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Review

Tight junction and polarity interaction in the transporting epithelial phenotype

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Abstract

Development of tight junctions and cell polarity in epithelial cells requires a complex cellular machinery to execute an internal program in response to ambient cues. Tight junctions, a product of this machinery, can act as gates of the paracellular pathway, fences that keep the identity of plasma membrane domains, bridges that communicate neighboring cells. The polarization internal program and machinery are conserved in yeast, worms, flies and mammals, and in cell types as different as epithelia, neurons and lymphocytes. Polarization and tight junctions are dynamic features that change during development, in response to physiological and pharmacological challenges and in pathological situations like infection.

Keywords: Tight junction; Claudin; Zonula occludens; ZO-1; MDCK; Na,K-ATPase; PAR; Polarized transport; Epithelium; Polarity; Apical; Basolateral

Contents

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1. Introduction

Life depends on asymmetry at all levels: (a) the vectorial flow of energy across the biosphere (as opposed to classical models based on the supply of energy), transformed life from an incredibly improbable organization of matter [1], into an unavoidable phenomenon [2,3]. (b) In most animal oocytes, the point where the spermatozoid enters defines two poles, and in subsequent steps conditions the asymmetric diffusion of gene products, creates an anterior–posterior axis and determines the development of the whole animal plan. (c) The nucleus, Golgi apparatus, microvilli, exocyst, flagellum, etc. are asymmetrically located. (d) Whether the mitotic spindle polarizes in a horizontal or a vertical position with respect to the plane of the epithelium, proliferating cells produces a normal planar tissue, or originates a polyp [4].

"Polarity" refers to a difference in structure, composition or function between the two poles of a cell, such as apical/basolateral in an epithelial one, and axon/dendrites in a neuron. Yet in the present article we reserve "polarity" for the expression of a given protein species in a particular location of the plasma membrane, as evidenced by the distribution of Na^+,K^+-ATPase, lipid rafts, tights junctions (TJs), gap junctions (GJs), adherent junctions (AJs), etc. [5–7].

Initial attempts were made to form artificial transporting epithelia by sandwiching ascite tumor cells between two filters, and mount the assembly as a flat sheet between two chambers to see whether they would develop an asymmetry [8]. Alternatively, natural epithelia were treated with collagenase, and the population of single cells obtained was seeded on filters, hoping that cells would express polarity [9]. The failures of these approaches led investigators to resort to culture of epithelial cells. MDCK (Madin–Darby canine kidney) cells became widely used, because they synthesize, assemble and seal tight junctions (TJs) [10,11]; express intramembrane particles and Na^+,K^+-ATPase in a polarized fashion [12,13]; and when infected with FLU or VSV viruses, cells deliver the corresponding envelope proteins to the apical or to the basolateral membrane respectively but not to both [14]. This approach opened new avenues in the understanding of TJs and polarity, which is reflected in a mushrooming literature (analyzed in [15–18]).

2. Toolbox

There is a series of facts and concepts that should be kept in mind while studying TJs and polarity that will be mentioned here without much elaboration.

(1) The assumption that the cytoplasm is a well-stirred solution contained within a neatly defined plasma membrane 10 nm thick was a very useful one to measure membrane fluxes and electrical phenomena, yet it is not adequate to understand polarity and TJs. Electron microscopy and cytochemistry show that the cell is overstuffed with organelles, fixed electric charges and polar sites able to establish hydrogen bonds, so no molecule, not even water, is free to diffuse in the cytoplasm [16,19]. In this respect, it is extremely improbable – actually, impossible – that a given protein species would achieve a homogeneous distribution neither all over the cell nor in the plasma membrane.

(2) Nature does not rely on random distributions and fortuitous encounters but distributes molecular species according to their character. Furthermore, proteins do not just carry signals but are themselves signals due to a sequence, a configuration, a distribution of electric charges, anchoring to a lipid or another protein (see below), that are recognized and sent to the cellular location where they should reside [15,16,20–24]. Once there, further modifications due to phosphorylation, contact with a given subunit, or the influence of an ion that has just increased

its concentration (notably H^+ and Ca^{2+}), suffice to make it appear as a different molecule and, accordingly, transferred to some other specific location in the cell, secreted, degraded, etc. In this respect, polarity depends on addressing signals that are interpreted by mechanisms, that deliver the molecule accordingly to a specify target, and a scaffold of molecules that retains it in position [16,18,25].

(3) TJs and polarity are not "things", but frozen images of vertiginous processes of exchange of proteins between organelles. These displacements are clearly observed and understood in the adsorption and processing of IgA that, once bound to the extracellular moiety of specific receptors, is internalized, and addressed to other sites of the cell ("transcytosis") [26–28]. Furthermore, TJs change in structure and degree of sealing in response to physiological conditions and pharmacological challenge [29,30]. An MDCK cell expresses at least five different types of K^+ channels in an absolutely polarized way, which are lost upon harvesting with trypsin and restored after plating at confluence [31–35]. Interestingly, this restoration not only preserves the polarization of each channel species, but each of them follows its own kinetics [36], presumably depending on the physiological role of each channel type. Therefore, polarization does not merely consist in expressing a given molecule in a specific place, but also at a specific time.

(4) The TJ is constituted by proteins [30,37–40] as well as lipidic microenvironments [41–44], whose arrangement does not concern us here. It is sufficient to say that this structure should have a considerable plasticity, as epithelia and endothelia are exposed to severe deformations, passage of leukocytes through the intercellular space, and their molecular components may have different half lives, which undergo active replacement without exposing the organism to the perils of a breakage of epithelial and endothelial barriers [17].

(5) The TJ is a promiscuous structure, as it can be established between epithelial cells derived from different organs and even different animal species, provided the two of them are able to make TJs [45,46]. In a way, this was expected, as epithelia frequently incorporate different cell types (i.e., goblet, mitochondria-rich, mucous, etc.). Thus the digestive tract, from the mouth to the anus, or the nephron, from the glomerulus to the collecting duct, not only have different cell types, but the transepithelial electrical resistance (TER) of their segments vary widely, from a few tenths of ohms per square centimeter in the proximal tube and the ileum, to 5–10 $K\Omega.cm^2$ in the urinary bladder and the colon [43,47].

(6) Accordingly, genetic programs to make TJs are highly conserved and ready to use whenever the cell requires an impervious barrier between two compartments. The mechanism that adjust the permeability of a given epithelium to the gradient of substances across it is mostly unknown [47,48].

(7) All occurs as if every cellular component was instantly informed of the status of other parts of the cell and could respond accordingly. No wonder the cytoskeleton and the cohort of molecules associated to it play a central role in the fate of TJs and polarity [15,17,49].

3. TJs as gate, fence, bridge and obstacle that can be bypassed

Since the TJ is placed at the limit between the basolateral side of epithelial cells and the apical domain, for a while it was assumed that the TJ was responsible for polarization. Yet a TJ can, at most, maintain a polarity that was already achieved by other means, but has no way of sorting molecules. On the contrary, today there is solid information that TJs can be eluded, depending on the molecular species.

3.1. The TJ as a gate

The TER of a given epithelium increases with the degree of elaboration of the strands of the TJ observed in freeze fracture replicas. Yet this increase is not linear, as it would be expected if strands acted as simple ohmic resistors in series (Fig. 1) [50–52]. Strands frequently anastomose, producing a complex pattern of electrical compartments [53,54]. While a diffusing substance gains access to successive compartments one-at-a-time, regardless of whether channels in other strands are transiently closed, the pulse of current delivered to measure TER requires that all channels in successive strands be open simultaneously. This arrangement would be in keeping with the exponential relationship between TER and the number of strands [6,50–52,54,55].

MDCK-I and Fisher rat thyroid cells spontaneously express high TER in normal culture conditions, and the resistance decreases following treatment with several fungal metabolites [56]. Interestingly, this was not accompanied by any structural change in the strands, as observed in freeze fracture replicas, suggesting that changes in conductance depend on changes at the molecular level [56]. These changes seem to have a considerable specificity, as changing the composition of phospholipids, sphingolipids, cholesterol and fatty acids, does not alter either TER or the structure of the strands, yet enrichment with linoleic acid does increase the paracellular flux of dextran without modifying either the TER or the complexity of TJs' strands [44].

Following the demonstration that mutations in claudin-16 abolish the paracellular resorption of Ca^{2+} and Mg^{2+} in the thick ascending limb of Henle's loop [57], claudins were identified as the molecules constituting the flickering channels in Fig. 1 (see also [58,59]).

In summary, the strands observed in freeze fracture replicas probably act as resistors spanned by gates that can be in the open or close configuration, and that may be constituted by claudins.

3.2. The TJ as a fence

When MDCK monolayers are treated with EDTA or EGTA, the TJ opens, TER falls, and apical membrane markers invade the basolateral side and vice versa [60–62] (Fig. 2B). Oddly

