Moreover, the stability of E-Cadherin at the plasma membrane and paracellular barrier function both depend on trans interactions of the Na^+/K^+-ATPase ß-subunits [75]. Because E-Cadherin and the Na^+/K^+-ATPase do not directly interact, their positive reinforcement may stem from binding to Ankyrin, which restricts their mobility at the basolateral membrane [57, 76]. This mode of positive feedback and basolateral stabilization may be particularly crucial for maintaining adhesion and barrier function during periods of mechanical stress caused by lumen inflation.

The studies highlighted above provide a framework for understanding, in a minimalist fashion, how a lumen can rapidly form *de novo* within an unpolarized group of cells (Fig. 1), as often occurs during animal development. Cell contacts mediated by homotypic interactions of Cadherins across cells positionally establish a lateral domain. Formation

of adherens junctions promotes cohesion and compaction of epithelial cells, which then facilitates trans interactions of the Na$^+$/K$^+$-ATPase, stabilizing it at the lateral domain. Selective stabilization of Cadherin and the Na$^+$/K$^+$-ATPase, in essence, provides a means for asymmetric transport prior to the expression of a robust polarity network that is present in more mature epithelia. The formation of TJ adjacent to the lateral domain then allows for selective ion transport to establish osmotic gradients. Paracellular fluid transport can then generate hydrostatic pressure to separate the newly forming apical domains of cells lined by microlumina. This model of lumen formation contrasts with others describing apical membrane polarization as a prerequisite that is inextricably linked to lumen formation [2, 77]. However, as explained in later sections, apical secretion has many important roles in lumen growth, particularly in invertebrates. The basic principles described here may represent the minimal requirements for lumen opening in unpolarized organ precursors or those that polarize slowly, such as those that give rise to the mammalian blastocoel and zebrafish gut.

5.   Roles and regulation of CFTR and Cl⁻ transport in lumen formation

While the Na$^+$/K$^+$-ATPase generally provides the driving force for fluid transport in most organs [1], other channels and transporters are also required for lumen formation. Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a vertebrate apical Cl⁻ and HCO$_3^-$ channel that functions in concert with the Na$^+$ gradient established by the Na$^+$/K$^+$-ATPase to drive fluid secretion and regulate mucus properties [78]. CFTR function is of great interest biomedically because inactivating mutations are the cause of cystic fibrosis, a life-threatening disease that affects respiratory, gastrointestinal, and urogenital organs [79].

In addition to its roles in human disease, CFTR-dependent fluid transport is also critical for regulating lumen size during development. In the zebrafish embryo, Kupffer's vesicle (KV), the organ of laterality that controls left-right asymmetry, is a transient fluid-filled cyst-like structure lined by a ciliated epithelium. Lumen formation in KV is critical for establishing asymmetric calcium signaling [80], which depends on cilia motility in the lumen to drive fluid flow [81]. Mutations in *cftr* impair lumen expansion in KV, but not cilia formation or motility, and results in near randomization of organ laterality [82]. Lumen growth in KV relies on coupled secretion of Na$^+$ and Cl⁻ because chemical inhibition of the Na$^+$/K$^+$-ATPase mimics the effect of *cftr* mutations, while chemical activation of CFTR causes hyper-inflation [82].

Apical localization of CFTR is essential for its function in Cl⁻ and fluid secretion. In KV, lumen growth is associated not only with CFTR-dependent apical fluid secretion but also proliferation. Interestingly, these cell divisions have an important role in targeting Cftr apically. Prior to KV formation, precursor cells arrange into a rosette formation and undergo cell division. During cytokinesis, daughter cells sever midbody remnants at what will form the apical poles underlying the lumen. These midbody fragments seem to establish a polarized domain by recruitment of Rab11, a GTPase critical for apical trafficking. In fact, optogenetic clustering of Rab11 in KV is sufficient to sequester Cftr and inhibit lumen formation [83]. Thus, KV illustrates another way of how newly polarizing cells target membrane proteins asymmetrically during lumen morphogenesis.

