



## Review

# Epithelial cell–cell junctions and plasma membrane domains

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## ABSTRACT

Epithelial cells form a barrier against the environment, but are also required for the regulated exchange of molecules between an organism and its surroundings. Epithelial cells are characterised by a remarkable polarization of their plasma membrane, evidenced by the appearance of structurally, compositionally, and functionally distinct surface domains. Here we consider the (in)dependence of epithelial cell polarisation and the function of smaller plasma membrane domains (e.g. adherens junctions, gap junctions, tight junctions, apical lipid rafts, caveolae, and clathrin-coated pits) in the development and maintenance of cell surface polarity. Recent evidence of cross-talk and/or overlap between the different cell–cell junction components and alternate functions of junction components, including gene expression regulation, are discussed in the context of cell surface polarity.

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## 1. Introduction

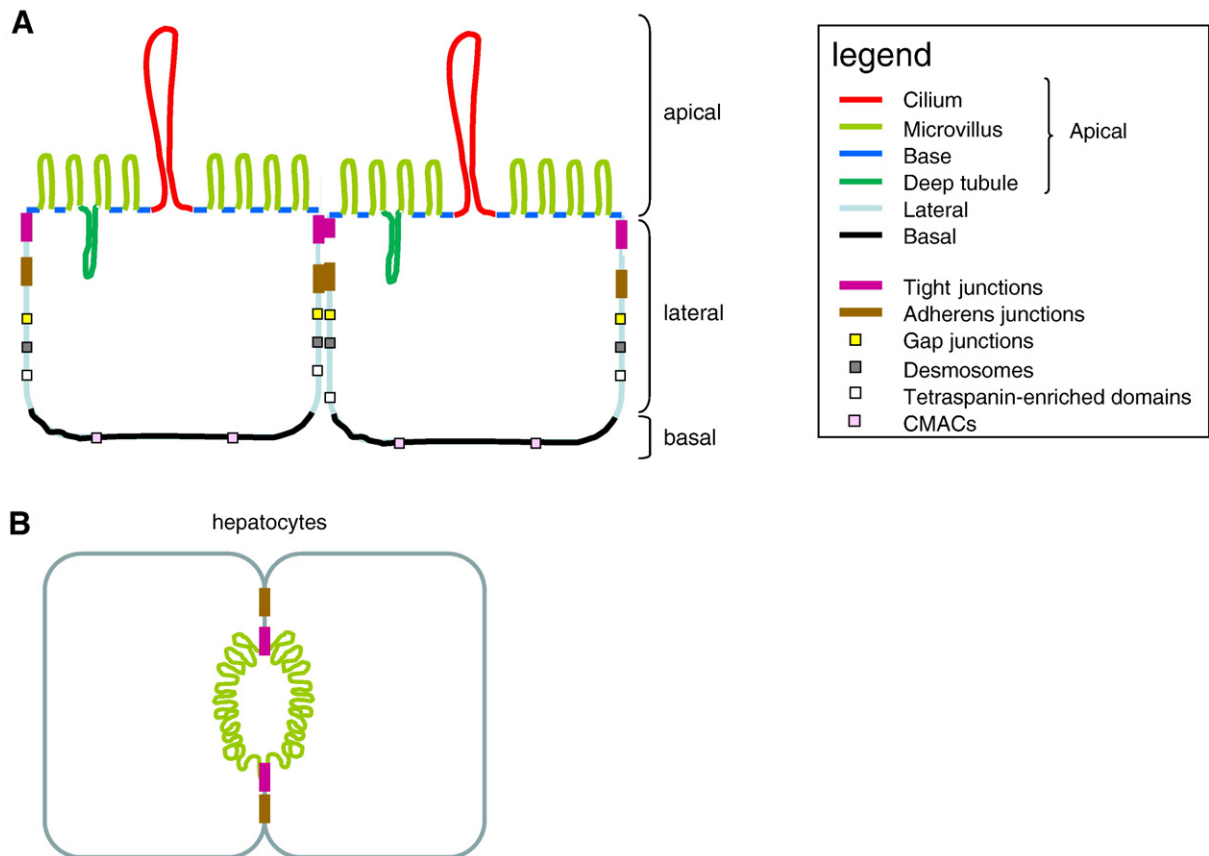
The ability of individual cells to differentiate their plasma membrane to form specialised domains with distinct protein and lipid compositions is crucial for many cell biological processes. These include cell adhesion, signalling, directed migration, asymmetric cell division and cell fate determination, the development of neuronal networks, and the development of functional epithelial and endothelial barriers [1,2]. Distinct mechanisms have been implicated to contribute to plasma membrane differentiation: (i) Lipids and non-integral membrane proteins can be exclusively or predominantly synthesised or metabolised at defined areas of the cell surface. (ii) Proteins and lipids can be trafficked exclusively or predominantly from intracellular sites such as the Golgi apparatus and/or endosomes to defined areas of the cell surface. (iii) Proteins and lipids can be retained at – or repelled from – defined areas of the cell surface. These mechanisms likely act in concert, allowing cells to establish and maintain plasma membrane domains with steep compositional and functional boundaries.

While most cell types establish cell surface domains transiently, others, including neurons [3], oligodendrocytes [4], and epithelial cells [5] maintain a more permanent segregation of plasma membrane proteins and lipids. Here, we consider two types of epithelial plasma

membrane domains: large multi-micrometer-scale domains (e.g. apical, basal/lateral domains; section 2) and, within these, smaller submicrometer-scale domains. In this article we discuss the functional interrelationship between these diverse plasma membrane (micro) domains and their formation, and address emerging cross-talk between junctional microdomains.

## 2. Epithelial plasma membrane differentiation

Epithelial cells form semi-permeable sheets that line most body cavities and separate the organism from the outside environment. The subcellular localisation of proteins has been experimentally visualised by light microscopy and electron microscopy, and steep structural and compositional gradients of plasma membrane proteins in epithelial cells have been demonstrated. These gradients give rise to several epithelial surface domains (Fig. 1A), discerning (a) a basal surface domain facing the extracellular matrix and underlying tissue or, in case of hepatocytes, the sinusoidal blood, (b) a lateral surface domain facing adjoining cells, and (c) an apical surface domain with numerous finger-like projections called microvilli facing the epithelial lumen or outside environment. The apical surface can be further subdivided into two compositionally and biophysically distinct domains (I) the apical base membrane and (II) apical microvilli membranes [6,7]. In



**Fig. 1.** A. Cartoon depicting the different plasma membrane domains in epithelial cells. B. Cartoon showing the distinct polarised geometry displayed by hepatocytes.