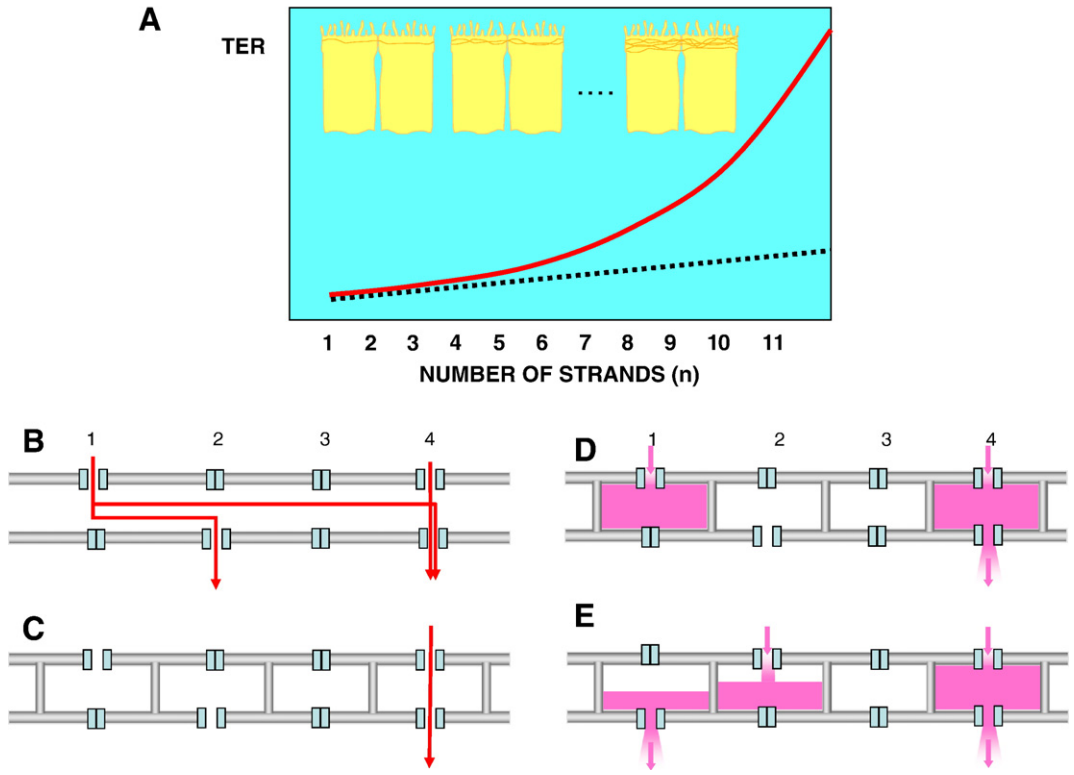

Fig. 1. The relationship between junctional structure, TER and permeability of the TJ. (A) TJs vary in the number of strands (*n*). If strands were simple ohmic resistors, then TER would increase with *n* in a linear manner, as indicated by dotted line. Yet TER increases exponentially with *n*, as indicated by red line. (B) Flickering channels (1, 2, 3 and 4) would not solve the problems though, because ions (red lines) may cross a given strand by any channel that happens to be open at the time. (C) Yet strands have frequent anastomoses and trabeculae, so that for current to flow it is necessary that channels on the two upper and lower strands be open simultaneously. Only compartment 4 would be conducting in this example. (D) Diffusion would instead follow a different set of rules. Thus a diffusing substance like mannitol or dextran (pink) may flow into compartment 1 in spite of the lower channel being closed. (E) When this channel opens, the solute would leave the compartment regardless of whether the channel in the above strand remains open or closed. The difference between electrical and solute permeability mechanism is that, while the first requires that all pathways in the series simultaneously open, solute permeation does not.

enough, these phenomena that occur simultaneously are frequently misinterpreted to be a sequence, in which opening of the TJ causes a loss of polarity. As we shall discuss below, Ca^{2+} triggers junction formation and polarization, and the information discussed in the present review strongly suggests that although both phenomena usually occur simultaneously, they can be experimentally separated.

Even if it remains tightly sealed, the TJ does not act as a barrier for all lipid species, in particular if the molecule of lipid can spontaneously jump from one leaflet of the plasma membrane the other ("flip-flop"). Fluorescent lipid probes added to the outer leaflet of the apical membrane of MDCK cells, are restricted to remain there, unless they can flip from the external to the cytoplasmic leaflet, circumvent the TJ, diffuse towards the basolateral domain, and flop back to the extracellular leaflet (Fig. 2C) [63,64].

Viruses and microorganisms can bypass the fence imposed by the TJ (Fig. 2E). This bears enormous importance for human health, as rotaviruses are a major cause of diarrhea in young animals including humans [65]. Integrins, which act as receptors for retroviruses [66], are expressed in the basal and lateral domains [67,68]. Although a virion is too big to cross the TJ and reach integrins, protein VP8 in the viral spikes, which is similar to the extracellular domain of occludin and claudins, can transiently open TJs, allowing integrins to access the apical membrane and meet viruses there [69]. This ability of VP8 was employed as a tool to enhance enteral administration of macromolecules like insulin [69,70].

The *Listeria monocytogenes* take advantage of the temporary opening of the TJ in the apoptosis sites of intestinal epithelium where the bacteria receptor, E-cadherin, is transiently exposed to the lumen [30,71].

3.3. The TJ as a bridge between neighboring cells

The liposoluble probe dipicrylamine diffuses, with the aid of an applied voltage, from the cell membrane of a previously loaded cell to the plasma membrane of the neighboring one (Fig. 2D) [72]. Photobleaching studies of MDCK cells, that were previously loaded with a lipid fluorescent probe, showed that this probe can diffuse to neighboring cells, provided temperature is kept above the melting point of the hydrophobic chains [73]. Likewise, PIP_2, ganglioside G-M1, and cholera toxin B move between the cells of a colony [74]. The transfer was inhibited by pre-treatment with poly-L-lysine and polyethyleneimine, suggesting that the TJ plays an important role, perhaps by permitting diffusion of lipids and their protein "cargo" across these cell-to-cell contact points [74].

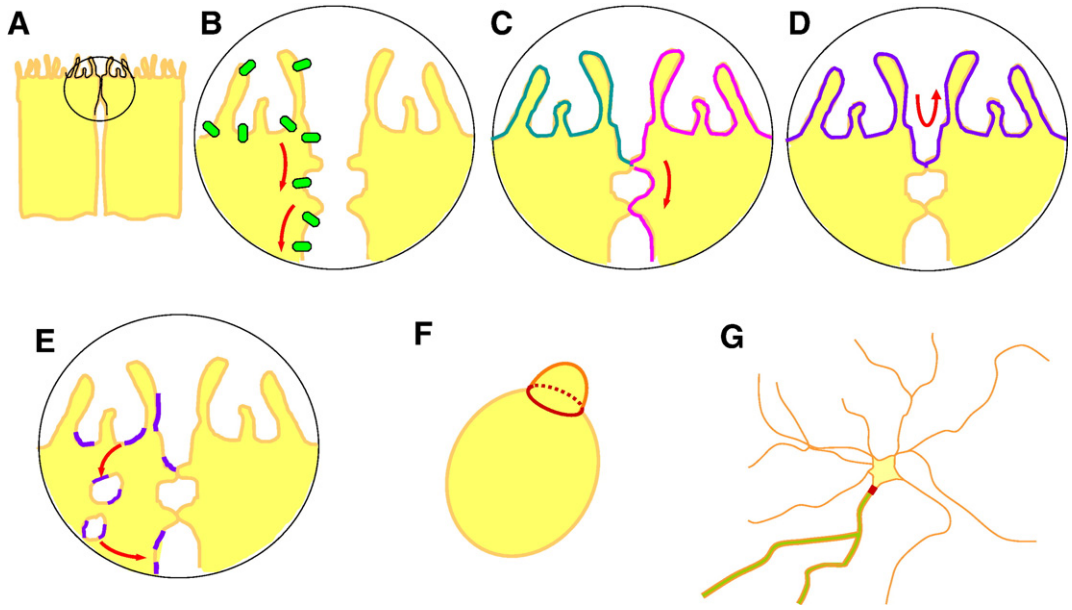

Fig. 2. TJs as fence, bridge and barrier than can be bypassed. (A) A TJ at the corner of two neighboring cells. (B) An apical marker (green) that had been previously restricted to the apical domain by the TJ, gains access to the basolateral domain (red arrows) when the junction is opened with a Ca^{2+} chelators. Total opening of the TJ occurs during diapedesis, when leukocytes cross the endothelial barrier. (C) In the cell on the left, the lipidic probe (green) does not diffuse past the TJ. In the cell on the right, instead, the lipidic probe (pink) that can flip-flop from the outer leaflet of the plasma membrane towards the inner leaflet, bypasses the TJ, and diffuses to the basolateral domain. (D) A lipid probe added to the apical domain of the cell on the left (violet) diffuses through the lipidic matrix of the strands of the TJ and reaches the apical side of the cell on the right. (E) A large molecule trapped on the apical side is removed and reinserted on the lateral side. (F) Yeasts have no TJs, nevertheless the septin ring (red) forms a fence that prevents the diffusion of proteins between the mother and the emerging bud membranes. (G) Neurons are polarized not only at the functional level (vectorial transmission of the nerve impulse), but the composition of the plasma membrane in the soma and dendrites is drastically different from that in the axon. Yet neurons do not have TJs or other anatomic barriers. Nevertheless membrane molecules in the area marked with a red square are anchored to the underlying cytoskeleton, and clog the passage of molecules diffusing in the plane of the plasma membrane.

In some cases, stimulation of a single cell induces the activation of phospholipase C that cleaves PIP_2 to produce a sudden increase of IP_3 which, in turn, induces Ca^{2+} release from intracellular stores to the cytoplasm. This signal frequently expands 5 to 10 cells beyond the stimulated one, a distance that would require a vast production of IP_3 (1.0 mM) in the stimulated cell, to deliver it to the neighboring cells via GJs [75]. Such IP_3 quantity is higher than most estimates of the precursor PIP_2, though. An alternative possibility would be that TJ bridges (Fig. 2D) would allow cells to obtain PIP_2 from its neighbors by membrane lateral diffusion, and enough IP_3 for the coordinated response [74]. Other signaling lipids, like ganglioside G-M1, might conceivable diffuse through the same route as well. Nevertheless, when MDCK cells expressing the glycosphingolipid Forssman antigen (GFA) are co-cultured at confluence with non-expressing cells, GFA remains in tagged cells [76]. It was argued though, that when the lipid carries a bulky attachment, it may hindrance the diffusion at the crevice between two cells.

3.4. The TJ can be bypassed

The possibility exists that instead of diffusing, a molecule would be retrieved from the plasma membrane on one side of the TJ and reinserted on the other side (Fig. 2E). Thus, in MDCK cells maintained in Ca^{2+}-free medium, Na^+,K^+-ATPase is randomly distributed over the whole plasma membrane [77]. When this ion is added, and the TJ forms, as shown by the increase of TER, part of the enzyme becomes trapped on the apical (wrong) domain. Yet in spite of the well-established TJ, the enzyme is removed from the apical and new enzymes are addressed to the basolateral [77]. Another mechanism, termed transcytosis, is responsible for relocating immunoglobulins [21,22,26], insulin, vitamins, iron and even whole cells between two environments without altering the unique compositions of those environments [28]. Transcytosis is not exclusive of epithelial and endothelial cells, it is also present in cultured neurons [78] and osteoclasts [79].