In addition to Rab11, many other factors are known to regulate CFTR's apical targeting. Unlike most apical membrane proteins, during biosynthetic transport, CFTR is randomly sorted for apical and basolateral membrane transport [84]. Apical enrichment of the channel derives from its more rapid removal from the basolateral membrane, much of which is redirected apically by transcytosis [85]. The apical CFTR population is also subject to internalization but undergoes recycling [84]. The relative stability and recycling of the apical CFTR pool depends on its cytosolic PDZ (PSD-95, disks large, zona occludens 1)-interacting domain to bind to scaffolding proteins such as NHERF1/2 [86, 87] and PDZK1 [88]. Apical recycling of CFTR also depends on its phosphorylation by Protein Kinase A (PKA) [89, 90], which is traditionally better known for its influence on CFTR channel gating and activity [91]. In fact, commonly used approaches for CFTR activation, such as Forskolin treatment, act indirectly through the production of cAMP, which stimulates PKA. Thus, while CFTR's sorting is not robust, its apical localization is reinforced by interactions with scaffolding networks, and the same signaling network that activates its channel activity (cAMP and PKA) also regulates its trafficking by stimulating recycling after apical internalization. CFTR's steady state polarization, which relies heavily on transcytosis and recycling of existing intracellular pools, contrasts with that of the Na^+/K^+-ATPase, whose basolateral localization is tightly regulated and reinforced by transcellular interactions and cytoskeletal stabilization (refer to section 4). These differences may reflect the more common and ancient role of the Na^+/K^+-ATPase in lumen formation compared to CFTR, whose function in lumenogenesis is unique to vertebrates.

Although regulation of CFTR activity and trafficking has been extensively characterized, few *in vivo* studies have addressed the relevance of these mechanisms to lumen formation. One surprising discovery is that CFTR can be regulated non-cell autonomously to promote lumen formation [34]. During morphogenesis of the mouse salivary glands, the submandibular gland (SMG) becomes innervated by parasympathetic nerves. This innervation is required for lumen resolution in the SMG, which is ultimately dependent on CFTR-dependent fluid secretion. The mechanism by which parasympathetic ganglia regulate CFTR is through the release of vasoactive intestinal peptide (VIP), which signals through the VIPR1 receptor on SMGs. VIPR1 is a G protein-coupled receptor subtype whose activation causes cAMP production, thereby activating CFTR through PKA. Exogenous VIP treatment to isolated SMGs in culture is sufficient to rapidly induce lumen formation, and the effect is blocked by inhibition of PKA or CFTR [34].

Additional insight into CFTR regulation *in vivo* came from an unbiased forward genetic screen in zebrafish for mutants exhibiting impaired intestinal morphogenesis [92]. Prior studies had established that while the main driving force of fluid accumulation in the zebrafish gut is active Na^+ secretion by the Na^+/K^+-ATPase, Cl^- transport also promotes lumen growth in the intestine by further drawing Na^+ and water into the lumen [11]. From the genetic screen, mutants for *cse1l* were found to exhibit hyper-expansion of the gut lumen that phenocopies CFTR activation. Loss of Cse1l causes the gut lumen to increase in volume several fold within a period of hours, and this effect is suppressed by inhibiting CFTR activity. Physical interaction of Cse1l and CFTR in mammalian cells support a model in which direct binding causes negative regulation of channel activity [92], although the exact mechanism remains unknown. Interestingly, *cse1l* mutants undergo early gut

morphogenesis normally and do not show aberrant lumen expansion until nearly a day after lumen formation is complete. These findings indicate that CFTR's channel activity, which is present only after gut lumen formation [92], must be tightly regulated to limit organ growth, suggesting that the channel is otherwise poised in an active state without Cse1l in the zebrafish intestine. Additional regulation of CFTR comes from its subcellular localization, being mostly in subapical vesicles in CFTR high expresser cells (CHEs) in the absence of PKA-mediated stimulation, which promotes cell surface localization [93]. Interestingly, CHEs have been found in the rat [93] and zebrafish gut (our unpublished results), the mammalian lung [94, 95], fish gills [96] and skin [97] and may be major drivers of fluid secretion in those tissues.

Finally, although CFTR has traditionally been described as a vertebrate-specific gene, recent analysis indicates that *Drosophila* has a distant *ABCC4* ortholog, *CG5789*, that is functionally related to CFTR. Mutants for *CG5789* exhibit ionic and osmotic phenotypes in the intestine, and these can be suppressed by the expression of human *CFTR* [98]. It remains unknown whether *CG5789* regulates lumen morphogenesis in *Drosophila* similarly to vertebrate CFTR.

