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Lysosomal Degradation of Junctional Proteins

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Abstract

Epithelial cells develop tight junctions (TJs) and cell polarity. Both properties are sensitive to environmental signals such as the epidermal growth factor (EGF) and the cardiotoxic steroid ouabain. EGF is regarded as the main protector against injuries in epithelia, and ouabain is a hormone that regulates blood pressure, natriuresis, cell survival, and cell adhesion. After treatment with epidermal growth factor or ouabain, epithelial dog kidney MDCK cells undergo a drastic remodeling that includes changes in the transcription, translation, localization, and degradation of cell junction proteins. Degradation of these proteins involves selective and nonselective autophagy as well as endocytic lysosomal and proteasomal routes. The remodeling mechanism of tight junction's proteins includes the activation of Src and ERK1/ERK2 kinases, the phosphorylation and translocation into the nucleus of the transcription factor STAT3, the activation of PKC to induce the endocytosis of claudin-2, and the delivery of this protein to the lysosomes. Whole communicating junctions and desmosomes are internalized by one cell and sent to degradation by nonselective autophagy. Nonselective and selective autophagies in epithelial cells are very context dependent; nevertheless, it is clear that, together with endocytic lysosomal and proteasomal degradation, they play a key role in the remodeling and functioning of cell junctions.

Keywords: claudin-2, ouabain, EGF, MDCK, tight junctions

1. Introduction

When multicellular organisms left the sea of constant chemical composition to conquer the land, it became necessary to develop mechanisms to maintain a constant internal milieu

similar to the sea that was left behind. *Epithelia* were fundamental in terrestrial conquest because these tissues avoid the loss of water, transport vectorially substances to introduce nutrients into the body, eliminate waste substances, and protect the individual from pathogens and their toxins [1]. These functions of epithelia depend on two basic characteristics of the cells that constitute them; the first one is *plasma membrane polarity*, which consists on the division of the plasma membrane in an apical, a lateral, and a basal domains, each one with characteristic structure, composition, and function [2]. Due to its continuity, the lateral and the basal domains are often referred as the basolateral domain. The second characteristic of epithelia is *cellular junctions* that bind epithelial cells to each other and to the substrate (**Figure 1**) [1, 3]. Adhesion between neighboring cells depends on the *junctional complex*, which is a group of intercellular junctions that includes the tight junctions (TJs), adherens junctions (AJs), desmosomes (Des), and communicating or GAP junctions (GAPJs) [4]. Attachment to the substrate requires the formation of two additional cellular junctions: focal adhesions (FAs) and hemidesmosomes (HDes) (**Figure 1**) [5, 6]. *Cellular junctions* and *plasma*