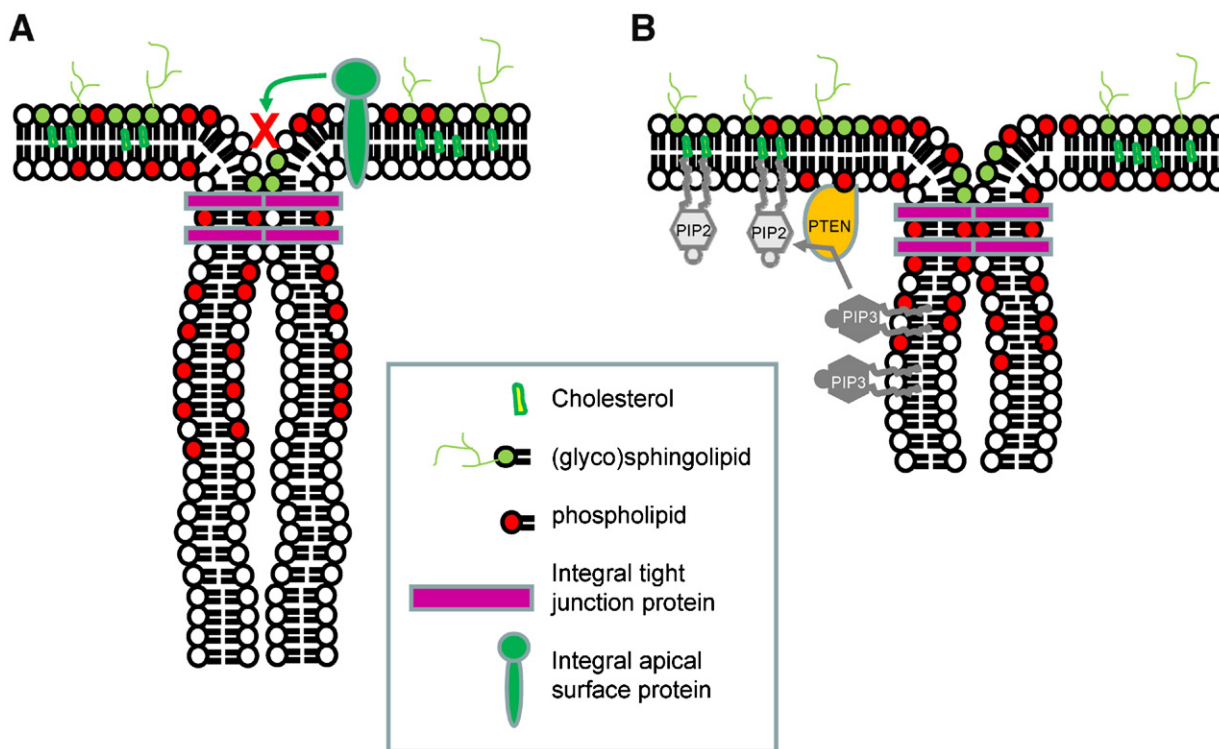
addition, the apical surface of most epithelial cells contains a primary cilium, which is structurally, compositionally, and functionally distinct from the surrounding apical membrane. Experiments with Laurdan staining (Laurdan is a dye that intercalates between lipids and its emission spectrum is affected by membrane fluidity) suggested that the ciliary membrane has a highly condensed bilayer domain at its base that could function as a fence to separate the ciliary membrane from the surrounding apical membrane [8]. At least in intestinal epithelial cells, the apical surface also consists of up to 1  $\mu\text{m}$ -deep tubular membrane invaginations between adjacent microvilli. This might be the only part of the apical surface sterically accessible for intracellular membrane fusion/budding events [9,10] (Fig. 1A).

The position of the apical and basal/lateral surface domains relative to each other is not strict. This is exemplified by hepatocytes that form apical plasma membrane domains at the lateral cell surface between two neighboring cells [11] (Fig. 1B), a feature that is not typically found in other epithelial cells and controlled by polarity protein (Par-1) kinase activity [12]. Intercellular adhesions [13,14] and the adhesion of cells to the extracellular matrix [15] also determine apical plasma membrane domain positioning.

The earliest level of polarised membrane organisation is already prominent in single cells [16]. Two apical plasma membrane proteins, the highly negative charged and anti-adhesive transmembrane sialomucin podocalyxin (a.k.a. gp135) and the sodium-proton exchanger regulatory factor (NHERF) are segregated exclusively to the free cell surface immediately following attachment of a single epithelial cell to the substratum. Thus a defined localisation appears prior to formation of lateral surface domains and cell–cell junctions. Depletion of podocalyxin from the cells by RNAi leads to defects in cell surface polarisation, evidenced by the inability of the cells to segregate the  $\text{Na}^+/\text{K}^+$ -ATPase beta-subunit and the carcinoembryonic antigen-related cell adhesion molecule (sialoglycoprotein gp114), which are basolateral and apical plasma membrane marker proteins, respectively [16]. Podocalyxin induces the formation of

apical surface microvilli, and controls epithelial morphology [17]. It has been proposed that the early polarised distribution of podocalyxin–NHERF forms a preapical domain during polarisation that functions as an apical scaffold to recruit other molecules and macromolecular complexes such as the cytoskeleton that help further shape and stabilise the apical surface domain [16]. Whether the (pre) apical domains also help shape the lateral and basal surface domains is not understood. In favor of this, depletion of apical transport proteins perturbed epithelial cyst formation, indicating the important role of apical membrane biogenesis in epithelial organization [18]. Moreover, overexpression of podocalyxin in polarised epithelial cells stimulates growth of the microvilli-lined apical surface domain with a concomitant loss of lateral surface domains and cell–cell junctions [17]. Apical microvilli formation and membrane growth thus influences epithelial morphology and lateral surface domain dynamics, which might be partly explained by the anti-adhesive properties of the podocalyxin protein. However, its associating partner NHERF can bind to cell–cell junction-associated  $\beta$ -catenin and the protein and lipid phosphatase PTEN (for phosphatase and tensin homologue deleted on chromosome ten). PTEN activity contributes to membrane asymmetry in the inner leaflet of the plasma membrane (discussed below).