In live HepG2 cells, soon after basolateral endocytosis of CD59 (a GPI protein), a fraction of the integral membrane protein associated to rafts (MAL2) is redistributed into peripheral vesicular clusters that concentrate CD59, and that is accessible to transferrin (Tf) receptor, a basolateral recycling protein [80]. Following Tf receptor segregation, the clusters fuse in a globular structure containing MAL2 and move towards the apical surface for CD59 delivery.

Transcytosis is also involved in pathological conditions. For example *Streptococcus pneumoniae* binds specifically to the uncleaved pIgR on the apical membrane, and uses the transcytosis machinery to invade epithelial cells [81].

Neurotropic viruses are able to cross the blood/brain barrier and infect brain cells. Thus, rabies virus (RVG) specifically binds to the acetylcholine receptor [82] and undergoes transcytosis through the endothelia [83] and reaches brain tissues. Intravenous injection of chimeric peptide derived from RVG bounded to small interfering RNAs against flavivirus, can cross

the mouse blood/brain barrier, silence the expression of this virus and protect against it [84].

4. Polarized distribution of molecules that do not depend on TJs

Polarization of unicellular organisms constitutes a clear demonstration that it may be utterly independent from TJs. Although studies performed in different cell types tend to show a clear homology of the mechanisms involved, it may be premature to mix the observations in different systems. Accordingly, we shall classify them into the main cell types where original observations were made.

4.1. Yeast

Yeast cells proliferate by budding. It would be terribly inefficient to insert the necessary molecules anywhere in the membrane, leaving their encounters to chance. Of course yeasts address these molecules to budding sites. Genetic approaches have uncovered a large number of genes involved in these processes, yielding a working model for the establishment of cell polarity (discussed in [85]).

Yeasts buds beside the scar left on the membrane by the previous division, where they form a patch that concentrates GTPase Cdc42p, and is surrounded by a contractile ring formed of acto-myosin and the GTP-binding protein septin [86]. Like the TJ, the septin ring constitutes a fence (Fig. 2F) that prevents mixing of membrane components between the mother and the bud [87]. The components for the emerging bud are delivered via a bundle of actin cables precisely oriented and controlled by the Cdc42p GTPase [88]. All the cargo is transported by cytoskeleton motors to the exocyst, a multi-protein complex that tethers the vesicles first to the tip and later on to the septin ring [85]. As reviewed below, the exocyst in epithelial cells is located at the TJ and in neurons is involved in neurotransmitter release.

4.2. Neurons

Ever since Ramón y Cajal, neurons were recognized to have an asymmetric structure that results in "anterograd" stimulation. The axon bears a molecular analogy with the epithelial apical membrane (e.g., GPI protein Thy-1, hemaglutinin viral HA, fowl plague virus are expressed in both), and dendrites with the basolateral domain (They both express G protein of the vesicular stomatitis virus) [89,90]. This correspondence is further supported by the fact that, when expressed in MDCK cells, axonal markers like GABA transporter GAT-1 are targeted to the apical membrane of MDCK cells [91,92].

Even though there are no TJs in neurons, there seems to be a functional fence localized at the axonal initial segment membrane [93] that prevents mixing of lipids [94] and proteins [95] (Fig. 2G). This fence is formed by the accumulation of membrane proteins anchored to submembrane cytoskeleton [93,96] and may be a case of a universal mechanism for formation of diffusion barriers in the cell membrane (Fig. 2F, G).

At an early stage, neurons from the hippocampus cultured *in vitro* produce several neurites which are in principle able to develop into axons. Yet, at a later stage, only the one which is closest to the centrosome becomes an axon [97], and the rest are inhibited from pursuing this pathway [98,99]. This process involves recruitment of "partition defective polarity complex" (aPKC/PAR, see below) and disheveled (Dvl) to the growing axon tip [100]. Besides of forming autotypic TJs, GAPJ and AJs between adjacent lamellas within the myelin sheet, of a given cell [239], glial cells establish heterotypic septate like junctions with the axon, at the Ranvier nodes' borders [351]. These junctions participate in the saltatory transmission of the electrical impulse by restricting the diffusion of axonal proteins like ion channels [352].

4.3. T Lymphocytes and neutrophiles

T cell polarization is required for processes such as leukocyte migration, proliferation, activation in response to antigen presentation, and cytotoxicity.

In migration induced by chemokines, leukocytes polarize in two different regions: the leading edge, where actin is concentrated, and the uropod, which is a pseudopod-like projection, that may anchor the cell to the extracellular matrix while the cell front pulls forward the cell body [101]. The uropod concentrates several adhesion molecules and aPKC, and its location is determined by the microtubule organizing center (MTOC) [102,103]. Cdc42 is a key player in the orientation of polarity, as leucocytes expressing a dominant-negative Cdc42 are able to migrate but do not polarize in the direction of the gradient, therefore abolishing chemotaxis [104].

When pathogen components are exhibited onto the major histocompatibility complex (MHC) antigen-presenting cell and the T cell interact forming a microdomain known as the immunological synapse (IS). This structure is initially formed with a central region of integrins surrounded by a ring of engaged MHC–peptide complexes. Over a period of minutes this organization is inverted, resulting in a mature IS characterized by a central group of activated T cell receptors (TCRs) surrounded by a ring of adhesion receptors (reviewed in [105]). The MTOC (as well as its associated vesicles), talin and PKC-θ are enriched in these contacts [106] while other components, including ezrin and moesin and the cell surface protein CD43, redistribute to the pole opposite to the synapse (reviewed in [107]). The IS is essential for the correct activation of T cells and for the generation of an asymmetric division that gives rise to effector and memory cells. This is accomplished by the tether of PKC-ζ to the opposite pole of the IS and segregation of Numb (an inhibitor of Notch pathway) away from PKC-ζ positive zones, resulting in asymmetric mitosis and the generation of different lineages in a similar way as happens in early development of invertebrates [108,109].

5. Proteins that play a major role in cell polarity

This section is focused on prominent sets of proteins complexes that participate in polarity (Fig. 3 and Table 1 for the nomenclature used), and that are activated by asymmetric spatial

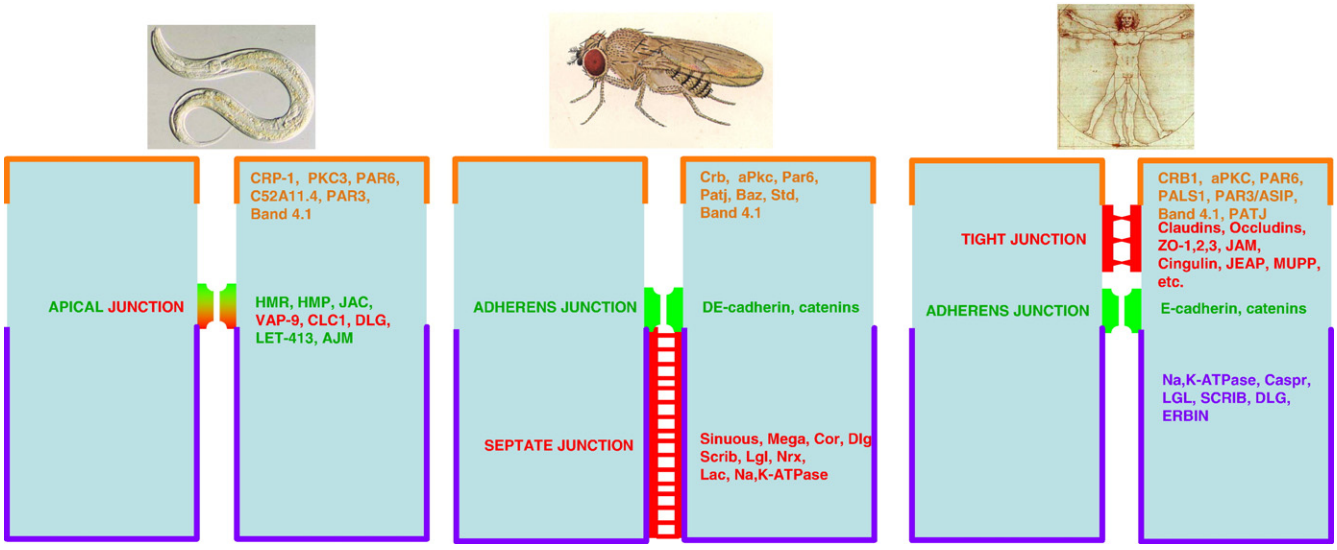

Fig. 3. Prominent proteins from the epithelial transporting phenotype, from *Caenorhabiditis elegans*, *Drosophila* and mammals. The apical and basolateral membranes are represented in orange and violet respectively. Occluding junctions (TJ in mammals and septated junctions – SJ – in invertebrates) are in red, adherens and adherens-like junctions in green. Notice that mammals have the adherens junction below the TJ (i.e., towards the basolateral), but *Drosophila* have adherens junctions above the SJ; worms have apical junctions with characteristics of adherens and tight junctions. Furthermore, the Na^+,K^+-ATPase is within the SJ in *Drosophila*, but it does not occupy an equivalent position in the TJs of mammals, where it is instead a basolateral protein [49,163,267,350,351].

cues. Following these spatial cues, evolutionary-conserved protein complexes distribute asymmetrically and form a scaffold that interacts with downstream effectors, which consequently modify actin and tubulin cytoskeletons, and the sorting machinery, promoting asymmetric cell division, cell phenotype determination or the establishment of apical–basal polarity [49,110].

5.1. *Caenorhabditis elegans* and the PAR proteins

The anterior–posterior (AP) axis of *C. elegans* is specified by sperm entry into the egg, and it is established in the one-cell embryo through reorganization of cytoplasmic and cortical structures [111]. Genetic screens in *C. elegans* for maternal effect lethal mutations that disrupt this axis specification identified six genes called *par* (for partitioning defective) and protein kinase C3 (*pkc3*), which encodes an atypical protein kinase C (aPKC) [109,112–114]. PAR1 and PAR4 (also known as LKB1) are ser/thr protein kinases. PAR2 is a ring finger protein. PAR3 and PAR6 are PDZ domain scaffold proteins involved in multiple protein–protein interactions. PAR5 is a 14-3-3 protein [109,112–115].