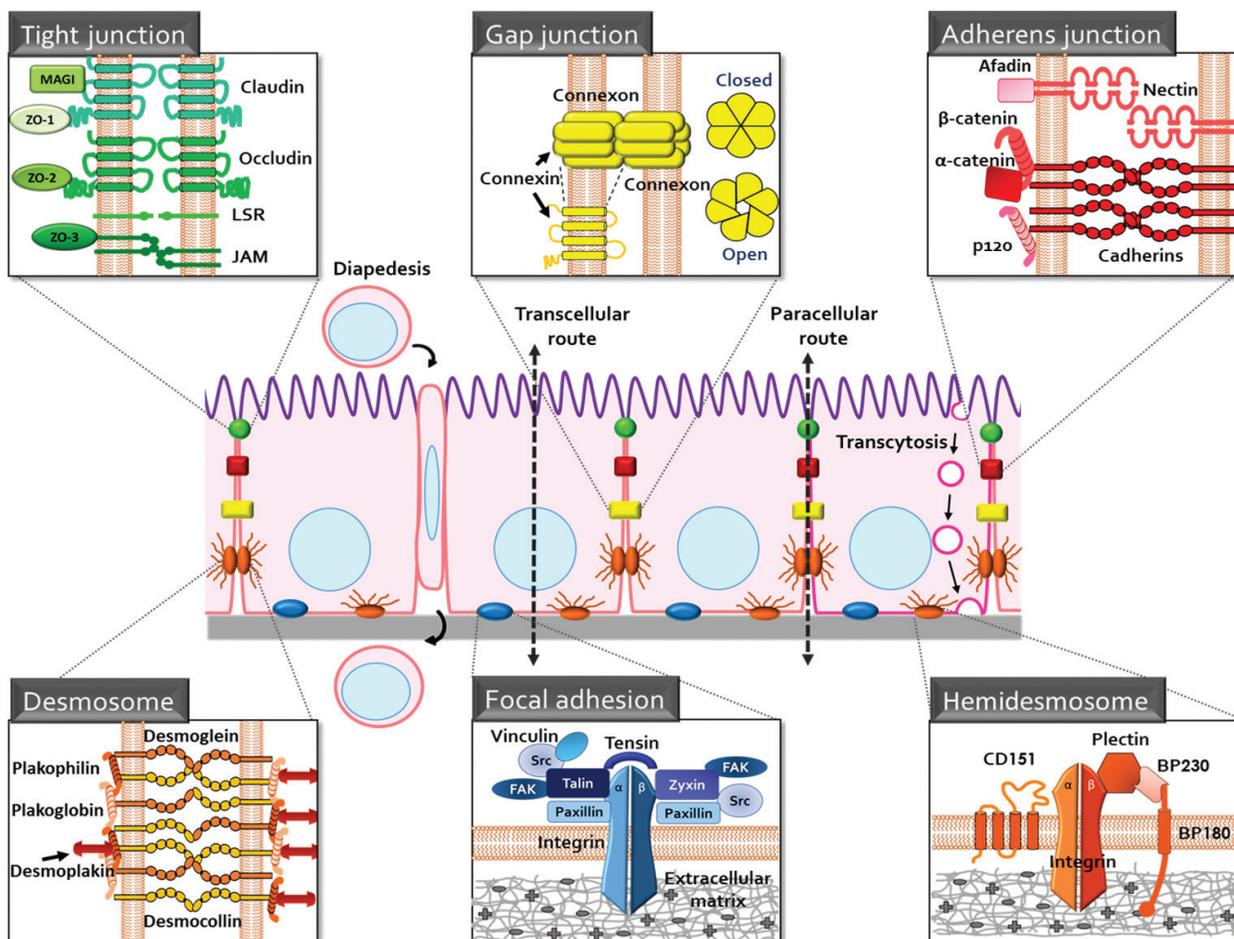


Figure 1. Cell junctions and polarity in epithelial cells. Substances cross the epithelial layer through the transcellular or paracellular routes or by transcytosis. Each scheme depicts the main molecular components of cellular junctions and its organization in the membrane. MAGI are inverted membrane-associated guanylate kinase-like proteins, ZO-1, -2 and -3 are Zonula Occludens-1, -2 and 3, Src is the protein homologous to the Rous sarcoma virus kinase, FAK is the focal adhesion kinase, LSR stands for the lipolysis-stimulated lipoprotein receptor, JAM is the junctional adhesion protein.

membrane polarity are crucial requirements for epithelial function and influence each other in complex ways, for example, the formation of a specific cell junction is the result of the polarized expression of many proteins and lipids; conversely, the development of cell polarity requires cell adhesion, initially to the extracellular matrix and then to the neighboring cells [7–10], and the development of AJs is, in general, a prerequisite for the formation of the other intercellular junctions [11, 12].