The polarised distribution of lipids is equally important as that of proteins, as first demonstrated for sphingolipids. In comparison to the combined basal and lateral membranes, the apical membranes of epithelial cells generally display a significant enrichment in glycosphingolipids and sphingomyelin at the expense of phosphatidylcholine ([19], and references therein; Fig. 2A). Sphingolipids display a unique distribution pattern in the apical membrane. For instance, two different complex glycosphingolipids, the gangliosides GM1 and GM3, localise preferentially to the microvilli membranes and apical base membrane, respectively [20]. By clustering, (glyco)sphingolipids and cholesterol can change the biophysical properties of the immediate membrane environment, which is thought to give rise to (plasma)



**Fig. 2.** A. Cartoon showing a plasma membrane lipid bilayer with tight junctions. An apical protein with significant extracellular domain cannot pass the tight junctions. B. PTEN generates polarised distribution of phosphoinositides in the inner plasma membrane leaflet. Sphingolipids (green) are enriched in the apical domain, whereas phospholipids (red) are not.

membrane domains or rafts that attract specific molecules. Subsequent oligomerisation of raft proteins may stabilise and promote larger raft domains [21]. Raft clustering has been proposed to be crucial in the biogenesis of apical membrane domains during development [22]. However, not the entire apical surface domain displays raft-like properties and at least two different lipid bilayer phases in the apical plasma membrane of epithelial cells coexist [16]. Particularly membranes of the microvilli and deep apical invaginations are enriched in sphingolipid–cholesterol raft domains [7,9].

Sphingolipids typically reside in the exoplasmic outer leaflet of the plasma membrane, which is in agreement with their prior sorting in organelles such as the Golgi complex and endosomes [23]. Phospholipids such as phosphatidylethanolamine that also reside in the cytoplasmic inner leaflet of the plasma membrane do not display enrichment at either the apical or basolateral domains (Fig. 2A). Studies in which liposomes carrying fluorescent (non-raft) lipid probes were fused to the apical plasma membrane domains of polarized epithelial cells, demonstrated that only fluorescent lipids that were inserted into the outer leaflet of the plasma membrane bilayer are restricted from diffusing out of the apical plasma membrane domain. In contrast, lipids inserted into the inner leaflet can freely diffuse between apical, lateral, and basal domains [19]. These results indicate that some physical intramembrane diffusion barrier or fence segregates apical from lateral and basal plasma membrane lipids in the outer leaflet of the plasma membrane.

Lipids in the inner leaflet of the plasma membrane also display a polarised distribution between apical and basal–lateral surface domains. This is well illustrated by the phosphoinositides, highly bioactive phospholipid components in the cytosolic side of plasma membranes. Mostov and coworkers demonstrated that phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub> or PIP<sub>3</sub>) is stably localised at the lateral and basal surface domains of epithelial cells, but excluded from the apical cell surface domain [24]. In contrast, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub> or PIP<sub>2</sub>) localises exclusively at the apical plasma membrane domain of epithelial cells [25] (Fig. 2B). PIP<sub>3</sub> can be generated by phosphatidylinositol 3-kinase, via phosphorylation of PIP<sub>2</sub>. PIP<sub>2</sub> can be formed by PTEN, via dephosphorylation of PIP<sub>3</sub>.

PTEN was found to be recruited to the apical surface as soon as epithelial cells start to develop apical polarity [25]. Possibly, this early recruitment of PTEN is mediated by the podocalyxin/NHERF scaffold (see above). PTEN activity can be regulated by sphingolipids [26]. This might lead to the cooperation of sphingolipids and phosphoinositides at the early apical domain. Downregulation of PTEN in epithelial cells by RNAi resulted in a homogenous distribution of PIP<sub>3</sub> and PIP<sub>2</sub> in the plasma membrane. This suggests that PTEN is required for the enrichment of PIP<sub>2</sub> and the concomitant removal of PIP<sub>3</sub> from the apical plasma membrane domain [25]. While other phospholipids can diffuse freely between apical and basolateral domains (see above), segregation of PIP<sub>2</sub> and PIP<sub>3</sub> to the apical plasma membrane domain versus the basolateral membrane appears to be controlled locally by synthesis and metabolism (Fig. 2B).

Polarised segregation of PIP<sub>2</sub> and PIP<sub>3</sub> by PTEN seems to be critical for the further development of cell surface polarity. The addition of PIP<sub>3</sub> to the apical surface of epithelial cells resulted in an apical-to-basolateral transformation of the plasma membrane, and inhibition of phosphatidylinositol 3-kinase, responsible for the production of PIP<sub>3</sub>, resulted in abnormally short lateral surface domains [24]. PIP<sub>3</sub> therefore appears to regulate the formation of the basal and/or lateral plasma membrane in epithelial cells. Conversely, the ectopic insertion of PIP<sub>2</sub> into the basal plasma membrane domain resulted in the relocalisation of apical proteins to the basal and lateral surfaces, suggesting that PIP<sub>2</sub> regulates apical plasma membrane formation [25]. Apically localised PIP<sub>2</sub> recruits annexin-2 which can cluster PIP<sub>2</sub>; the GTPase Cdc42 which organises the apical cortical actin cytoskeleton and may promote apical-directed exocytosis; and the polarity

proteins atypical protein kinase C (aPKC) and Par6, all to generate the apical plasma membrane domain [25]. Par proteins have typically been found to localise to cell–cell adhesion junctions (see [27,28] for recent reviews on Par proteins and cell polarity). Mostov et al. [25] proposed that distinct Par protein complexes exist for the establishment of epithelial polarity: a complex at the apical surface and involved in the formation of this domain; and a complex at the tight junctions and required for their formation. Although Par protein complexes have typically been found to associate with tight junctions and adherens junctions, significant portions of these, which may not necessarily form obligate complexes, also localise to the apical surface and other cellular locations (e.g. [29–32]). Par protein complexes might act as molecular organisers to connect the acquisition of apical–basal polarity with the positioning and formation of junctional structures [30].

### 3. Cell junctions, other plasma membrane microdomains, and their constituents

The segregation of apical from basal plasma membrane constituents is widely believed to be mediated and controlled by lateral cell–cell adhesion junctions, which are therefore often considered to be the primary epithelial polarity landmark [5]. By definition, cell–cell junctions are part of the lateral surface domain. Because of their relative stability and complex multi-component nature, cell–cell junctions can be considered membrane microdomains themselves. Within the lateral surface domain, strand- and/or belt-forming cell–cell junctions such as adherens junctions and tight junctions (see sections 3.1. and 3.2.) display a fixed localisation relative to each other in a given organism (e.g. vertebrates, insects), while spot-like cell–cell junctions appear more randomly distributed. The different cell–cell junctions can be distinguished by their molecular composition (but see below) and by their structural morphology at the electron microscopic level. All cell–cell junctions contain transmembrane proteins that in a homo- or heterotypic manner can bind to their counterparts on adjacent cells, and associate with cytoplasmic proteins and the cytoskeleton via which cell–cell junctions relay signals to the cell interior and vice versa. The size of most plasma membrane junctions discussed here are below or just at the resolution of light microscopy. These junctions typically have been discovered by electron microscopic studies decades before the broad introduction of molecular biology and fluorescent microscopy as general lab tools. Although the discovery of junction-associated molecules is leveling off, new constituents are still being discovered. In recent years the constituents of different junctions are also found acting at other subcellular sites than their major microdomains and dynamic cross-talk between the different junctions and molecules is an emerging phenomenon.