Table 1
Proteins that play a major role in epithelial polarity

		Drosophila	Mammals
PAR complex		Baz (Bazooka)	PAR3/ASIP
		Par6	PAR6A to PAR6D
		aPkc	aPKC
	Location	Marginal zone	TJ
Crb complex		Crb (Crumbs)	CRB1 to CRB3
		Patj (Pals-associated TJ protein)	PATJ, MUPP1
		Sdt (Stardust)	PALS1
	Location	Marginal zone	TJ
Scrib group		Lgl (Lethal giant larvae)	LGL1, LGL2
		Dlg (Disc large)	DLG1 to DLG3, SAP97
		Scrib (Scribble)	SCRIB1, ERBIN
	Location	Lateral membrane, beneath the AJ	Lateral membrane

Location refers to the position that the complex occupies in mature epithelia. Marginal zone is the most apical region of *Drosophila* epidermis.

After fertilization, PAR3 and PAR6 together with the PKC-3, form a complex in the anterior half of the *C. elegans* zygote. The Ser/Thr kinase PAR-1 and the ring finger protein PAR-2 occupy the posterior half. PAR4 and PAR5 proteins are uniformly distributed. These proteins are required for subsequent asymmetric segregation of P granules (RNA-rich organelles) and the asymmetric placement of the first mitotic spindle and mutations in any one of these genes disrupt polarization of the zygote. In this way, the asymmetric distribution of PAR proteins transforms a spatial cue into stable positional coordinates that direct the localization of cytoplasmic factors (reviewed in [109,112,116,117]).

5.2. *Drosophila*

Following the discovery of the PAR proteins in *C. elegans*, subsequent studies revealed evolutionary conserved functions of these proteins in other organisms. Moreover, studies carried mainly in the fruit fly lead to the finding of other two groups of proteins (Crb complex and Scrib group) that regulate cell polarization (Table 1).

5.2.1. PAR–aPKC complex in *Drosophila*

The Par proteins are also necessary in the generation of AP-axis determination in *Drosophila*, but we focus here on the participation of these proteins in the generation of epithelial polarity, so we refer the reader to recent reviews on the former theme [49,115,116].

The first epithelium that emerges during *Drosophila* development is the blastoderm through a process known as cellularization, in which the membrane invaginates around each nucleus in the syncytium produced by nuclear divisions in the egg. Hence, cytokinesis and epithelium formation go hand in hand, allowing the study of epithelial polarization that occurs coordinately in ~5000 cells (reviewed in [118]).

Par6 and aPkc form a constitutive complex that can associate with, and phosphorylate, Baz (PAR–aPKC complex) [119]. The PAR–aPKC concentrates at a subapical region (the marginal zone) above the AJ in *Drosophila* epithelia and is required for proper development: mutants in aPkc as well as Par-6 result in defects similar to those of Baz, i.e., disruption of apical–basal polarity [119–121]. During cellularization, Baz establishes apical complexes along cleavage furrows in the absence of AJs and it is required for positioning DE-Cad (*Drosophila* E-cadherin) [122] indicating that Baz acts upstream of AJ formation. Baz itself is positioned by two cues, an apical scaffold and dynein-mediated transport, and its recruitment to the cell cortex is dependent on Par6 binding to Cdc42-GTP [123]. Baz recruits AJ proteins in a subapical region while helping direct aPkc to the extreme apical region, resulting in the formation of a stratified apical domain, with Baz and AJs localizing basally to aPkc and Par6 [120,124]. This contrast with the polarization hierarchy of mammalian cells, in which the nectin-based junction and AJs formation are necessary to recruit mammalian PAR3 and precedes the generation of polarity (see below) [125–128].

5.2.2. Crb complex in Drosophila

Crb complex, identified first in *Drosophila*, localizes at the marginal zone and includes the transmembrane protein Crb, as well as Std (a MAGUK protein) and Patj (a multi-PDZ domain protein) [118]. Crb protein is an apical membrane determinant as its insertion is sufficient to confer apical characteristics to the plasma membrane and its over-expression induces an expansion of the apical domain [129]. This activity might be due to the linkage provided by Crb to Dmoesin and to the spectrin/actin cytoskeleton [130,131]. Crb in concert with Sdt and Patj are also required for the biogenesis of the AJ and maintenance of epithelial integrity [132–137], because mutants of these proteins show degeneration of ectodermally derived epithelia [137–139]. Furthermore, Sdt could be downstream of Crb since over-expression of Std could restore some defects observed in Crb mutants [1139].

During early epithelial morphogenesis the PAR–aPKC complex functions upstream of Crb and is required to establish the apical localization of Crb [140,141]. The aPkc binds to both Crb and Patj, and phosphorylates Crb. The phosphorylation of Crb is functionally significant because a non-phosphorylatable protein behaves as a dominant negative [142]. Nevertheless, Crb is necessary to maintain Baz at apical membranes after gastrulation [141].

5.2.3. Scrib group in Drosophila

This group of proteins localizes to the lateral membrane and comprises Scrib (a LAP protein [143,144]), Dlg (a MAGUK protein) and Lgl (a myosin II binding protein with WD-40 domains). In *Drosophila*, Scrib shows a genetic interaction with both Dlg and Lgl [145,146], and loss-of-function mutants in these three genes have very similar developmental phenotypes, including loss of apical–basal polarity, impaired cell cycle exit and tissue overgrowth that results in larval lethality (reviewed in [147]). However, there is no evidence of physical interaction between these proteins in epithelia, although in *Drosophila* neuronal synapses, Scrib associates with Dlg through a protein termed GUK holder [148].

Scrib group, PAR–aPKC and Crb complexes interact to set limits in apical and basolateral membrane domains and in positioning the AJ. As described above, the PAR–aPKC complex is required for the formation of the AJ and the localization of the Crb complex to the marginal zone. The Scrib group in turn establishes the lateral domain and suppresses the generation of apical identity on the lateral domain by antagonizing the Crb complex. On the other hand, the Par complex inhibits the "lateralization" of the apical domain by antagonizing with the Scrib group [115,124,140,141,145,146]. Thus, aPkc located at the marginal zone phosphorylates and inactivates the Lgl protein on the apical side [123,149]. On the other hand, Lgl is active on the lateral domain and excludes Par6 preventing the activity of the PAR–aPKC complex on the lateral domain [123].

5.3. Mammalian

5.3.1. PAR–aPKC complex in mammalian cells

In fully polarized mammalian epithelial cells, the PAR–aPKC complex (PAR3–aPKC–PAR6) localizes to the TJ region [150–152]. The recruitment of this complex to its location has been studied during junction formation in subconfluent cultures or using the Ca^{2+} switch assay [54] (see Synthesis and assembly of the TJ). This approach has revealed that the initial adhesions between cells triggers the trans-interactions of nectins and E-cadherin to form AJs and then recruit JAMs to the apical side of AJs [125,153–155]. The recruitment of PAR3 to nascent cell–cell contacts could be mediated by JAM and nectins-1 and -3 [126–128,156,157]. Moreover, the trans-interactions of nectins and E-cadherin activate Cdc42, which binds to PAR6-aPKC and induces the activation of aPKC [150, 158–162] which is necessary in the formation and establishment of TJs and epithelial polarity [158,163,164]. Interestingly, in endothelial cells both PAR3 and PAR6 associate directly with vascular endothelial cadherin (VE-cadherin), but this complex lacks aPKC and expression of VE-cadherin in epithelial cells affects tight junction formation. This suggests that in endothelia, another PAR complex exists and does not promote cellular polarization through aPKC activity [165].

LKB1, the mammalian PAR4, is a kinase whose activation generates apical–basal polarization and some attributes of TJs in intestinal epithelial cells, even in the absolute absence of cell–cell contacts, i.e., single cells [166]. LKB1 facilitation of TJ formation involves the phosphorylation of AMP-activated kinase (AMPK) [167]. LKB1 also can phosphorylate PAR1, which in turn can regulate PAR3 stimulating its binding to the 14-3-3 protein (PAR5), and thus regulating the activity of the

PAR–aPKC complex [113,168,169]. ATP depletion in MDCK cells causes the formation of a PAR3-Tiam1 complex which makes them unable to activate aPKC and Rac respectively resulting in TJ disassembly [170].

Interestingly, pathogenic CagA protein from *Helicobacter pylori* binds to and inhibits PAR1, causing junctional and polarity defects that may liberate the cells from growth inhibitory cues, and thereby promote infection and gastric carcinogenesis [171,172].

The role of the PAR–aPKC complex in the generation of polarity has been confirmed in many cell systems (reviewed in [115,173–175]). In axon determination in cultured neurons aPKC accumulates at the tip of the neurite, which will eventually become the axon. Axon growth depends upon kinesin-mediated transport of PAR3 to the tip, a step promoted by glycogen synthase-kinase 3β (GSK3β) and adenomatous polyposis coli (APC) protein. Once there, PAR3, in conjunction with Tiam1, activates Rac, which provokes an intense modification of the cytoskeleton [176,177]. This signaling cascade may be triggered by neurotrophic factors such as NGF, BDNF, NT3 [99].

In migrating astrocytes cell polarity is characterized by the orientation of a pseudopodium-like structure (protrusion) and the reorientation of the MTOC, the microtubule cytoskeleton, and the Golgi to face the direction of migration. The correct orientation of the protrusion as well as the MTOC is achieved by the activation of integrins at the front of the cell which leads to the activation of Cdc42 and the recruitment of the PAR–aPKC complex at the leading edge [178]. The PAR–aPKC complex then inactivates GSK3β and, as a consequence, the APC protein associates with the plus ends of microtubules [179]. On the other hand, the PAR–aPKC complex also induces the recruitment of DLG1 at the plasma membrane where it interacts with the microtubule-bound APC to polarize the microtubule network and the MTOC [180].

5.3.2. Crb complex in mammalian cells

In mammalian epithelia, PALS1 acts as an adaptor mediating the indirect interaction between CRB and PATJ, which localizes to tight junctions [181,182]. This complex is important for epithelial polarity biogenesis. Over-expression of CRB3 or RNA interference mediated inhibition of PATJ and PALS1 delays formation of the TJ and disrupts cell polarity [183–186].

These effects might be due to the interactions between PAR–aPKC and Crb complexes. The two complexes can associate with one another through a PALS1–PAR6 interaction and this association is enhanced by activated Cdc42 [187] and the loss of PALS1 and PATJ decrements the localization of aPKC to the TJ during polarization, which may result in the perturbation of downstream polarity signals [181,186,187].