6. Secreted and tethered luminal cargos implicated in lumen growth

In contrast to vertebrates, in the *Drosophila* trachea, the Na^+/K^+-ATPase functions in lumen formation by regulating the apical secretion of cargoes. This was established by forward genetic screens for mutations affecting morphogenesis of tracheal tubes, which undergo lumen expansion and elongation to reach proper size [3, 99]. These genetic screens uncovered surprising interactions of seemingly disparate pathways - septate junctions (SJ; which are analogous to vertebrate TJ), the Na^+/K^+-ATPase, and secreted polysaccharides [99].

Early studies found that the *megatrachea* mutation [10] affects a member of the Claudin family of membrane proteins [100], which are traditionally known for regulating paracellular barriers in vertebrate TJ. This led to the conclusion that SJ function analogously to TJ in lumen morphogenesis by controlling paracellular flux [100]. However, as the name suggests, *megatrachea* mutants have a phenotype contrary to what would be expected if its relevant role in lumen formation was paracellular barrier formation. Rather than exhibiting smaller lumina, *megatrachea* mutants and mutations affecting other drosophila Claudins have enlarged and elongated tracheal tubes [101]. Other studies found that mutants of the Na^+/K^+-ATPase phenocopy those of SJ [31], that the Na^+/K^+-ATPase is required for proper organization of SJ [31, 102], and that the role of the Na^+/K^+-ATPase in tracheal growth is independent of its catalytic pump activity [103]. Thus, although *Drosophila* requires conserved machinery to regulate lumen growth, they paradoxically utilize a different mechanism than is found in vertebrate models.

Subsequent work established that the Na^+/K^+-ATPase and SJ regulate tracheal lumen growth by controlling the apical secretion of enzymes that modify luminal chitins [104], which are invertebrate oligosaccharide polymers required for proper tracheal tube size and shape [13, 14, 105, 106]. These enzymes, which are called Chitin-binding, LDLRa-containing

deacetylases (Ch-LD) are thought to modulate the physical properties of chitins to control lumen expansion [104, 107]. In addition to SJ, the biosynthetic and endocytic pathways, in general, contributes to apical secretion, resolution of luminal chitin, and tracheal lumen growth [108]. While it was postulated that apical secretion regulates tracheal lumen expansion by promoting swelling of the luminal chitin matrix [108], mosaic analysis of mutants deficient in ER-to-Golgi trafficking showed that apical secretion promotes lumen secretion cell-autonomously, even though secreted proteins readily diffuse in the lumen [109]. It was therefore concluded that secretion promotes tracheal lumen growth by promoting apical membrane expansion rather than through fluid accumulation and matrix swelling, and that the luminal chitin matrix acts as a physical cue to shape the tracheal lumen [109, 110]. Recent analysis supports this conclusion and indicates that the mechanical properties of chitinous ECM influence the composition of the apical cytoskeleton to shape lumen growth through an active feedback mechanism [111, 112]. Collectively, these studies suggest that adaptation of phyla-specific genes may influence the different mechanisms regulating lumen morphogenesis among different animals (e.g. mechanical properties of chitin in invertebrates versus CFTR-dependent fluid secretion in vertebrates).