The major molecular constituents that have been identified in traditional cell–cell junction and other microdomains are outlined in the next section. For a complete and in depth discussion including transient interactions at microdomains, for instance with signaling molecules, the reader is referred to excellent recent reviews discussing the separate topics in (molecular) detail.

#### 3.1. Tight junctions

Tight junctions appear as a branching network of sealing strands that provide a physical intercellular barrier that restricts paracellular transport. Tight junctions in mammalian cells are typically observed precisely at the boundary between the apical and lateral surface domains, while adherens junctions and other cell–cell adhesion junctions are typically oriented more to the basal side. However, in insect epithelial cells, adherens junctions are at the apical apex and septate junctions (the insect equivalent of tight junctions) localise more to the basal domains.



The essential transmembrane proteins of the tight junctions are claudins (at least 23 family members are present in humans). The integral membrane proteins occludin and junction adhesion molecules (JAMs) are also present at high levels in tight junctions. At the cytoplasmic face the ZO-1, ZO-2 and ZO-3 (zona occludens) proteins are highly enriched (an alternative name for tight junctions is “zona occludens”, hence their name). The ZO-proteins might play a role in junction formation [33] as well as establishing a connection to the actin cytoskeleton, via alpha-actinin [34]. The cytoplasmic side of tight junctions recently has been suggested to act as an important microdomain that regulates gene transcription, with the scaffold ZO-1 protein playing a pivotal role (several studies of Balda and Matter and coworkers; see below). Recent reviews discuss in detail the transmembrane proteins [35]; interacting partners [36], interacting networks [37] and signaling networks [38] of tight junctions.

### 3.2. Adherens junctions

Adherens junctions are cell–cell microdomains that provide adherent strength. In mammalian cells adherens junctions localise to the basal side of tight junctions whereas in insect cells adherens junctions are at the apical most apex.

The integral membrane proteins are of the cadherin family, with E-Cadherin being most abundant in epithelia. Homotypic calcium-dependent interactions between cadherins in the extracellular space lead to strong cell–cell interactions. Cytoplasmic interacting proteins include alpha-catenin, beta-catenin, adenomatous polyposis coli (APC) and plakoglobin. Moreover, ZO-proteins have been found at adherens junctions. One function of the ZO-proteins and catenins in adherens junctions is linkage of the junctions to the actin and microtubule cytoskeleton [39,40]. The possibility of “Outside-in signaling”, well established for integrins, is now emerging concept for the cadherin-based adherens junctions [41–43]; see below).

### 3.3. Gap junctions

Gap junctions are unique cell-to-cell channels that allow diffusion of small metabolites, second messengers, ions and other molecules (< 1 kDa) between neighboring cells. Gap junctional communication is essential for electrical transduction, signaling and nutrition. Gap junction channels can be open or closed, a highly dynamic process regulated at multiple levels [44].

The integral membrane proteins forming these channels in vertebrates are the connexins of which over 20 family members have now been identified in humans, with connexin43 the most abundantly expressed connexin. While gap junctions are among the first identified microdomains by their typical electron microscopic appearance, cytoplasmic interactions at this traditional cell–cell junction only started to be uncovered a decade ago. Gap junction partners include ZO-1, acting as a scaffold to recruit signaling proteins [45], but also implicated in regulating gap junction size [46]. Moreover, like for other cell–cell junctions, cytoskeletal interactions with microtubules and indirectly with actin have been reported at gap junctions [47–49].

### 3.4. Desmosomes

Desmosomes help to resist shearing forces and mutations in desmosomal proteins give rise to skin and blistering diseases [50]. Desmosomes are adherent points that form a continuum of cells within tissues by linkage of their integral membrane proteins (desmocollin and desmoglein) via desmoplakins (plakophilin and plakoglobin) to intermediate filaments (reviewed in [51]). Desmosomes are crucial for tissue integrity by their very strong adherence, that resist calcium-depletion in developed tissue, but can be regulated by protein kinase C when dynamic remodelling of cell–cell adhesion is

required [51]. As for adherens junctions (see below) desmosomes have been implicated in regulation of Wnt signaling, but this is still controversial.

### 3.5. Cell–matrix adhesion complexes

Controlled interaction between the cells and the extracellular matrix (ECM) is essential for many processes, including normal development, migration and proliferation (reviewed in [52,53]). Integrin-mediated adhesion to the extracellular matrix are among the first adhesion junctions where bidirectional signaling occurs, i.e. from the cell to the ECM, so-called inside-out signaling; as well as in the opposite direction, so-called outside-in signaling. The integrin based cell–matrix adhesions are spot-like structures that are dynamically regulated to ensure communication between the cells interior and the ECM important for proper propagation of a variety of processes.

The transmembrane units are formed by heterodimers of the alpha- and beta-integrins (24 distinct heterodimers have been identified to date) that have a large extracellular domain a single transmembrane domain and relative short cytoplasmic tails. At the extracellular side integrins bind directly to the extracellular matrix (collagen, fibronectin, laminins etc.). Cytoplasmic partners include talins, paxillin, focal adhesion kinase and linkage to alpha-actinin and actin-stress fibers. These “focal adhesion complexes” control a variety of signaling pathways regulated by the interplay with the extracellular partners. Substantial cross-talk between the diverse cell–cell and cell–extracellular matrix junctions has been found, and the architecture of the epithelial monolayer is highly regulated by their concerted actions.

### 3.6. Tetraspanin-enriched microdomains

Tetraspanins (33 family members identified) gather a variety of transmembrane and cytoplasmic signaling proteins in “tetraspanin-enriched microdomains” (TEMs) [54–56]. TEMs form a complex network and have been functionally implicated in diverse signaling events, including those regulating apoptosis, proliferation and cytoskeletal organization, metastasis, viral entry and viral budding [57–61]. Tetraspanins have four transmembrane domains with their termini in the cytoplasm and typically have a larger 2nd extracellular loop than claudins and connexins. Although the latter proteins also contain 4 transmembrane domains, they do not belong to the “tetraspanin-family”. Like connexins and claudins, tetraspanins have conserved cysteine residues, and in addition also contain glycosylation sites. Although TEMs have a distinct molecular composition, they are related to lipid rafts. While TEMs are not classical cell–cell adhesion junctions, TEMs as mentioned here because cell–cell adhesion molecules such as claudins and EpCAM can associate with them.

### 3.7. Caveolae and lipid rafts

Increasing sophistication of imaging techniques to study cell surface compartmentalization has provided evidence for small, possibly relatively short-lived microdomains that may be less than 50nm in diameter. These include caveolae, lipid rafts, and clathrin-coated pits.