5.3.3. Scrib group in mammalian cells

The SCRIB, DLG and LGL proteins are highly conserved between *Drosophila* and mammals, localize to the lateral membrane and there are some evidence of its participation in the generation of polarity [188–193].

During cell polarization in cultured cells, mammalian LGL antagonizes the PAR–aPKC complex by competing with PAR3 for binding to the aPKC–PAR6 complex. When cell–cell adhesion activates to aPKC through Cdc42, aPKC phosphorylates and releases LGL allowing the formation of the PAR–aPKC complex [189]. Released LGL remains in the lateral region where it can interact with the basolateral exocytic machinery member syntaxin-4 [194]. This observation and the fact that a yeast homologous of LGL (Sro7) participates in the docking and fusion of vesicles to the plasma membrane [195] have prompted the hypothesis that the Scrib group may regulate membrane domain identity by regulating the delivery of transport vesicles containing basolateral proteins [110,194]. Interestingly, LGL is also required for the disassembly of apical membrane domains in depolarizing MDCK cells induced by Ca^{2+} depletion by the suppression of the PAR–aPKC complex activity [196].

The polarity disruption that produces some oncogenic viruses might be potentiated by effects of viral proteins on polarity proteins. Hence, the high-risk human papilloma virus (HPV) E6 proteins can lead to SCRIB and DLG1 degradation in a proteasome-dependent manner [191,197–199].

5.4. The exocyst complex

Also termed the "Sec6/8 complex", it is an eight-protein complex involved in polarized exocytosis from yeast to mammals. It localizes at the site of yeast budding, the neuronal synapse, and TJs [200,201]. Upon establishment of cell–cell contacts after addition of Ca^{2+}, the exocyst assembles and is recruited to TJs [200], in a region active in basolateral exocytosis [24,202]. Knockdown of PALS1 causes severe defects in TJ, together with mislocalization of adherens junction and exocyst [203].

The Sec6/8 complex is also present on trans-Golgi network (TGN) and plasma membrane in normal rat kidney (NRK) cells [204]. During neuronal development the exocyst complex sorts secretory vesicles to specific domains of the plasma membrane through its association with the microtubules, promoting neurite outgrowth [205]. Recently, Exo70 in HepG2 cells has been visualized at Golgi membranes and apparently at the microtubule-organizing center (MTOC) [206].

6. Synthesis and assembly of the TJ

TJ strands are mainly composed of two distinct types of proteins: occludin and claudins which traverse the lipidic matrix four times, and JAM proteins that belong to the Ig superfamily and only cross the plasma membrane once. In contrast zonula occludens proteins (ZO-1, -2, and -3), belonging to the MAGUK family, are TJ-members that form a submembrane scaffold through multiple protein–protein interactions [40]. There are other members of MAGUK family, such as Pals1, PAR3, PAR6, as well as transcription factors, such as ZONAB [207], and kinases such as PKC, PKA, etc. [208].

When epithelial cells are maintained in low Ca^{2+}, adhesion complexes do not form. Ca^{2+} addition (Ca^{2+} switch assay) triggers the epithelial transporting phenotype (TJs plus polarity) acting at the extracellular segment of E-cadherin [54,77] through a series of events involving two different G proteins, PLC, PKC

and calmodulin [208]. Ca^{2+} induces an initial phase of cell adhesion, where fibroblastic spot-like AJs containing E-cadherin and nectins as well as ZO-1 are formed as a nascent junctional complex [125,209,210]. Nectins recruit E-cadherins that subsequently form a cluster and become adhesive with those of neighboring cells [125,153,211,212]. E-cadherin also interacts with several cytoplasmic proteins (α-, β-, and γ-catenin) and the cytoskeleton to form the AJ. Acting together, these intra- and extracellular interactions provide the basis for many functions of the AJ, like morphogenesis, differentiation, growth control, etc. [213–217].

E-cadherin can stimulate polarization as its transfection into fibroblasts induces the movement of Na-K-ATPase from a uniform distribution on the plasma membrane, to sites of cell–cell contact, in a manner reminiscent of polarizing epithelial cells [218].

Components of the PAR–aPKC complex are recruited after the establishment of the initial spot-like AJs to which TJ components such as JAM, occludin and claudin-1 are recruited (see PAR–aPKC complex in mammalian cells). Thereafter, as epithelial polarization progresses, ZO-1 dissociates from E-cadherin, which separately forms the epithelia-specific belt-like AJ, and gradually colocalizes with occludin at cell–cell contact sites to form TJs [219]. In the following section we focus on the fundamental studies addressed to elucidate the molecular mechanism responsible for targeting the integral membrane proteins to the TJs.

6.1. Occludin

It is localized at TJ and directly associates with ZO-1. Early studies suggested that the association of occludin with underlying cytoskeletons through ZO-1 is required for its localization at TJs [220]. Later on, studies using chimeras of occludin C-terminal cytoplasmic domain with the transmembrane portions of connexin suggested an important role for cytoplasmic proteins, presumably ZO-1, ZO-2, and ZO-3, in localizing occludin in TJ fibrils [221].

The C-terminal domain appears to direct intracellular transport of occludin through the basolateral membrane as an obligatory intermediate in its transport to TJs [222]. Occludin lacking the first or second extracellular loop colocalizes with ZO-1 at regions of cell–cell contact when expressed in fibroblasts, demonstrating that interactions with ZOs are sufficient to cluster occludin even in the absence of TJs. In MDCK cells, both full-length and occludin lacking the first extracellular loop colocalized with ZO-1 at the TJ. In contrast, proteins lacking the second, or both, extracellular loops are absent from TJs and found only on the basolateral cell surface. These results suggest that the second extracellular domain is required for stable assembly of occludin in the TJ [223].

6.2. Claudins

Claudins (cln) are a family of tetra-span transmembrane proteins that represent the major constituents of epithelial and endothelial TJs. Intracellular C-terminal domain of claudins is connected via a PDZ-binding motif with several TJ-associated proteins.

Analyses of the distribution of a series of C-terminal deletion mutants of the cln-1 and -5 showed that residues located through C-terminal to the last transmembrane domain are required for the proper targeting to TJs [224]. While claudin derivatives lacking only the very C-terminal PDZ-binding motif continue to localize to TJs, mutants lacking the entire C-terminal juxtamembrane sequence do not associate with TJs and accumulate in intracellular structures. This indicates that crucial determinants for stable TJ incorporation of claudins reside in a cytoplasmic C-terminal sequence which up to now has not been implicated in specific protein–protein interactions.

Expression of a tailless cln-6 (CΔ187) in the suprabasal layer of the mouse epidermis results in the accumulation of cln-6, cln-10, cln-11, and cln-18 in the cytoplasm and elicits histological and biochemical perturbations in epidermal proliferation and differentiation that were evident after 1 week of age and that persisted throughout life. These results confirmed the importance of the cytoplasmic tail domain of cln molecules in membrane targeting and hence their function in TJs *in vivo* [225].

6.3. JAM

Junctional adhesion molecule (JAM) is an integral membrane protein that colocalize with the TJ proteins occludin, ZO-1, and cingulin [226]. Thus, transfection of Chinese hamster ovary (CHO) cells with JAM (either alone or in combination with occludin) results in enhanced junctional localization of both endogenous ZO-1 and co-transfected occludin. Additionally, JAM co-precipitates with ZO-1 in the detergent-insoluble fraction of Caco-2 epithelial cells. A putative PDZ-binding motif at the cytoplasmic C-terminus of JAM is required for mediating the interaction of JAM with ZO-1, as assessed by *in vitro* binding and co-precipitation experiments. JAM also co-precipitates with cingulin, another cytoplasmic component of TJs, and this association requires the N-terminal globular head of cingulin [227].

AF-6 (ALL-1 fusion partner from chromosome 6) is a Ras target that participates in the regulation of cell–cell contacts, including TJs, via direct interaction with ZO-1 downstream of Ras. The binding of AF-6 to JAM requires the presence of the intact C-terminus of JAM, which represents a classical type II PDZ domain-binding motif, suggesting that JAM can be recruited to intercellular junctions by its interaction with the PDZ domain-containing proteins AF-6 and possibly ZO-1 [228].

6.4. ZOs

ZO-1 is not normally present at the lateral plasma membrane. However, in most polarized epithelia, a significant fraction of the transmembrane proteins like occludin and claudins are normally resident in the lateral plasma membrane, although they are not organized into strands. Early studies on ZO-1 mobilization during TJ assembly [229] showed that catenins mobilize ZO-1 from the cytosol to the cell surface early in the development of TJs. Afterward, it was shown that ZO proteins play a crucial role

in the recruitment and localization of occludin [221], claudins [225,230–232] and the CRB complex [181] to the TJs. However, the N-terminal PDZ domain-containing half of ZO-3 delays the assembly of both TJ and AJ via a mechanism involving the actin cytoskeleton, ZO-1, β-catenin and the small G protein RhoA [233,234]. Recently, ZO-1 was shown to control the location of TJ integral membrane proteins, revealing complex protein binding and targeting signals within the SH3-U5-GUK-U6 region of this scaffold protein [235].

ZO-3-null mice prove to be viable and fertile with no significant abnormalities, indicating that ZO-3 is dispensable *in vivo* in terms of individual viability, epithelial differentiation, and the establishment of TJs [236]. Moreover, a mouse epithelial cell line lacking the expression of all ZO proteins (Eph4 ZO-null cells) displays well-polarized architecture in terms of the differentiation of apical–basolateral membranes and formation of belt-like AJs but lacked TJs completely in the confluent state. The exogenous expression of N-terminal PDZ1–3 domains of ZO-1 was inefficient to rescue the formation of TJs in Eph4 ZO-null cells; however, when N-terminal PDZ1–3 domains of ZO-1 were forcibly recruited to the lateral membrane, claudins were polymerized in Eph4 ZO-null cells, indicating that dimerization of the PDZ domains of ZO-1 determine whether and where claudins are polymerized in epithelial cells [231]. Thereafter, careful examination of the junctional complexes formation in parental and Eph4 ZO-null cells using the Ca^{2+} switch assay revealed that ZO-1 plays crucial roles not only in TJ formation, but also in the conversion from "fibroblastic" AJs to belt-like "polarized epithelial" AJs during epithelial polarization. Furthermore, this study also demonstrated that the acidic domain of ZO-1 is required for the proper segregation of belt-like AJs and TJs [232]. Altogether these observations strongly support the hypothesis that ZO-1 plays a direct scaffolding role in organizing proteins within the TJ and AJ.