The complex organization and regulation of cell junctions and cell polarity in epithelial cells are adaptations to perform vectorial transport. A given substance crosses epithelial layers either through the *transcellular pathway*, using polarized channels, transporters, and pumps [13, 14], the *paracellular pathway*, constituted by TJs and the intercellular space [15], or both (**Figure 1**). The contribution of each pathway depends on the kind of substance transported, the cell type that executes the transport, and the physiological state of the transporting cell. In bowels and kidneys of mammals, the transcellular pathway accounts for most of the glucose transport [13], while flying vertebrates, perhaps because they have a smaller intestine than other vertebrates, rely in a majoritarian paracellular transport [16]; most Ca^{2+} is reabsorbed at the thick ascending limb of Henle's loop through the paracellular pathway by a specific type of claudin (CLDN), claudin-16, a TJ protein that forms Ca^{2+} - and Mg^{2+} -selective pores [17], while glucose is transported predominantly through the transcellular pathway by polarized SGLT and GLUT transporters driven by the Na^+ concentration gradient [13, 18]. There are also two more processes to cross epithelia and endothelia: *whole cells cross epithelia*, as shown in spermatogenesis in which immature sperm cells open its way through the junctional complex of epithelial Sertoli cell monolayers [19] and in *diapedesis*, where neutrophils and leucocytes open transitorily the junctional complex and accede to the internal media [20]. Moreover, some substances and even viruses cross epithelia by *transcytosis* (**Figure 1**), a process that consists in the incorporation of the material to a vesicle generated in one plasma membrane domain that is, later on, translocated and fused to the opposite membrane domain where it delivers its content [21].

Recent research demonstrated that *cell junctions are signaling stations* that inform the adhesion status of the cells to cytoplasmic and nuclear mechanisms so they can induce proper responses [22, 23]. *Contact inhibition* and *healing of a wound* illustrate this property of cell junctions: loose AJs, or their absence by the lack of E-cadherin, an essential transmembrane protein of this cellular junction, trigger a period of high cell proliferation and migration through the activation of the Hippo signaling pathway and stop only when the normal size of the organ and a strong adhesion between epithelial cells are reached, a process referred to as *contact inhibition* [24]; when an epithelium is wounded, the cells at the front of the wound lose their cell junctions, start to proliferate, and migrate to heal the wound and extracellular protective factors, such as the epidermal growth factor (EGF), ouabain (OUA), and marinobufagenin, favor this reparation [25, 26]. Several membrane-associated proteins of cell junctions, notably β -catenin [27], a protein of the AJs, and zonula occludens-2 (ZO-2), a protein of TJs [28], are transcriptional cofactors that regulate the expression of proliferation-related genes, for instance, cyclin D1 and myc; conversely, transcription factors such as Jun, Fos, and ZONAB reside transitorily at the cytoplasmic region of the TJs [29, 30] and, at least ZONAB, downregulates paracellular permeability [30].

Cellular junctions and plasma membrane polarity are highly regulated. For example, a progressive conversion of renal intercalated cells of the collecting tubules from α to β type comprises the inversion of the apical H^+ -ATPase and a basolateral Cl^-/HCO_3^- exchanger polarity, in response to the increase in the expression of hensin, a protein of the extracellular matrix [31, 32], and cysts embedded in collagen displace their TJs from the vicinity of the lumen toward the proximity of the external surface [33, 34].

Cell junctions and plasma membrane polarity are crucial for the normal physiology of the organism, and its failure in several pathologies has disastrous consequences. To start with, it is common that the genetic elimination of crucial proteins, such as E-cadherin from the AJs or ZO-2, is lethal at embryonic stages [35, 36], but whenever an epithelial adhesion protein is not expressed, epithelia compartmentalization and vectorial transport are lost. For example, in hereditary familial hypomagnesemia with hypercalciuria and nephrocalcinosis, the lack of CLDN-16 and CLDN-19 impairs Ca^{2+} and Mg^{2+} reabsorption in the kidney [17, 37]; in cholestatic children's liver disease, the absence of ZO-2 and TJs provokes the invasion of bile salts into the blood [38]; in pemphigus vulgaris, the depletion of Des by autoantibodies against the desmosomal cadherin desmoglein-3 results in the formation of skin blisters [39], which can also appear if HDes are disassembled by mutations in the integrin $\beta 4$, an adhesion molecule of this cell junction [40]; loss of adhesion and augmented proliferation in colon cancer are elicited by mutations that increases the cytosolic and nuclear pools of β -catenin [27]; infection and inflammation boost *diapedesis* [20]; rotaviruses that cause diarrheas open TJs and accede to their basolateral receptors by exposing its VP8 protein, which bear small peptides with sequences identical to some regions of occludin, an integral membrane protein of the TJs [41]; and the exogenous expression of the E6 oncoprotein of the high-risk papilloma virus type 16 induces the translocation of β -catenin into the nucleus to increase proliferation in the skin [42].