Caveolae are signaling microdomains named after the characteristic presence of its structural protein caveolin. Caveolae are 50–100nm large flask-shaped membrane microdomains that exist in most cell types. They are important for uptake of extracellular and integral membrane molecules via clathrin-independent endocytosis and transcytosis. Caveolae contain high concentrations of sphingolipids and cholesterol and a variety of signaling proteins, including lipid-modified extracellular proteins (GPI-anchored) and lipid-anchored

cytoplasmic proteins [62]. Lipid rafts share the cholesterol-dependent detergent insolubility of caveolar membranes but do not contain caveolin.

### 3.8. Clathrin-coated pits

Clathrin-dependent internalisation is the classical form of extracellular uptake, which has been discovered decades before clathrin-independent internalization via lipid rafts and caveolae. Clathrin is recruited to the plasma membrane and forms so-called “clathrin-coated pits”. In addition, evidence suggests that clathrin-coated pits and other classes of endocytic vesicles might assemble from pre-designated plasma membrane domains, interconnected and positioned by an actin cytoskeletal network, that provide a platform for the rapid production of multiple vesicles [63]. The assembly of clathrin results in formation of “triskelions”, typical structures important in promoting membrane curvature. The pinching of vesicles, resulting in internalization in the cytoplasm, is mediated by the protein dynamin. Numerous other proteins contribute to the initiation of endocytosis, early endosome formation and lysosomal targeting or receptor recycling, a very dynamic process with high turnover, reviewed in detail in [64]. While clathrin-coated pits are not classical signaling domains, they in fact are major regulatory sites for receptor-mediated signalling. Clathrin-dependent endocytosis is important for down-regulation of signaling via transmembrane receptors, attenuation of signaling or increasing activity during internalisation.

## 4. Cell–cell junctions and other plasma membrane microdomains in the segregation of apical and basal/lateral plasma membrane components

The maintenance of cell surface polarity is a complex and dynamic process, which is influenced by regulatory signals, including those from extracellular growth factors, cytokines, nutrients and cell–cell junctions. Much progress has been made in understanding the molecular interactions of the core proteins at classical cell–cell junctions, some of which aid in the well-established function of these junctions including intercellular communication (gap junctions), barrier formation (tight junctions) and cell–cell connections (desmosomes and adherens junctions). However, evidence now accumulates that typical cell–cell junction proteins might have alternate functions at other subcellular sites.

### 4.1. Cell–cell junctions

In this section we discuss the involvement of cell–cell adhesion junctions in the segregation of apical and basal/lateral cell surface components. We consider such involvement by means of their (proposed) ability to form intramembraneous fences, to separate distinct extracellular environments, and to regulate transcription and proliferation.

#### 4.1.1. Intramembraneous fences

In the late 80s, tight junctions were proposed to provide the intramembraneous lipid and protein fence between apical and basolateral cell surface domains. Indeed, because there is hardly intercellular space at tight junctions, integral membrane proteins with extended extracellular domains would have difficulty passing from basolateral to apical surface domains and vice versa [65]. A role for tight junctions as a fence to restrict lipid diffusion was in agreement with the at that time more popular lipid-based model of tight junction structure, in which tight junctions were viewed as hexagonal lipid rods that inherently would prohibit mixing of lipids between the outer leaflets of apical and basolateral plasma membranes. When this lipid-based model was replaced by the current transmembrane protein-based data, the idea of tight junctions as intramembraneous fences was

upheld. This idea was mainly based on experiments in which the depletion of calcium from the culture medium resulted in a loss of functional cell–cell adhesions and a concomitant diffusion of apically applied lipids probes to the basolateral plasma membrane. In other studies, plasma membrane proteins were observed to redistribute between the apical and basolateral membranes following removal of extracellular calcium, which was attributed to a loss of tight junction fence function. Here we discuss the current view of the identity of the fence. Can loss of apical plasma membrane polarity following the depletion of extracellular calcium be attributed to the disassembly of tight junctions? Although loss of extracellular calcium ions impairs tight junctions [66], this effect may not be direct and predominantly mediated through adherens junctions [67]. However, the discovery of the cell polarity-controlling PAR gene products [68] and the association of these with tight junctions [69,70] fueled the relationship between tight junctions and cell surface polarity development (see below).

Doubts were raised as to the requirement of tight junctions for epithelial cell surface polarity by various labs. Rodriguez-Boulant reported polarised viral budding from the plasma membrane before tight junctions were established [71]. Detailed electron microscopy studies of the development of the rat pancreas suggested that the diffusion of apical plasma membrane glycoconjugates is restricted prior to the formation of complete tight junctions [72]. Furthermore, Fleming and colleagues reported that in the developing mouse embryo plasma membrane polarity can be uncoupled from the presence of tight junctions [73]. In *Xenopus* blastomeres the formation of tight junctions was suggested to be mediated by intrinsic processes manifested by the polarised phenotype established during cytokinesis [74]. In these studies, tight junction formation was thus observed to follow rather than initiate cell surface polarity. Clearly, unambiguous evidence for the function of tight junctions function as an intramembraneous fence that restricts the mixing of apical components with other plasma membrane domains was expected to be provided once the genes encoding for integral tight junction proteins were identified.

The first putative integral tight junction protein identified was occludin [75]. However, occludin-deficient embryonic stem cells normally differentiated into polarised epithelial cells, indicating that occludin is not part of the intramembraneous fence. The next integral tight junction protein discovered was claudin [76] but, mainly because of the many claudin isoforms expressed in cells, depletion of claudins could not provide unambiguous evidence for a role for claudins as an intramembraneous fence. Tsukita and colleagues then suppressed the expression of the two oldest-known non-integral cytoplasmic tight junction-associated proteins, zona occludens (ZO)-1 and ZO-2 in epithelial cells. Suppression of ZO-1 and ZO-2 resulted in a striking intracellular accumulation of all tight junction proteins known and no tight junctions were formed [77]. Remarkably, despite a complete lack of tight junctions, these cells were well-polarised with distinctive apical, lateral, and basal plasma membrane domains [77]. In all, the available data strongly suggest that tight junctions are not vital for the asymmetrical distribution of proteins in the plasma membranes as such. It remains an open question whether lipids in the outer leaflet of the plasma membrane remain asymmetrically distributed at the apical and basal/lateral surface domains in epithelial cells lacking tight junctions. The lateral diffusion of lipids may also be restricted by intramembraneous fences other than tight junctions. For instance, the lateral diffusion of proteins and lipids in the plasma membrane of polarized neurons, which lack tight junctions, have been suggested to be restricted by rows of densely packed anchored protein pickets [78] and/or submembraneous spectrin/actin/ankyrin skeletons [79]. As mentioned above, in the apical surface of epithelial cells highly condensed bilayer domains have been postulated to act as fences that separate the membrane of the primary cilia from the rest of the apical membrane [8].