6.5. PATJ

PATJ localizes to the TJ, where it interacts with ZO-3 via its sixth PDZ domain, and with claudin-1 via its eighth PDZ domains [181,182,237]. PATJ missing the 6th PDZ domain was found to mislocalize away from cell contacts. Hence, the PATJ/ZO-3 interaction is likely important for recruiting PATJ and its associated proteins to tight junctions. MUPP1, a paralog of PATJ, contains 13 PDZ domains and also interacts with tight junction proteins, including claudins and JAM [238,239].

7. TJs and polarity that change in response to physiological requirements and pharmacological conditions

7.1. Polarity reversal in epithelial cells

TJs and polarity are highly resilient to the deformations of the epithelium, proliferation, differentiation, migration, diapedesis of leukocytes, changes in tissue architecture that accompany embyogenesis, hormonal challenge, etc. A number of these phenomena have been reproduced in the laboratory and can be used to investigate pharmacological challenge and pathological conditions. Thus, hog thyroid cells maintained in suspension, form hollow spheres with TJs towards the medium and the basolateral domain towards the lumen [240]. When gelatin or collagen is added to suspension cultures, cells reverse its polarity [241], without opening the paracellular pathway [242,243]. Similar experiments were carried with MDCK cells [244]. Colonies of MDCK overlaid with collagen, change the position of apical marker gp135, and basolateral markers like Na^+,K^+-ATPase, E-cadherin, α-catenin and β-catenin [245]. These observations show the high degree of plasticity of polarity and the role of spatial cues (i.e., contact with extracellular matrix) which serve as signals to segregate apical and basolateral components.

These changes in polarity may play paramount physiological roles. Acid/Basic regulation in the organism requires a drastic adaptation of kidney cells, which in turn adapt by expressing two interconvertible forms of intercalated cells (IC): α-IC, that expresses H^+-ATPase in the apical domain, and band-3 protein in the basolateral one, and β-IC that expresses the opposite polarity [246]. Changes in pH induce this reversal of polarity through the secretion of hensin [247–250] and its polarization by galectin-1 [251].

7.2. Epithelial adjustment to a changing environment

Epithelial cells have to cope with environments that may change drastically in a short time. Such are the cases of epithelial cells in the intestine that are exposed to the vicissitudes of food intake and digestion, and epithelial cells in the nephron that have adapted to a severe dehydration, yet suddenly the animal drinks a considerable volume of fresh water. These adaptations are expected to involve changes in the transporting epithelial phenotype. Thus we have found that urine contains epidermal growth factor (EGF), that before being excreted has been in contact with different segments of the nephron [47,48]. EGF treatment of monolayers of MDCK cells increments TER through the reduction of claudin-2 expression and increment of claudin-4. This factor also changes the localization of both claudins-4 and -7 over the lateral domain, suggesting that it regulates the polarized distribution of different proteins [47]. Indeed, it has been shown that EGF stimulation induces the phosphorylation of PAR3 through c-Src and c-Yes and this is necessary for the optimum EGF effect on TJs [252]. These effects appear to be mediated by the ErbB1 receptor, because activation of ErbB2 in MDCK cells induces the relocalization of gp135 and ZO-1 to the lateral membrane, reinitiates proliferation and promotes multilayering [253]. These changes are mediated by the disruption of the PAR–aPKC polarity complex through the interaction of ErbB2 with aPKC–PAR6, which also leads to an anti-apoptotic effect [254]. Interestingly, ErbB1 activation does not disrupt polarity [253] nor affects the interaction between the members of the PAR complex [254]. Thus, oncogenic cell transformation involves both direct induction of cell proliferation (through Ras-ERK pathway) and direct disruption of cell polarity (through disruption of Par polarity complex) [254].

The role of EGFR signaling in epithelial integrity maintenance appears to be highly conserved. During tracheal

development in *Drosophila* EGFR signaling regulates the maintenance of tracheal epithelial integrity by modulating, at least in part, post-transcriptionally cadherin-based cell adhesions [255].

Moreover, ERBIN (a homologous of mammalian SCRIB and *C. elegans* LET-43) interacts directly with the C-terminal domain of ErbB2 and is necessary for the polarized distribution of the receptor [143]. ERBIN also interacts with active Ras acting as a negative regulator of the MAPK pathway [256]. In addition, ERBIN interacts with plakophilin-4 (a member of the p120 catenin subfamily — [257]) colocalizing at AJs and desmosomes. Disruption of this interaction leads to alteration of cell morphology and loss of cell–cell contacts suggesting that ERBIN plays a role in the establishment and the maintenance of cell–cell adhesion [258,259].

7.3. Lumen morphogenesis

When MDCK cells are maintained in a low Ca^{2+} medium they do not form adhesion complexes [77], but accumulate apical proteins in an intracellular organelle called the "vacuolar apical compartment" (VAC [260,261]) and accumulate basolateral proteins in a similar vacuole (VBLC). At this stage, microtubules of MDCK cells radiate from the center of the cell towards the plasma membrane. Induction of E-cadherin cell–cell contacts by Ca^{2+} addition reorients the microtubule cytoskeleton that becomes parallel to the substrate and induces the fusion of VACs with the plasma membrane. In this way, neighboring cells form a transient intercellular lumina surrounded by apical membranes. Hepatocytes constitute a clear example of this process. Later on, lateral membrane enlargement seems to push the TJ away from the basal membrane, microtubules undergo further reorientation to a position perpendicular to the support, and cells acquire the columnar epithelial phenotype [260–264]. At initial steps after intercellular lumina formation, the newly synthesized apical proteins are sorted by transcytosis (i.e., they are initially delivered to the basolateral membrane and subsequently re-addressed to the intercellular lumina), which is reminiscent of the hepatocyte sorting system. After the reorientation of microtubules and the acquisition of the columnar phenotype, the protein delivery becomes addressed directly to the apical membrane. These processes are controlled by the ser/thr kinase PAR1b [263–265]. During the development of cysts in three-dimensional cultures, lumen formation is controlled by the complex annexin2-Cdc42-aPKC that is recruited to apical domains enriched in PI P_2 by the phosphatase PTEN [266].

Defects in the *Drosophila* TJs Sinous, megatraquea and nervana proteins, provoke decreased lumen formation [267]. In zebra fish, intestine develops from a rod of cells with no epithelial characteristics. These cells develop polarity and TJ, a step that requires aPKCλ [268] prior the formation of multiple small lumina. Later on, they fuse to develop a single and continuous intestinal lumen. This process involves the expression of the transcription factor tcf2, which in turn induces the expression of cln-15 and the Na^+,K^+-ATPase that transport ions and fluids to promote the coalescence of the small multiple lumen into a single one [269]. This mechanism resembles early stages of lumen development in *Drosophila* tracheal system, in which tracheal epithelial cells deposit a chitin matrix that forms a framework lining the primordial cavity. This framework expands the lumen and contributes to specify the length of the trachea to be finally eliminated at later stages [270].

7.4. A bacterium requests the door it needs to enter the cell

Pseudomona aeruginosa enters the cell through the basolateral side. Yet since it arrives via the lumen of the digestive tract, it only "sees" the apical side of intestinal cells. Interestingly, upon contact of this bacterium with the apical domain, the cytoskeleton undergoes a growth processes that transforms a patch of the apical membrane into a patch of basolateral one, by summoning the participation of phosphatidylinositol 3-kinase (PI3K), an enzyme that produces PIP_3, as if it were in the basolateral side [271]. After this local transformation of a microdomain of the apical pole into the patch of basolateral side, the microorganism has the gate it needs to gain access to the organism.

7.5. Leukocyte transmigration (endothelia)

As stated in Toolbox 4, the TJ should have a considerable plasticity, as transmigration of lymphocytes requires an intercellular gap of some 1–2 μm, which does not appear to involve proteolytic degradation of TJs or adherens junctions [272]. Neutrophil transmigration occurs chiefly at post-capillary venules which have relatively simple TJ [273] but also at other vessel types that have well-developed and continuous TJs; nevertheless, it occurs preferentially at tricellular corners were TJ are discontinuous [272,274,275]. However, leukocytes also can use the transcellular route for diapedesis, although this occurs at a much lower frequency than the paracellular migration [276,277].

About the molecular events that take place during leukocyte transmigration it has been shown that during polymorphonuclear (PMN) transmigration across human umbilical vein endothelial cell (HUVEC) monolayers, ZO-1, ZO-2, and occludin remain associated with cell borders and are not degraded [278]. In TNF-activated endothelial cells, transmigration induces the disappearance (but not degradation) of VE-cadherin, α- and β-catenin, and plakoglobin from endothelial adherens junctions [279,280]. Real-time observation of GFP-occludin during monocyte diapedesis through a brain endothelial cell line revealed that this process is associated with local disappearance of occludin and increased permeability of the monolayer [281].

The JAM family of proteins is key player in the regulation of leukocyte transmigration. These proteins also function in the regulation of polarity generation, as they tether the PAR–aPKC complex to nascent cell–cell contacts [127,128,157,282] which make them ideal mediators of the structural changes that must take place during leukocyte transmigration. Thus, an anti-JAM-A monoclonal antibody inhibits monocyte transmigration in both *in vitro* and *in vivo* models [226]. JAM-A acts as a ligand for the β_2-integrin lymphocyte function-associated antigen 1 (LFA-1) and contributes to the LFA-1-dependent transendothelial migration of T cells and neutrophils [283].

Likewise, an anti-JAM-C antibody, or a soluble JAM-C molecule, blocks transendothelial migration by interfering with the interaction between the leukocyte β2-integrin Mac-1 and endothelial JAM-C [284,285]. JAM-C mediates an increase in permeability by modulating actomyosin-based contractility and VE-cadherin-mediated adhesion through down-regulation of Rap1 activity shifting the equilibrium towards the disruption of VE-cadherin-mediated adhesion [286].