2. Cell junctions have a general common layout

All junctions have a similar structural layout: they have transmembrane proteins that are the receptors for adhesion, and a series of membrane-associated proteins that bind the cytoplasmic aspect of transmembrane receptors to the actin, tubulin, or cytokeratin cytoskeleton to provide mechanical strength. Besides cell adhesion, cell junctions are sensors that inform, in and out, the state of extracellular environment to modulate cell's proliferation, differentiation, and fate. Given that lysosomes are of paramount importance for cell junctions and plasma membrane polarity, it is necessary to briefly review the degradation routes where this organelle intervenes.

3. Lysosome degradation pathways

Lysosomes are major degradative organelles of eukaryotic cells. They were first identified as cell compartments enriched in hydrolases [43], but now they are also recognized as providers of building blocks during starvation and powerful stations to sense nutrients and regulate transcription and cellular homeostasis [44]. Lysosomes have a highly acid lumen (pH 4.5–5.0) produced by a vacuolar H^+ -ATPase. The acidic pH is necessary for the hydrolysis of waste materials and drives the transport of sugars, amino acids, nucleotides, and lipids, through the

single membrane of the organelle for recycling [45]. The lysosomal membrane owes its resistance against the activity of the hydrolases that it contains, to the expression of a prominent glycocalyx in its inner surface, formed by glycosylated transmembrane proteins such as the human LIMP-2 and its homologues in *Caenorhabditis elegans* SCAV-3 [46].

Many intracellular proteins are ubiquitinated and degraded in the proteasome (**Figure 2, 1**). There are also several routes to deliver cellular material into the lysosomes: an *endosomal-lysosomal* route, similar to the secretory route, that consists in the ubiquitin (Ub) or clathrin mediated endocytosis of the protein to be degraded, the posterior fusion of the vesicles containing this protein to the early endosomes (EE), which in turn may mature to late endosome (LE, 2) or produce multivesicular bodies (MVB, 3) and fuse with the lysosome (**Figures 2 and 3**) [47, 48]; the *microautophagy* degrades intracellular proteins engulfed by the lysosome (**Figure 2, 4**) [49], the nonselective *macroautophagy* degrades big intracellular waste (**Figure 2, 6**) [52, 53] (**Figure 2, 5**). *Endocytosis* requires the ESCRT machinery and the activity of several Rab GTPases that tether and dock the SNARE fusion machinery [54]. Rab5 participates in the fusion of clathrin-coated vesicles with early endosomes (**Figure 2, EE**) [55, 56], Rab4 in the recycling of proteins to the plasma membrane [57], and Rab7 in the transport from early to late endosome and lysosome biogenesis and maintenance [58]. *Nonselective macroautophagy* consists of the engulfment of intracellular waste material by a phagophore, a double-membrane structure possibly derived from the endoplasmic reticulum [53]. Since its appearance and through all its maturation, the phagophore incorporates the microtubule-associated protein 1A/1B light chain 3 (LC3). LC3 exist in a cytosolic form (LC3I) that, at the onset of