Although a chitin-based luminal matrix is mostly limited to invertebrates, apical secretion of other factors has important roles in lumen formation in vertebrates and invertebrates alike. In particular, proteoglycan secretion regulates lumen formation in many species. Secreted and tethered proteoglycans are thought to promote lumen opening and expansion by their anti-adhesive and water-adsorbing properties. In *Drosophila*, apical secretion of ECM proteoglycans proteins is required for lumen morphogenesis of the developing eye [113] and salivary gland [114]. Similarly, in the *Drosophila* hindgut, apical secretion of a large, O-glycosylated mucin-like protein, Tenectin, is required lumen expansion, and exogenous expression of Tenectin in other tubular epithelia is sufficient to increase lumen size [115]. As gel-forming mucins draw fluid, Tenectin is thought to promote lumen expansion by increasing hydrostatic pressure [115]. This notion is supported by experiments showing that Tenectin secretion can promote localized lumen expansion and epithelial cell flattening in multiple tubular organs, and it does so in a dose-dependent manner [115]. It is also formally possible that Tenectin could promote lumen expansion through biochemical signaling cues, such as by binding to ECM receptors or sequestering extracellular ligands [17, 116]. However, the more common and conserved roles of fluid transport in lumen expansion highlighted in this review suggests hydrostatic pressure as a simpler explanation. Similar roles for secreted proteoglycans and mucin-like proteins in lumen morphogenesis have been found in *C. elegans* [117, 118]. During lumen formation in the *C. elegans* vulva, expression of several secreted and tethered matrix proteins is dynamically regulated, and mutations in various apical matrix proteins lead to distinct phenotypes affecting lumen inflation, stabilization, and resolution [118]. In the mouse vasculature, apical secretion of Podocalyxin, a heavily sialylated tethered mucin, promotes lumen expansion by inhibiting the interaction of opposing apical membranes, which is thought to be mediated by electrostatic repulsion of negatively charged glycans [119]. Although apical delivery of Podocalyxin is required for lumen morphogenesis in MDCK cysts [21], the mouse vasculature [119], and likely also zebrafish vasculature [16], it does not seem to be universally required in all epithelial organs that it is expressed. In the mouse

kidney, for example, although Podocalyxin and its related CD34 family member Endoglycan are highly expressed at apical lumina of nephrons, double mutants of these sialomucins do not show lumen phenotypes in this tissue [120]. Because CD34 proteins have cytosolic PDZ-interacting domains that bind to and help organize apical scaffolding and polarity networks [21], Podocalyxin/Endoglycan double mutants fail to recruit the PDZ-scaffold NHERF1 to the apical pole of nephrons [120]. Given that NHERF1 is also required for lumen formation in MDCK cysts [21], it is remarkable that Podocalyxin/Endoglycan double mutants undergo lumen formation normally.

The mechanisms controlling apical trafficking of proteoglycans and mucins such as Podocalyxin have been extensively characterized in MDCK cell cysts [19–22, 121]. Early 2-cell stage cysts are initially unpolarized and localize Podocalyxin to the basolateral membrane. Cell-ECM integrin interactions establish basolateral identity and trigger internalization of Podocalyxin, which is then transported through recycling endosomes to the newly forming apical membrane (apical membrane initiation site, AMIS). Trafficking of Podocalyxin to the AMIS depends on GTPases such as Rab11 and Rab8, and domain identity of the AMIS is specified by polarity protein networks such as Par3/Par6/aPKC/ CDC42/Anxa2 and phosphoinositides [19–22, 121]. These mechanisms have been reviewed in detail previously [122, 123]. *In vivo* studies have provided additional mechanistic insight to apical membrane proteins sorting and trafficking, demonstrating roles for Rab8 [124, 125], Rab11, [126, 127], MYO5B [128], and the vacuolar H^+ATPase (V-H^+ATPase) [129], among others [28, 130–132]. A forward genetic screen for mutations affecting apical membrane biogenesis in zebrafish revealed that V-H^+ATPase activity is required for biosynthetic sorting of O-Glycosylated apical membrane proteins and for post-Golgi trafficking of all apical membrane proteins tested [129]. In this system, V-H^+ATPase activity regulates the acidic lumen of the TGN, and manipulation of luminal pH during TGN export is sufficient to cause randomized sorting. These studies suggest that acidification of the TGN may promote apical sorting of O-glycosylated membrane proteins by controlling their oligomerization [28]. This model is supported by studies showing that oligomerization of mucins is enhanced by the mildly acidic pH levels characteristic of the TGN [133].

Importantly, some organs, such as the zebrafish intestine, undergo lumen formation before apical protein sorting is tightly regulated, and the above mentioned unbiased genetic screen for mutations affecting apical membrane protein targeting in this organ failed to yield lumen phenotypes [129]. Additionally, even in *Drosophila*, some tubular organs regulate apical polarization independently of canonical apical polarity factors such as Par-6 and aPKC [134]. Therefore, more *in vivo* studies are needed to understand the roles and requirements of apical polarization in developing organs versus cell culture models.