Another novel regulator of cell–cell adhesion and leukocyte transmigration is the prion protein (PrP^C). This protein is well known for its role in prion diseases which affect different mammals species including humans [287]. PrP^C is a GPI-anchored glycoprotein associated with raft microdomains and is the first molecule of this class described to have basolateral location in epithelial cells [288]. In endothelia of various species, it is expressed at cell–cell junctions and plays a role in the transmigration of monocytes through brain endothelial cells [289]. Lymphoid cells also express substantial levels of PrP^C [290] and is also present in enterocytes, concentrated beneath the TJs colocalizing with E-cadherin in the lateral membranes [291]. This distribution shed some light on the pathogenesis of the infective prion disease as it makes ideal for the transmission of the infective prion protein from the intestine and through the circulation to the central nervous system.

7.6. Leukocyte transmigration (epithelia)

Leukocyte transmigration in epithelia proceeds in the basal to apical direction. During acute inflammation it can reach levels that produce epithelial disruption [292]. Occludin is a regulator of the transmigration of neutrophils across epithelial sheets. The N-terminal cytosolic domain of occludin, which is not critical for the barrier function, is nevertheless important for transmigration because its modification increases efficiency of transmigration without detectably affecting the morphology of the TJ. The first extracellular loop of occludin also has a function in neutrophil transmigration [293].

JAM-C is abundantly expressed in intestinal epithelial cells and is a component of epithelial desmosomes. JAM-C plays a role in regulating PMN transepithelial migration through direct binding interactions with Mac-1 [294], a situation similar to the one observed in endothelia (see above). Another molecule, CD47, a member of the Ig superfamily, is expressed along the basolateral surface of colonic epithelia and may serve to regulate TJ opening during integrin-mediated transepithelial migration as its inhibition results in accumulation of PMN within the epithelium [295]. JAML, a protein with expression largely restricted to granulocytes, binds to the Coxsackie and adenovirus receptor (CAR), a TJ component, and this interaction regulates PMN migration across epithelia [296].

The surveillance that dendritic cells carry all over the mucosal surfaces involves the opening of TJs between epithelial cells and the direct sampling of microorganisms. These operations are carried out without impinging the integrity of the epithelial barrier. Interestingly, this involves the formation of TJ-like structures containing occludin and claudin-1 between dendritic cells and epithelial cells [297].

8. TJ and polarity during development

8.1. TJ formation in the mammalian blastocyst (mouse)

Embryonic compaction is the process of increased cell adhesion mediated by the E-cadherin/catenin system in 8- to 16-cell embryos. It requires the formation of junctional complexes and results in a polarized distribution of cell-surface and intracellular components [298]. The differentiation of the trophectoderm (the first epithelium to form during mammalian development) starts during the 8-cell stage and is completed by the 32-cell stage. It is at the 32-cell stage that the trophectoderm first engages in vectorial transport to generate the blastocoel. At this point, TJ establishment between trophectoderm cells is essential to avoid the leakage of the fluid accumulated inside the blastocoel and to maintain the polarized distribution of different transport systems (reviewed in [299,300]).

The formation of TJs during trophectoderm development involves a series of steps that begin with the assembly of JAM-A at the membrane in pre-compact 8-cell embryos, where it is found weakly localized to cell–cell contact sites and clearly in advance of activation of E-cadherin adhesion at compaction and cell polarization [301]. The ZO-1 staining at cell–cell contacts changes from focal (8 cells) to zonular (16–32 cells) over a period consistent with the assembly of TJs [302], and is regulated by the spliced isoform expressed ZO-1α+ [302,303]. ZO-1α− assembly occurs from the 8-cell stage onwards, whereas ZO-1α+ expression and assembly occur immediately prior to blastocoel fluid accumulation suggesting that ZO-1α+ may act as a final rate-limiting step in the synthesis of the tight junction [304]. Cingulin is initially localized to the cytocortex and its assembly at TJs occurs after that of ZO-1 (from 16-cell stage) [305]. Occludin is critical for the formation of functional TJs in embryos [306]. Occludin first assembles at apicolateral membrane contact sites in late morulae always in association with the ZO-1α+ isoforms and colocalizes with ZO-1α+ within perinuclear sites before coordinated incorporation of both proteins occurs into putative TJs [307].

During the early phase of apicolateral junctional complex (AJC) formation at compaction, ZO-1α− transiently colocalizes with α- and β-catenin in a single, permeable, junctional complex. Subsequently, after occludin assembly in the late morula, the AJC segregates into two distinct structural domains (apical, TJ; subjacent, AJ), coincident with the establishment of a permeability seal and the formation of the blastocoel. Also, the rab13 GTPase, involved in vesicular traffic control, is intimately associated with the maturing AJC from the onset of assembly of TJ proteins at the terminal phase of epithelial differentiation in precise colocalization with ZO-1α− [308]. In general, the TJ and desmosomal proteins assemble in the sequence ZO-2, ZO-1α− isoform, ZO-1α+ isoforms, occludin and desmoplakin [309].

Different PKC isoforms have been implicated in the establishment of polarity during compaction and TJ formation in early development which denotes its conserved role in polarity establishment. For example, membrane assembly of both ZO-2 and ZO-1α+ depends upon different PKC isoforms activities which are regulated by the contact pattern of the cell

membrane and thus dictate where and when TJ biogenesis occurs [309–312].

8.2. TJ formation in the mammalian blastocyst (human)

In the human morula, TJs are found between all cell pairs, at all levels of cellular apposition [313]. Claudin-1, two occludin isoforms, JAM and the ZO-1α− isoform are ubiquitously expressed throughout pre-implantation development, whereas expression of ZO-2, desmocollin-2 and ZO-1α+ isoform predominates at late stages of cleavage and associated to blastocyst formation [314].

8.3. TJ formation in the Xenopus embryo

Blastula formation and the development of the first epithelium in *Xenopus* are under the control of maternally encoded mRNAs and proteins, whereas mammalian blastocyst formation is regulated by the embryonic genome [300]. In *Xenopus* blastomeres the first TJs are generated during the late phase of the zygote division cycle and culminate in the formation of a diffusion barrier in the 2-cell embryo establishing the nascent blastocoel. TJ biogenesis is achieved by sequential membrane assembly of cingulin, ZO-1 and occludin. From the 2-cell stage, maternal cingulin assembles into the regions of cell–cell contact that corresponded to the future TJ area. Calcium-dependent cell–cell adhesion is not necessary to assemble cingulin-containing fences at the boundary of distinct membrane domains [315]. Occludin dephosphorylation is temporally coincident with the biogenesis of 'mature' TJs in *Xenopus* embryos suggesting that occludin dephosphorylation is an important step in the physiological assembly of mature TJs in a native epithelium [316]. About the site where TJ formation occurs, there have been contrasting results. Thus, one study [317] reported that TJs do not initially form at the interface between the apical and basolateral membranes, but rather assemble at variable locations up to hundreds of microns form the apical surface. Moreover, C-cadherin and the Na^+,K^+-ATPase localization along the lateral domain was found apical and basal to the TJs. On the other hand, other study [318] showed that nascent TJs are formed precisely at the border between old (apical) membrane derived from the oolemma and new (basolateral) membrane generated during cytokinesis. Both studies agree that the mechanism of TJ assembly involves intrinsic signals independent of any cellular interactions or adhesion, demonstrating that the prior establishment of cell polarity at cytokinesis defines the future site of the TJ which contrast with the phenomena observed in the mammalian embryo.

9. The special case of Na^+,K^+-ATPase polarization and its functional coordination with cell adhesion

Until Skou [319,320] described the Na^+,K^+-ATPase, and Koefoed-Johnson and Ussing [321] offered a plausible model for Na^+ transport across the frog skin, in which this enzyme was assumed to be placed polarized at the basolateral membrane (Fig. 4A), the electrical and transporting asymmetry of epithelia posed several intricate conundrums (reviewed in [7,322]). Yet, in spite of these works, the reasons for the polarized distribution of this enzyme proved to be very hard to unravel. Thus at variance with the H^+,K^+-ATPase that exhibits clear sorting signals [323,324], no such cues were detected in Na^+,K^+-ATPase.

The α-subunit is essential for enzymatic and pumping activities [325,326]. The role of the β- one is essential, as the enzyme cannot work without it, but was not an obvious one. Then several cues were discovered. First, it was found [327] that the chicken fence pattern of Na^+,K^+-ATPase in epithelia (Fig. 4B) can be split into two moieties by treating the monolayer with 2.0 mM EGTA, indicating that each green line is due to the contribution of enzymes from two neighboring cells (Fig. 4C). A second cue was that in mixed monolayers of MDCK and epithelial cells from a different animal species, MDCK cells express Na^+,K^+-ATPase in all homotypic MDCK/MDCK borders, but not in heterotypic ones (MDCK/other, Fig. 4D, E). A third cue was afforded by expressing dog β-subunit (i.e., the MDCK cells are of canine origin) into other cell types (e.g., CHO cells from Chinese hamster ovary), which prompts MDCK cells to express Na^+,K^+-ATPase in heterotypic borders (Fig. 4F left) [327]. A fourth cue was that α- and β-subunits are intimately bound together from the earliest moment of synthesis in the endoplasmic reticulum so the α- one would follow the fate of the β- one [328]. A fifth one was that β-subunit has all the characteristics of an attaching molecule (Fig. 4G) [329]. Actually, cells transfected with dog β-subunit do in fact become sticky [327]. On these bases the model depicted in Fig. 4H was proposed. It accounts for all major characteristics of Na^+,K^+-ATPase polarity, one of them being that this enzyme is not actually basolateral, but only lateral, i.e., it is expressed only at locations where enzymes in the neighboring cell can interact through their β-subunit.

In summary, TJ nature and Na^+,K^+-ATPase polarity, which are the two essential features of transporting epithelia, offer a plausible model for vectorial net transport of ions (Fig. 4I) [322]. Given that net transport of glucose, amino acids, and several ions (notably H^+, K^+, Ca^{2+}, Cl^-) are often operated by the driving force generated by the Na^+ transport elicited by the Na^+,K^+-ATPase, this model accounts in principle for the net transport of most biologically valuable substances.