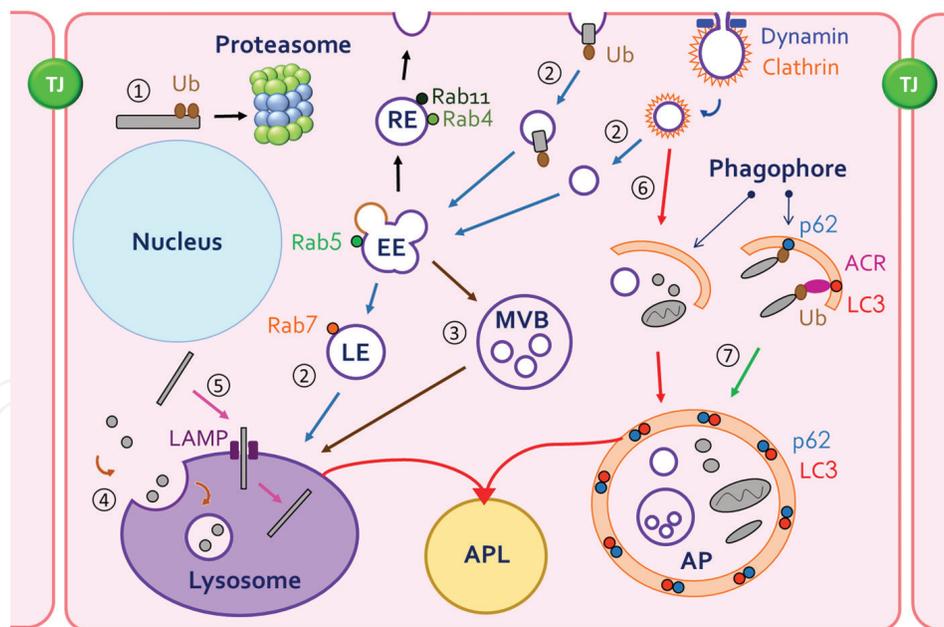


Figure 2. Protein Degradation pathways. (1) *Proteasome pathway*, used for degradation of the ubiquitin (Ub) bound cytosolic proteins. (2) *Endocytic pathway*, involving endocytic vesicles that fuse to the early endosome (EE), progress to the late endosome (LE), multivesicular bodies (MVB, (3) and finally to the lysosome. (4) *Microautophagy*, the lysosome engulfs some cytosolic proteins. (5) *Chaperon mediated autophagy*, allows the passage of cytosolic proteins via translocons. (6) *Nonselective macro autophagy*, large intracellular waste material is engulfed by the phagophores that becomes, later on, the autophagosome (AP). This organelle then fuses with the lysosome to form the autophagolysosome (APL). (7) *Selective macro autophagy*, intracellular waste material is ubiquitinated, bound to autophagic cargo receptors (ACR), LC3 or p62, and then engulfed by the phagophores to continue the autophagy route. RE is the recycling endosome.

autophagy, is conjugated with the lipid phosphatidylethanolamine to form LC3 type II (LC3II). *Selective macroautophagy* is triggered with the purpose to degrade a specific protein. This protein is first ubiquitinated by ligases such as Beclin1, which is then bound to the ubiquitin-binding sequestosome 1 protein (p62), which in turn binds to LC3II. The protein can also be bound, indirectly, to p62 through the autophagic cargo receptor protein (ACR, **Figure 2, 7**). Progressive encirclement of the waste material by the developing phagophore conduces to the formation of the organelle called autophagosome, which later on fuses with the lysosome to become an autophagolysosome. Fusion of the autophagosome to the lysosome requires a low acidic pH of the lysosome and is inhibited by drugs that access the lumen of this organelle and neutralize its pH, such as chloroquine and NH_4Cl [51], or inhibit the lysosomal V type H^+ -ATPase, as bafilomycin A1 [59]. The lysosome itself may invaginate small cytoplasmic waste materials, in a process similar to the formation of multivesicular bodies from the late endosome [60]. In the chaperone-mediated autophagy, cytosolic unfolded proteins that contain a KFERQ-like pentapeptide bind to the lysosome receptor Lamp-2 and are directly translocated into the lumen of the lysosome through a multimeric translocation complex [50, 53].

A central regulator of lysosomal activity, particularly autophagy, is the target of rapamycin (mTOR), a multi-protein complex that includes the kinase mTOR itself, inhibited by rapamycin, the raptor adaptor, two intrinsic inhibitors of mTOR activity, DEPTOR and PRAS40, and a G-protein. The mTOR complex senses energy and nutrient availability, growth factors, and stress conditions to modify cell growth and proliferation. In normal conditions, mTOR localizes in the cytosol and triggers anabolic programs, like mRNA translation. Under starvation, mTOR is translocated to the cytosolic side of the lysosome membrane, where it initiates catabolic processes like autophagy [44].

A growing body of evidence suggests that lysosomes can function as Ca^{2+} stores and contact intimately to the endoplasmic reticulum, the peroxisome, and the mitochondria to deliver necessary lipids [61, 62] and that lysosomes can fuse to the plasma membrane to pour hydrolytic enzymes in the extracellular media that modify the extracellular matrix and induce differentiation [59]. Lysosomes induce cell death when its membrane is permeabilized and hydrolases such as cathepsin B, a Ca^{2+} -sensitive protease, are released in the cytoplasm. Cell death induced by lysosomal damage is observed in tissue remodeling, elimination of excessive intracellular waste or metals, and the immune response to intracellular pathogens and neurodegenerative diseases [63, 64].