7. Conclusions and perspectives

The work highlighted here shows the tremendous progress made in understanding the roles of polarized transport in lumen morphogenesis. Though vastly different in scale and complexity, lumen formation throughout many different organs and animals relies heavily on the polarized targeting of membrane and secreted proteins. Common themes described in this review are that secretion promotes lumen formation by generating hydrostatic

pressure in a variety of organs and organisms, and that phyla-specific genes appear to have been co-opted for regulation of lumen morphogenesis among invertebrates and vertebrates. Moreover, as argued here, self-organization principles appear to underly polarization of critical junctional complexes, ion pumps, and channels required for lumen opening and expansion in immature epithelia (Fig. 1). These insights may help to explain how single lumen formation can be achieved in newly forming epithelial organs *in vivo*.

While candidate-based studies of cell culture models have yielded major insights into the mechanisms of lumen morphogenesis, unbiased genetic approaches *in vivo* are needed to better define the roles of epithelial polarization, sorting, and trafficking in lumen formation. Of particular importance is to obtain genetic evidence on the mechanisms controlling sorting and polarization of the Na^+/K^+-ATPase *in vivo*. *Drosophila* and *C. elegans* have traditionally showcased the power of using unbiased genetics to study lumen formation, providing many detailed mechanisms described here. However, the amenability of some vertebrate models such as zebrafish and medaka to forward genetics [135, 136], sophisticated live imaging [41], and genetic engineering [137, 138] should also be leveraged to uncover vertebrate-specific mechanisms controlling lumen formation.

Acknowledgements

Abbreviations

ABCC4	ATP Binding Cassette Subfamily C Member 4
AJ	Adherens Junction
AMIS	Apical Membrane Initiation Site
Anxa2	Annexin A2
AP	Adaptor Protein
aPKC	Atypical Protein Kinase C
cAMP	Cyclic Adenosine Monophosphate
CDC42	Cell Division Cycle 42
CD34	CD34 Molecule
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CG5789	*Drosophila* Dmel\CG5789
CHEs	CFTR High Expressing Cells
Ch-LD	Chitin-binding, Low-Density Lipoprotein Particle Receptor Class A-containing deacetylase

CHO	Chinese Hamster Ovary
Cldn15la	Claudin15-like A
COP-1	Coat Protein 1
Cse1l	Chromosome Segregation 1 Like
ECM	Extracellular Matrix
Ehbp1	EH Domain Binding Protein 1
ER	Endoplasmic Reticulum
GEF	Guanine Exchange Factor
GPI	Glycosylphosphatidylinositol
KV	Kupffer's Vesicle
MDCK	Madin-Darby Canine Kidney
Na$^+$/K$^+$-ATPase	Sodium-Potassium Pump
NHERF1	Na(+)/H(+) Exchange Regulatory Cofactor NHE-RF1
Nkcc1	Sodium-Potassium-Chloride Cotransporter 1
Par3	Partitioning Defective 3 Homolog
Par6	Partitioning Defective 6 Homolog
PDZ	PSD-95, Disks Large, Zona Occludens 1
PDZK1	PDZ Domain Containing 1
PKA	Protein Kinase 1
SJ	Septate Junction
SMG	Submandibular Gland
Tcf2	Transcription Factor 2
TGN	Trans-Golgi Network
VIP	Vasoactive Intestinal Peptide
V-H$^+$ATPase	Vacuolar-Proton ATPase
VIPR1	Vasoactive Intestinal Peptide Receptor 1
ZO-1	Zona Occludens 1