The role of adherens junction in the establishment and maintenance of apical–basal/lateral cell surface polarity is not clear. Using a gene knock-out strategy, E-cadherin was shown to be essential in the establishment of cell polarity during the process of compaction [80]. In contrast, in two recent studies with cultured cells that either were depleted of E-cadherin by siRNA [81] or in which the trafficking of adherens junction components to the cell surface was inhibited [82], adherens junctions were not detected. Yet these cells developed apical–basal/lateral cell surface polarity and functional tight junctions, although with a delay [81–82]. Of interest, hepatocytes lacking adherens junctions show changes in polarised protein trafficking [82], which is in agreement with a role for cell–cell junctions in recruiting intracellular trafficking routes [5]. Not only cells lacking specific cell–cell adhesion junctions, but also single intestinal epithelial cells have been shown to be able to completely polarise and develop apical and basal/lateral cell surface domains [83]. Thus, upon Par4/LKB1 activation, single epithelial cells rapidly remodel their actin cytoskeleton to form an apical brush border, junctional proteins redistribute in a dotted circle peripheral to the brush border in the absence of cell–cell contacts, and apical and basolateral markers are segregated to their respective membrane domains [83]. These studies were performed with cultured cells and it remains an open question whether in vivo cell–cell adhesion is critical for cell surface polarity establishment. In all, despite substantial evidence that cell–cell adhesion junctions influence epithelial surface polarity dynamics (reviewed in [84]), recent evidence in cultured cells suggests that epithelial cell–cell adhesions may not be essential for the segregation of apical and basolateral plasma membrane components per se.

#### 4.1.2. Separating extracellular environments

Once tight junctions have been formed between adjacent epithelial cells and provide a barrier that restricts the paracellular transport of solutes, they may contribute to the maintenance of cell surface polarity by keeping receptors and ligands separated to the apical and basal/lateral domains. Polarised epithelial cells display a relative low proliferation rate, which is a prerequisite for the establishment and/or maintenance of a polarised phenotype. This has been demonstrated in well-differentiated human hepatoma cells, which develop apical surface domains only when in G1 phase when p27<sup>Kip1</sup>-mediated inhibition of cyclin-dependent kinase 2 allows regulated trafficking between recycling endosomes and the apical surface domain [85]. Indeed, loss of cell surface polarity and carcinogenesis often go hand in hand. Vermeer et al. [86] showed that in differentiated human airway epithelia, heregulin- $\alpha$  is present exclusively in the apical membrane and the overlying airway surface liquid, physically separated from its receptor ErbB2-4, an oncogenic receptor tyrosine kinase which localises to the basolateral membrane. The ligand cannot bind its receptor in polarised epithelial monolayers. When cells lack polarisation, or when tight junctions between the adjacent cells are open, heregulin- $\alpha$  rapidly activates the ErbB2-4 receptor leading to cell proliferation. Similarly, epidermal growth factor, the ligand for ErbB1, which is present in the apical fluid of some epithelia, is segregated from its receptor by the tight junction barrier [87]. Interestingly, activation of ErbB2 induces a relocation of the cell–cell adhesion junction-associated ZO-1 and apical resident protein podocalyxin (section 2.) to the lateral plasma membrane domains, reinitiates proliferation and causes cell multilayering [88]. Moreover, activation of ErbB2 in polarised epithelial cells disrupts the segregation of apical and basal surface components by associating with the Par polarity complex proteins Par6 and aPKC, in a manner that is uncoupled from proliferation control [89]. Therefore, tight junctions may not be required for the differentiation of apical and basal/lateral plasma membrane domains by acting as an intramembrane fence (section 4.1.1.), but may allow and/or contribute to the establishment and maintenance of cell surface polarity by acting as a barrier for paracellular transport, i.e. by virtue of its “gate” function [90]. This

might explain why extracellular calcium depletion and loss of adherens and tight junctions leads to dedifferentiation of the epithelial cell surface, while a lack of cell–cell junctions as such does not prevent cell surface differentiation (section 4.1.1.). It may also explain the notion that genetic interference with tight junction-regulatory proteins in whole organisms generally inflict more pronounced effects on epithelial cell polarity.

#### 4.1.3. Regulators of transcription

Typical cell–cell junction proteins might have alternate functions at other subcellular sites. This raises the intriguing possibility that cell–cell junctions control cell proliferation via the well-known but poorly understood phenomenon of “cell–cell contact inhibition”. Below we discuss from a molecular viewpoint the possible effect of cell–cell junctions as transcriptional “enhancer/silencer subdomains”, and how this may affect epithelial cell surface polarity.

Interactions at the cytoplasmic side of specialized cell–cell junctions may help to control the genetic program leading to altered cell proliferation: adherens junctions have been suggested to sequester catenins to prevent their nuclear action. Although  $\beta$ -catenin is a well-established transcriptional cofactor in the Wnt signaling pathway, the cross-talk between  $\beta$ -catenin and other shared components between adherens junctions (or desmosomes) and the Wnt signaling pathway is still controversial [91–93]. Also the gap junction protein connexin43 has tumor suppressive-like effects unrelated to channel function (reviewed in [47,48,94,95]). Recently, connexin43 has been implicated in regulating transcription factors (“SMADs”) [96]. Alternatively, connexins might use ZO-1 as an intermediate for regulation of gene transcription as described at tight junctions: junctional ZO-1 can sequester the transcription factor “ZONAB” and thereby regulate cell proliferation [97,98].

Stable cell–cell junctions might thus form a “magnetic bar” [97] that sequesters transcription factors or their modulators in contacted cells. When junctions are lost, proteins translocate to the nucleus and alter gene transcription. Noteworthy, ZO-1 [99], its relative ZO-2 [100] and ZONAB [97,101,102] typically present at junctions in contacted cells, have all been found nuclear in sparse proliferating cells. Conversely, the protein symplekin is abundant in the nucleus in a variety of cells, but is also found at tight junctions in polarised epithelial cells. Interestingly, ZONAB transcriptional activity is controlled by symplekin via direct interaction ([103] and references therein). Moreover, ZO-1 and ZONAB are essential for cyst formation, pointing to a pivotal role for these proteins in epithelial polarisation [102,103] for more in depth reviews see [97,98]). ZONAB interacts with promoter sequences of genes encoding for ErbB2 and cell cycle regulatory proteins including p27<sup>Kip1</sup> [104] which influence cell polarity (section 4.1.2.).