To maintain compartmentalization and vectorial transport in epithelial cells, the synthesis and degradation of adhesion proteins must be closely coordinated. Nevertheless, epithelial cells must have certain degree of plasticity to modify cell junctions in response to the variable environment. Lysosomal activity is crucial in both situations.

4. Role of lysosomes in the degradation of tight junction proteins

4.1. Biology of tight junctions

TJs, also known as *zonula occludens* (ZO), are formed at the most apical region of the lateral plasma membrane domain [4], constitute the frontier between the apical and the basolateral domains, and obliterate the intercellular space at this region, converting the epithelia

in an effective barrier against the free diffusion of ions and other substances [3]. This cellular junction has two main functions: it is a *gate* that transports ions and other substances through the paracellular pathway [65–67] and a *fence* that impairs the diffusion of integral membrane proteins and lipids from the apical to the basolateral domain and the other way around [68, 69], a property that helps to maintain cell polarity [8]. In the case of lipids, its impairment is restricted to those from the exoplasmic leaflet of the plasma membrane. In some experimental conditions, TJs can be a *bridge* that allows the diffusion, in the plane of the membrane, of membrane liposoluble probes and of phosphatidylinositol bisphosphate (PIP2), from one epithelial cell to its neighbors [70–74]. TJs are composed of three main types of integral proteins that perform the task of adhesion receptors: the four-transmembrane-domain family of CLDNs [75] and TAMP proteins, that includes occludin and tricellulin [76], the single transmembrane domain proteins of the junctional adhesion molecule (JAM) family and the lipolysis-stimulated lipoprotein receptor (LSR) (**Figure 1**) [77–82]. CLDNs constitute the paracellular ion and water channels and barriers [17, 65, 83] and confer to the TJs and the epithelia its specific paracellular permeability and selectivity, depending on the type of CLDNs expressed [17, 65, 83–85]. The integral membrane proteins of the TJs bind to a cluster of three cytoplasmic proteins of the ZO, named ZO-1, ZO-2, and ZO-3 (**Figure 1**, green). These proteins belong to the MAGUK family and are platforms for protein-protein interaction [86–88]. ZO's plaque proteins in turn bind transmembrane once to the actin cytoskeleton [89]. There are a number of other integral and associated proteins that are nonessential or reside transiently at TJs but, nevertheless, are important for vesicular trafficking and signaling [78]. In insects and mollusks, the paracellular pathway is controlled by the septate junctions, structures that have different molecular compositions and structures and are located below the AJs [90]. Based on the crystal structure of the euglena tetraspan protein IP39, another member of the group of proteins to which CLDNs belong, the PMP22/EMP/MP20/Claudin superfamily, it has been suggested that CLDNs may be originated from an ancestral four-transmembrane-domain protein, similar to IP39, which may have played a role in the peculiar tortuous movement of this photosynthetic protist [91].

Epithelia adjust the permeability of their paracellular route in response to physiological requirements, pathological conditions, and pharmacological challenges. One simple way to gauge epithelial permeability is to measure the transepithelial electrical resistance (TER) [92]: the higher the value of TER, the lower the paracellular permeability. On this regard, the renal system is very illustrative. Human kidneys filtrate 170 l of plasma but secrete only 1.7 l of urine. Water, proteins, sugars, and ions from the glomerular filtrate are reabsorbed, and the filtrate is steadily concentrated along the nephron. The epithelia that line this tubular surface in vertebrate species gradually increase their TER from approximately $10 \Omega \text{ cm}^2$ at the proximal convoluted tubule [93, 94] to several thousands of $\Omega \text{ cm}^2$ at the collecting duct [95, 96] and up to hundreds of thousands of $\Omega \text{ cm}^2$ at the bladder [97, 98]. A number of epithelial cell adaptations account for this TER gradient: increments in cell size, reduction of the junctional membrane tortuosity, a progressive increase in the structural complexity of TJ strands, and the expression of a specific set of CLDNs in each nephron segment [15, 65]. CLDN-2 induces a low TER phenotype in renal MDCK cells [99], from cation and water-selective channels [83, 100, 101], and it is expressed in proximal tubules [102–104], where it is necessary for the uptake of Na^+ , water and, likely, Ca^{2+} [105]. CLDN-4 induces a high-resistance phenotype upon the epithelial cells that express it [106–108], including those at the distal nephron segment epithelium [102–104].