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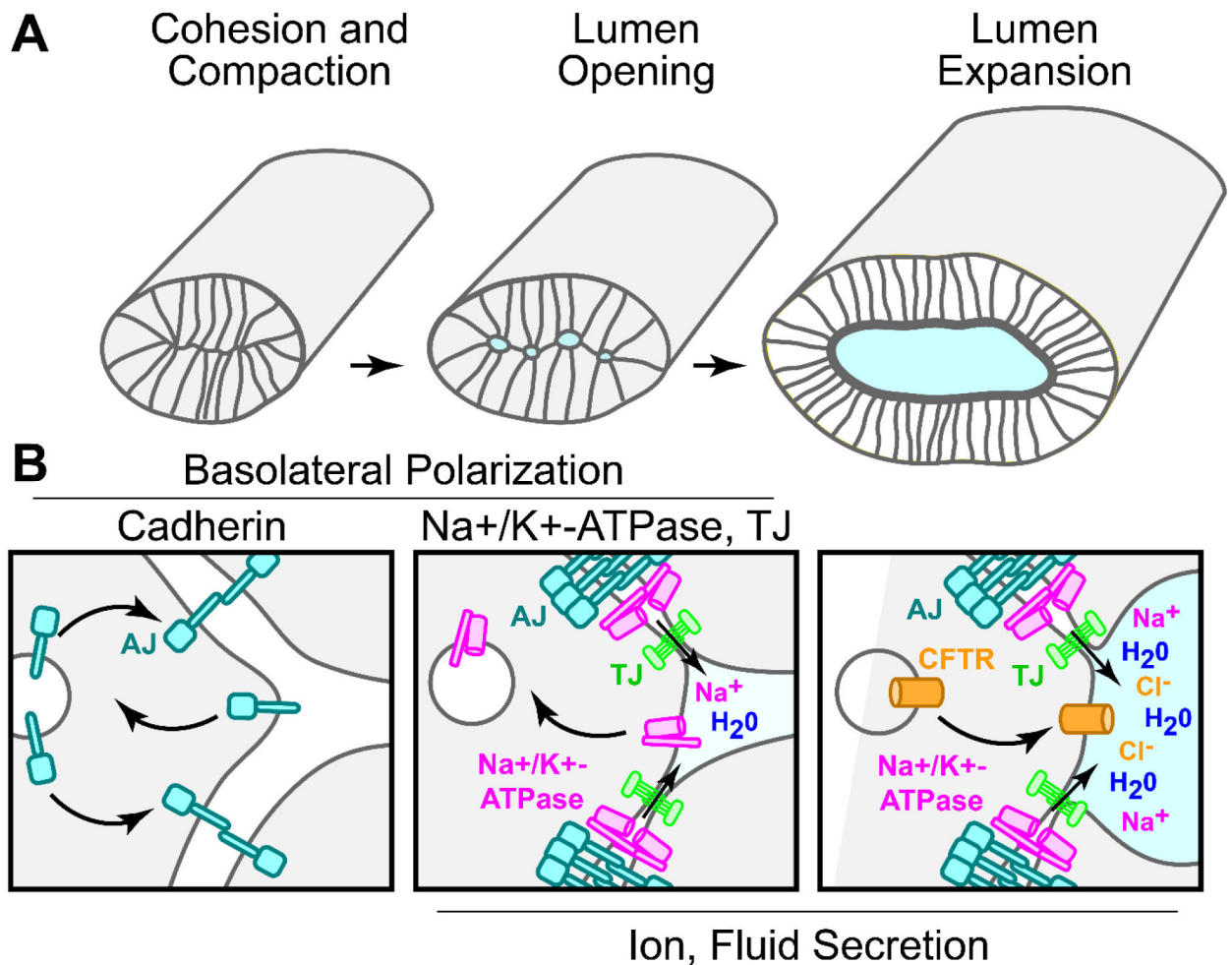
Fig. 1. Proposed mechanisms of lumen initiation and growth in vertebrate unpolarized epithelial organs.

(A) Basic morphogenetic events occurring during *de novo* lumen formation in newly polarizing organ precursors. Cell adhesion is a fundamental requirement that establishes positional domains required for proper development of other critical adhesion complexes.

(B) Minimal molecular events that may facilitate lumen morphogenesis. Left panel: After differentiation, epithelial cells express adherens junction (AJ, cyan) proteins such as E-Cadherin that mediate cohesion. Transcellular interactions of cadherin extracellular domains stabilize the AJ at the newly forming basolateral domain. Cadherins lacking trans-interactions undergo internalization (inward arrow) and recycling (outward arrow). Middle panel: Cohesion and basolateral polarization facilitates transcellular interaction of Na^+/K^+-ATPase (magenta) ß-subunits, thereby stabilizing the complex. Like cadherins, Na^+/K^+-ATPase lacking trans-interactions are subject to internalization (inward arrow). Basolateral polarization also promotes the formation of tight junctions (TJ, green), allowing the epithelium to establish selectively permeable paracellular barriers to mediate ion and fluid transport. Hydrostatic pressure commences lumen opening. Right panel: Continued

activity of ion channels such as basolateral Na^+/K^+-ATPase and apical CFTR in vertebrates drives enhanced fluid transport needed for lumen expansion.