The findings above lead to a general emerging concept in which some proteins that can bind to cell–cell junctions are upregulated and translocated to cell–cell junctions during differentiation [105], and impairment of cell–cell contacts can lead to activation of transcription factors by junction-associated proteins [100,106,107]. The high, sometimes almost crystalline, enrichment of proteins in microdomains makes these structures unique sites within the cells. The “magnetic bar” function of classical cell–cell junctions might be crucial for cell–cell contact inhibition via regulation of proliferation. Interference with adhesion might change the magnetic bar and thus lead to altered gene expression with consequences for epithelial cell polarity. Adherens junction strength can be regulated by several factors, including the action of the epithelial cell adhesion molecule EpCAM.

The calcium-independent non-classical cell adhesion molecule EpCAM (for Epithelial Cell Adhesion Molecule) was discovered as an epithelial cancer marker and has long been associated with epithelial tumors [108–110]. EpCAM can upregulate c-Myc leading to cell proliferation. Overexpressing EpCAM results in a reduced dependency on growth factors. Conversely, EpCAM knock-down decreases

proliferation rate in cancer cells proliferation [111,112]. Initially, EpCAM was studied as part of cell–cell contacts. Litvinov et al. found homophilic lateral and intercellular interactions mediated by the extracellular (ecto) domain of EpCAM [113]. These interactions weaken the adherence of neighboring cells by direct interference with adhesion junction strength [114]. The effect might be due by competition for actin-cytoskeleton anchorage via alpha-actinin, which has been reported to bind to EpCAMs cytoplasmic tail [113]. In conclusion, EpCAM is a non-classical transmembrane protein that is present at cell–cell junctions and affects proliferation. EpCAM seems to alter proliferation by direct feedback on the strength of adherens junctions strength and/or expression levels.

#### 4.2. Caveolae, lipid rafts, and clathrin-coated pits

Caveolae and clathrin-coated pits are membrane microdomains that compartmentalise intracellular signalling pathways, and regulate the expression levels of membrane proteins and lipids at (the different domains of) the cell surface. Similar as cell adhesion junctions translate physical signals from neighboring cells and/or the extracellular matrix, caveolae and clathrin-coated pits also define how cells respond to the extracellular environment. In this paragraph we discuss the potential involvement of caveolae and clathrin-coated pits as membrane microdomains in epithelial cell surface differentiation.

##### 4.2.1. Caveolae and lipid rafts

Cell migration requires the asymmetrical organisation of cell surface activities [115], a process that is critical for tissue development and wound healing. Caveolin-1, the principal protein component of caveolae, is excluded from the leading edge from migrating cells and displays a polarised localisation the cell rear, linked to the cytoskeleton [116]. Caveolin-1-deficient cells lose cell polarity and exhibit impaired wound healing [117], and loss of caveolin-1 polarity impedes polarisation and directional movement [118]. Knock-out experiments revealed that caveolin-1 establishes cell surface polarity by coordinating diverse signalling pathways [117]. Specifically, it was proposed that caveolin-1 stimulates Rho GTP loading [117]. Rho family GTPases are well-known for their involvement in regulating cell–cell adhesion junction dynamics, polarised intracellular trafficking and epithelial cell polarity (see Van Aelst [119] for a review). Possibly, caveolae signalling plays also important roles in the establishment and/or maintenance of surface polarity in epithelial cells.

Exposure of differentiated human hepatoma HepG2 cells to the cytokine oncostatin M stimulates the biogenesis of apical plasma membrane domains, in part by controlling polarised trafficking from recycling endosomes [85,120]. Binding of oncostatin M to its receptor at the basolateral surface domain causes recruitment of the signal-transducing co-receptor gp130 into cholesterol-dependent and detergent-resistant membrane domains [120]. Removal of plasma membrane cholesterol abolishes this recruitment and inhibits polarised trafficking and apical plasma membrane biogenesis in response to oncostatin M [120]. These data provide a clue for a molecular mechanism that couples the biogenesis of an apical plasma membrane domain to the regulation of intracellular trafficking in response to signalling in lipid microdomains at the basolateral surface.

##### 4.2.2. Clathrin-coated pits

Endocytic traffic controls both apical–basal polarity and cell proliferation [121]. Clathrin-coated pits have essential roles in intracellular trafficking and cellular signalling processes at the plasma membrane. Of interest, clathrin mediates the endocytosis of epithelial apical junctional proteins E-cadherin, p120 and beta-catenins, occludin, JAM-1, claudins 1 and 4, and ZO-1 in T84 intestinal epithelial cells following depletion of extracellular calcium [122]. In a recent study, it was demonstrated that clathrin knock-down depolarised basolateral proteins, by interfering with their biosynthetic delivery

and polarised plasma membrane recycling, but did not affect the polarity of apical proteins [123]. Clathrin thus appears as a key regulator of basolateral polarity. The clathrin-binding epsins were shown to regulate Cdc42, a critical player in cell polarity in all eukaryotes, and epsins might therefore function as spatial and temporal coordinators of endocytosis and cell polarity [124].

### 5. Associations and feedback between cell–cell junctions and microdomains

A subset of the common cytoplasmic interactions at cell–cell junctions have been observed during discovery of the cell–cell junctions at the ultrastructural level, such as the intermediate filaments at desmosomes. The presence of signaling proteins, scaffold proteins and cytoskeletal interactions (discovered 1980s–1990s) at adherens junctions and tight junctions is also well established. Such interactions have only been discovered the last decade at gap junctions: during the identification of molecules at gap junctions it became clear that several partner proteins of connexins are not restricted to “their” cell–cell junction or the synthesis and degradation pathway. The distinct cell–cell junctions have several partners in common. For example gap junction proteins (connexins) have been found to be associated with structural and scaffold proteins typical present in tight junctions as well as with proteins previously discovered at adherens junctions [reviewed in [47–49]. Several of these interactions have a direct or indirect impact on epithelial cell polarity.

#### 5.1. Cross-talk between cell–cell junctions

Demarcation between molecular components of junctions might not be as strict as their structural appearance. Adherens junctions, tight junctions and gap junctions share several cytoplasmic partners, including the scaffold ZO-1, which makes this protein a putative candidate linker of junctional proteins from different structures. Besides such a common role for ZO-1 in the distinct junctions, a specific role in each junction, namely separation of adherens junction from tight junctions during establishment of epithelial polarity [125,126] or gating of gap junction channels [127] has also been proposed.

The timely development of the different cell–cell junctions appears to be an integrated process. For instance, in most epithelial cells, the formation of adherens junctions precedes and facilitates the formation of tight junctions [78]. In hepatic cells, junctional adhesion molecule-A modulates E-cadherin expression and is critical for the formation of apical surface domains [128]. Connexin32 expression in hepatic cells is in part related to induction of tight junctions through modulation of MAGI-1 expression [129]. The ability of squamous epithelial cells to organise desmosomal proteins into a functional structure depends upon their ability first to organize an adherens junction [130].