As mentioned above, the α- and β-subunits of Na^+,K^+-ATPase are firmly bound together in vertebrate cells [7,330]. Furthermore, while the enzyme is located at the lateral borders, the TJ is found at the outermost end of the intercellular space where it forces any fluid pumped into the intercellular space to move inwards. Yet in organisms such as *Drosophila* the enzyme is not only a component of septate junctions (SJs), perhaps an ancestor of TJs, but also is essential for the correct formation of SJs [331–333]. Thus, fly null mutants of both the α- and β-subunits show disruption of septate junction function and mislocalization of several SJ components [331,332]. This junctional activity of the Na^+,K^+-ATPase is mediated by the A, B and C α-subunit isoforms and by the extracellular domain of Nrv2 (β-subunit), but surprisingly the ion pump activity seems to be dispensable [334]. This contrast with the traditional view of the ATPase, as an ion pump required for ion homeostasis. A model for the role of the ATPase in SJ assembly, consistent

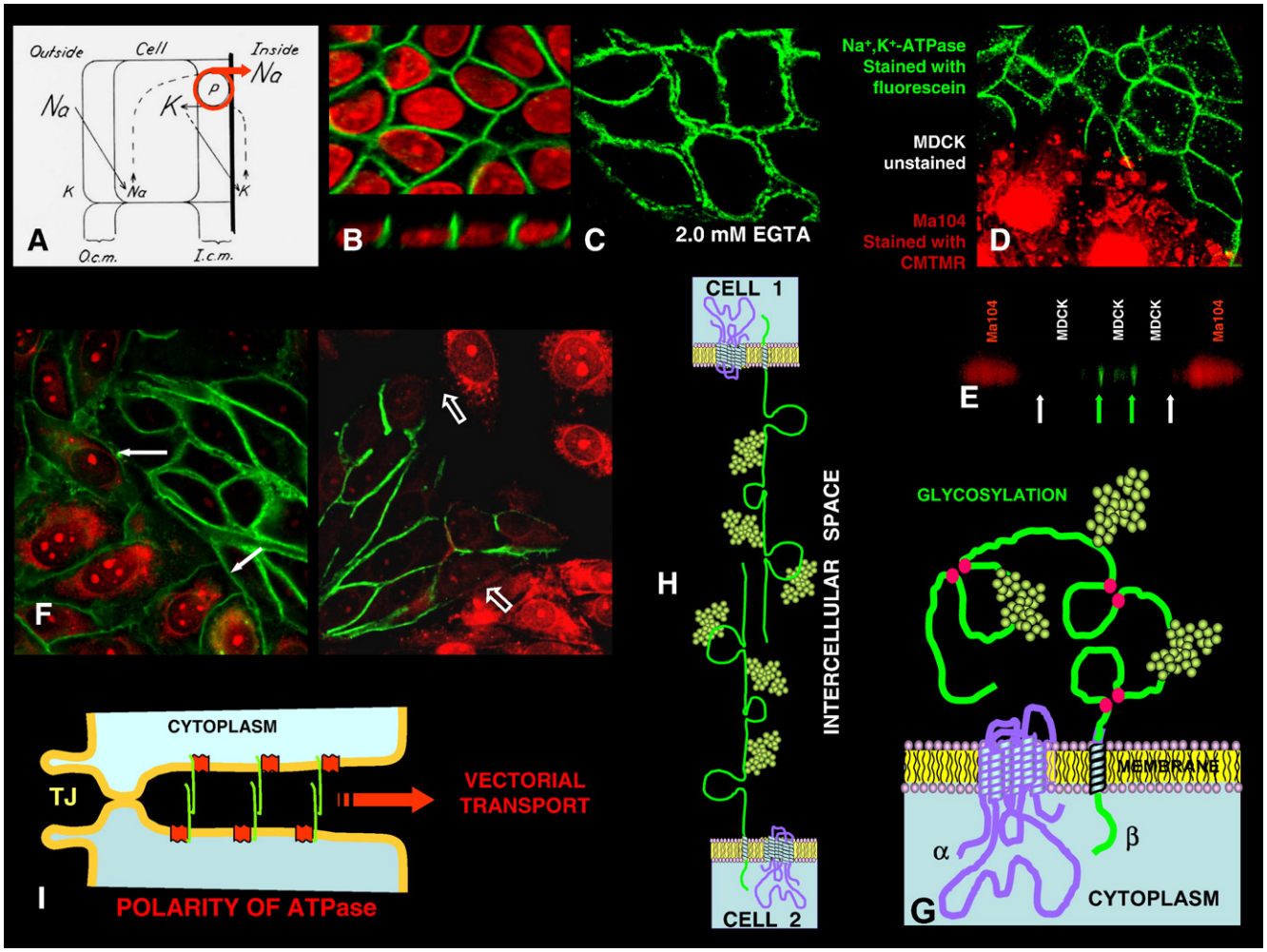

Fig. 4. The peculiar polarity of Na^+,K^+-ATPase. (A) The model of Koefoed-Johnson and Ussing which illuminated half a century of epithelial transport studies, assumed that the enzyme (P, red) is expressed on the inner barrier. (B) Confocal micrograph of a monolayer of MDCK cells showing that the enzyme (green) is not actually basolateral, just lateral, a situation particularly clear in the transversal optical section. (C) Treatment with EGTA splits the image of lines, indicating that each line represents the contribution of enzyme from each neighboring cell. (D) MDCK cells (unstained) co-cultured with Ma104 cells that have been previously stained with CMTMR (red). Na^+,K^+-ATPase (green) is expressed by MDCK cells only if the neighbor is another MDCK cell. Of course, Ma104 cells have their own Na^+, K^+-ATPase, yet they are not labeled by the antibody used, so the chicken fence image of Na^+,K^+-ATPase in Ma104 cells is not observed. Similar results can be observed by co-culturing MDCK cells with other cell types, e.g., CHO cells. (E) A mixed monolayer as viewed in transverse optical section, showing the sequence Ma104/MDCK/MDCK/MDCK/Ma104. Na^+,K^+-ATPase (green arrows) is only observed in homotypic contacts. Notice that first and third MDCK cells express the enzyme in contacts with the MDCK at the center, but not in their borders with Ma104 cells (white arrows). (F) (left) CHO cells transfected with dog β-subunit and co-cultivated with MDCK ones. Notice that now all cells express Na^+,K^+-ATPase in their borders; (right) MDCK cells co-cultured with mock-transfected CHO cells. (G) Na^+,K^+-ATPase (see text). (H) Experimental model [327] to interpret the polarity of Na^+,K^+-ATPase. Cells 1 and 2 are two epithelial cells that contact each other through their β-subunits, thereby anchoring themselves to the plasma membranes facing the intercellular space. (I) The combination of a TJ expressed at the outermost end of the intercellular space, and Na^+,K^+-ATPase expressed in the plasma membrane in contact with the intercellular space, combine to develop a phenotype appropriated to produce vectorial transport of Na^+ (after [7,322,327]).

with the combined results of the α- and β-subunit involvement in junction regulation, is that the extracellular domain of the β-subunit may interact with multiple extracellular SJ components to assemble an extracellular complex, whereas the α-subunit interacts with cytosolic proteins or intracellular portions of transmembrane proteins to promote junction formation, paracellular barrier formation and tracheal tube-size control [332,334]. An example of a protein that could interact with the α-subunit to organize junctions is the cytoskeletal protein Ankyrin, which has been shown to bind to two distinct sites on the rat α-subunit [335,336], sites which are conserved in the *Drosophila* ATPα [334].

The observations described above supports the hypothesis that the Na^+,K^+-ATPase has an evolutionary conserved role in the formation and maintenance of adhesion complexes [7]. This is further sustained by the fact that rat α1 isoform is able to rescue all junctional defects of *Drosophila* Atpα-null mutants and by the function of the Na^+,K^+-ATPase in the regulation of cell adhesion in other organisms. For example, in zebrafish Na^+, K^+-ATPase plays crucial roles in heart and retinal pigmented epithelium morphogenesis in a program that involves the participation of polarity proteins such as ZO-1, aPKC and Pals [268,337–339]. An intriguing hypothesis was posed by the prediction that in *C. elegans* the α- and β-subunits are not

bound together [340], which opens the possibility that in this organism the activities of pumping and adhesion of the subunits might be separated.

However, throughout evolution, the separation of the ATPase and the TJ was anatomical, but not necessarily functional, as the enzyme has a powerful effect on the TJ as well as other junctions. For example, the Na^+,K^+-ATPase plays a crucial role during TJ formation in the trophectoderm [341–344]. Furthermore, there is a mechanisms that regulates the adhesive state of the cell by signaling through the Na^+,K^+-ATPase. The so called P→A mechanism (from ***p***ump and ***a***ttachment), is triggered by ouabain binding to the Na^+,K^+-ATPase, which activates a tyrosine kinase (probably c-Src) which in turn trans-activates the EGFR leading to activation of the extracellular regulated kinases (ERK 1/2), which mediate the decrease of the small GTP-binding protein RhoA [345,346]. The activation of this pathway changes the degree of phosphorylation of adhesion associated molecules and induces its retrieval from the cell membrane (reviewed in [330]).

Furthermore, in the last years, ouabain (used for a couple of centuries as a toxic drug of vegetal origin), has turned to be a hormone whose physiological role remains to be elucidated [330,347,348]. Yet, it has been recently shown that ouabain affects cell–cell communication [349]. Using neurobiotin as an intracellular probe, these authors demonstrated that in monolayers of MDCK cells a given cell is connected through gap junctions to 3 neighbors (out of 6), a figure that increases to 5 neighbors when challenged with ouabain. This effect, due to a specific enhancement of conexin 32, plays a paramount role in the rescue of ouabain-sensitive cells by ouabain-resistant ones.

10. Concluding remarks

Metazoans depend on a multitude of highly specific fluxes of substances between a number of compartments, such as blood, lymph, cerebro-spinal fluid, as well as the different fluids contained in the eye, salivary glands, gallbladder, intestine, glomerular filtrate, etc. The cells operating these exchanges across epithelia and endothelia share a basic phenotype constituted by TJs and polarity. Both of them reflect the ability of their cells to endow a given region of their membrane with specific molecular species, a fundamental property already developed by unicellular organisms. It depends on the work of ancestral molecular complexes that recognize, retain, assemble or deliver molecules, and integrate a sort of intercommunicated network intimately linked to the cytoskeleton. As the overall pattern of these mechanisms is unraveled, we find a variety of diseases due to their malfunction, and conceive pharmacological tools to alleviate them.

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