4.2. Autophagy of CLDN-2 participates in the differentiation of epithelia induced by the epidermal growth factor and exerts a protective effect

The fluids that bathe apical membranes, such as urine, semen, and milk, are radically different from each other, but the interstitial milieu that contacts the basolateral membranes has a constant composition maintained by homeostatic mechanisms. This difference suggests that substances in the apical media might regulate specific epithelial properties. Several substances in the extracellular milieu induce TER changes in canine MDCK cells [109]. One of them is EGF [110], a substance previously known to increase the TER of epithelial kidney pig LL-CPK1 cells [111]. Urinary EGF reduces the cellular CLDN-1 and CLDN-2 protein level and increases CLDN-4 one [110]. EGF decreases the cellular level of CLDN-2 [112] through the simultaneous activation of Src kinase, extracellular regulated kinases 1/2 (ERK1/2) [113, 114], and the transcription factor STAT3 [114] that, in turn, may accelerate clathrin-mediated endocytosis and lysosomal degradation of CLDN-2 [113], block CLDN-2 [115], and trigger CLDN-4 [116] transcription in MDCK cells. In lung cancer cells though, EGF increases CLDN-2 through the activation of the EGF/EGFR/MEK and cFos pathway [117]. It would be interesting to find out the molecular mechanisms that fail in cancer and provoke the opposite response.

The response elicited by EGF is transient, reaches a maximal value of TER at 15 h, and slowly decreases to control values at 24 h. This downregulation is provoked by the induction of the synthesis of prostaglandin E_2 by the EGF itself that increases AmpC production, which in turn blocks the activation of ERK1/2 [118].

It is not clear which vesicular compartment participates in the CLDN-2 degradation induced by EGF. The observation that the knockdown of Rab14 induces the lysosomal degradation of CLDN-2 in MDCK cells [119] opens the possibility that EGF somehow be able to inactivate this Rab protein.

The induction of CLDN-2 downregulation by EGF is blocked by bafilomycin A1 and chloroquine, indicating that it may be performed by autophagy (**Figure 3**). A schematic representation of the mechanisms of EGF effect on CLDNs is shown in **Figure 4**. The induction of selective autophagy by EGF can be seen as a differentiation or protective effect. In this respect, autophagy has been observed in Caco-2 cancer colon cells deprived of nutrients, where selective autophagy of CLDN-2 is activated, resulting in an increase in TER [120]. Moreover, in porcine gut IPEC-1 epithelial cells, the deprivation of nonessential amino acids induces an apoptotic process that degrades CLDN-1 and ZO-1, but if autophagy is inhibited with 3MA, degradation of adhesion proteins and apoptosis is potentiated, indicating that autophagy has a protective role in these cells [121]. Finally, the injured spinal cord in rats induces the degradation of p120 and β -catenins, as well as CLDN-5 and occludin, in blood vessels of endothelia. This degradation is performed through selective autophagy, considering that these proteins associate to LC3II and p62. The administration of retinoic acid potencies autophagy and improves movement of the injured rats [122].