N-cadherin knock-out in neural crest cells from mice [131] or knock-down of connexin43 in fibroblasts [132] results in defective function of intercellular communication or loss of membrane localisation of adherens junction proteins, respectively. In the latter study, the defect of proper membrane localisation could be pinpointed to a defect in connexin43 trafficking in the absence of N-cadherin and vice versa, suggesting a coordinated insertion of distinct cell–cell junction proteins [132]. These data suggest that direct feedback between classical adherens junction proteins and gap junction proteins is required for their normal function in regulating cell behaviour (reviewed in [48]). Whether this phenomenon is general for connexin- and cadherin family members remains to be established. Another emerging cross-talk between classical cell–cell junctions in regulation of cell polarity is the induction of tight junctions by upregulation of a subset of connexins ([133] and references therein). Although the effects were found to be mediated



through both gap junction channel function dependent and independent effects, and evidence suggest a direct interaction between the compositional proteins of the distinct junctions, the exact mechanism of the cross-talk between gap junctions and tight junctions in contribution to cell polarisation warrants further investigation.

### 5.2. Cross-talk between cell junctions and tetraspanin-enriched microdomains

Adherens junction strength can be modulated by EpCAM (section 4.1.3). Moreover, EpCAM has recently been identified in the tetraspanin web (reviewed in [110]). Interestingly, another typical cell–cell contact protein, namely the tight junction protein claudin7, was identified in the EpCAM–TEM complex [109]. Biochemical experiments suggested that claudin7 and EpCAM might interact, but proteins like ZO-1 are putative linkers. Clinical studies showed a role for EpCAM/claudin7 in promoting metastasis [134], suggesting reversal of epithelial polarisation. Other tetraspanins have also been associated with loss of polarisation. Tetraspanin TM4SF5 overexpression results in loss of E-cadherin and epithelial to mesenchymal transition in hepatocarcinoma cells via a p27<sup>kip1</sup> dependent mechanism. The complete changing of polarisation, characteristics of differentiated cells, induced by TM4SF5 could be counteracted by downregulation of TM4SF5 itself, or its downstream effector p27<sup>kip</sup> as well as by increasing E-cadherin levels [135]. An opposite effect of TEMs in stimulating polarisation has been found by acting in concert with integrins resulting in gene expression leading to increased adherens junction strength [136].

Thus, cell–cell junctions, cell–ECM adhesion and other plasma membrane microdomains such as TEMs act closely together to regulate cell differentiation and polarity. The outcome of cell destiny is dictated by (i) the structural influence; (ii) direct feedback between the cell–cell junctions and (iii) regulation of the expression of gene products.

### 5.3. Apical location of cell–cell junction proteins: other functions?

#### 5.3.1. Connexin hemi-channels at the apical membrane?

Hemi-channels are half a gap junction channel consisting of six connexins that are in contact with the extracellular space and thus are not directly involved in cell–cell communication. Functional evidence for the existence of these channels is mainly derived from studies in neuronal tissues [94]. More recently, hemi-channels have been immunolabelled at the basal membrane in polarised epithelial cells, but their function in epithelia remains unclear [137]. Whether hemi-channels exist as isolated channels or form higher ordered structures of multiple channels is not known. While hemi-channels have been implicated in the release of molecules such as ATP, the precise function and general existence of these channels needs further investigation.

#### 5.3.2. Hijacking of microdomains by viruses

Recently, tight junctional claudins and tetraspanins have been found to be (ab)used in the life-cycle of diverse viruses. Entry of cells by a variety of viruses via attachment factors and/or receptors is well established and has now been described in detail. A crucial step in cellular entry is uptake, which often requires the above-discussed microdomains clathrin-coated pits, caveolae and lipid rafts (reviewed in [138]). Hepatitis C virus (HCV) entry was known to depend on factors like clathrin, low pH, temperature and two receptor proteins: the tetraspanin CD81 and the scavenger receptor BI. However, the presence of these two proteins was insufficient for viral entry. Evans and coworkers found that claudin-1 is critical for viral entry of HCV with crucial function of an extracellular loop [139,140]. Evidence suggests that the virus directly binds to claudin-1, but a direct interaction has not been found to date. Moreover, claudin-1 was found to colocalise with the tetraspanin CD81, the earlier identified co-

receptor for HCV [141]. More recently, the specific involvement of claudin-1 in HCV entry in hepatocytes was being confirmed, and Claudin-1, -6, and -9 were shown to be equally potent as a co-receptor with CD81 for HCV entry in endothelial cells. In independent studies, a direct association between claudin-1 and the tetraspanin CD9 has been found outside tight junction strands [142], and TEM-located claudins are likely targets for viral entry. Whether claudins in TEMs serve an alternative function in addition to barrier-forming in tight junction strands under physiological conditions remains to be established. In addition to virus entry, also virus budding has been associated with tetraspanin-enriched microdomains. In Jurkat cells, the tetraspanins CD9, CD63 and CD81 were found to be enriched at sites where human immunodeficiency virus (HIV) envelope proteins are enriched prior to budding [143,144]. The findings above illustrate that cell–cell junction proteins might have additional, receptor-like functions that is translated into signalling and thereby indirectly help to regulate cell destiny, including the process of cellular polarisation.

## 6. Concluding remarks

The surface of polarised epithelial cells has traditionally been viewed as two relatively large domains (apical and basolateral), each facing the distinct extracellular environments and separated by cell–cell junctions. Later, additional subdomains have been identified and/or received increasing attention in the recent years. These include cell–cell junction microdomains and microdomains involved in endocytosis and signalling. These findings have complicated our view with regard to the mechanism by which the cell maintains the structural, compositional and functional characteristics of all these membrane domains.

Powerful studies in which adherens or tight junctions were selectively removed from the cell surface prompt a revisiting of our thinking how cell–cell adhesion junctions contribute to the establishing and/or maintaining cell surface polarity. Single epithelial cells have been shown to organize membrane asymmetry and adherens junctions and tight junctions might not be crucial for formation of cell polarity. Recent developments indicate that sub-micrometer-sized cell–cell junctions, perhaps in part via their “gate function” or their function as signaling platforms, control transcription and in this way the genetic program that controls cell proliferation and polarisation, two tightly linked processes.

Recent developments in genetics, biochemical and dynamic molecular imaging techniques have provided evidence of cross-talk and/or overlap between the different cell–cell junction components. This, and the reported localisation and function of classical junctional proteins in low but likely significant concentrations elsewhere in the cell, are expected to pave the way to a better understanding of how the many different specialised cell surface domains cooperate to establish and maintain epithelial cell polarity and architecture.

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