4.3. Ouabain induces degradation of CLDN-2

In the eighteenth century, William Withering used extracts of the herb foxglove (*Digitalis purpurea*) to treat successfully what is now called congestive heart failure [123]. The active

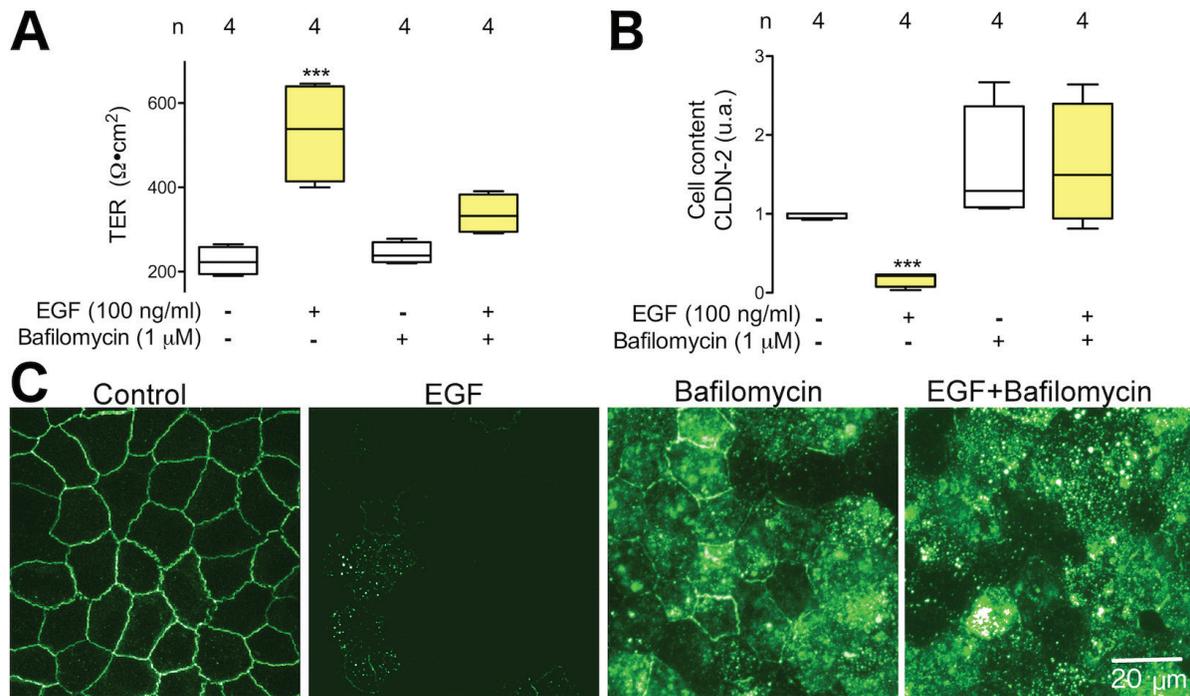


Figure 3. Epidermal growth factor (EGF) induces CLDN-2 degradation in a bafilomycin 1A-sensitive manner. Epithelial dog kidney cells (MDCK) confluent monolayer grown on filters were incubated 15 h in control condition, EGF, bafilomycin A1 (Baf), or EGF plus Baf. (A) Transepithelial electrical resistance measurements. (B) Densitometric analysis of the cellular content of CLDN-2 measured by immunoblot. (C) CLDN-2 Immunofluorescence of cells incubated in the indicated conditions.

principle involved in this therapy is digoxin, a member of the family of compounds named cardiotonic steroids, which bind and inhibit the Na^+, K^+ -ATPase [124] and of which *ouabain* is the prototypic compound. This enzyme is expressed in the plasma membrane of most animal cells, where it transports intracellular Na^+ out of the cells, in exchange of extracellular K^+ toward the cell interior, against their concentration gradients, in a reaction driven by ATP hydrolysis. The inhibition by OUA became the main criterion in determining whether a given flux depended upon an active transport. Animals, including humans, produce OUA and other similar substances such as marinobufagenin and proscillaridin that are synthesized in the hypothalamus [125] and the adrenal glands [126]. Cardiotonic steroids are now considered hormones [127, 128] that regulate salt-sensitive blood pressure [129], salt handling in the kidney [130], and sodium homeostasis [131]. OUA effects require binding to the Na^+, K^+ -ATPase and the activation of intracellular pathways and genes [132], including ERK1/2 [133] and the inositol (3,4,5)-tris-phosphate receptor [134, 135], indicating that Na^+, K^+ -ATPase is a receptor that transduces cardiotonic steroid occupancy into intracellular mechanism. Interestingly, signaling proceeds even when transport activity is completely inhibited [136–138].

The activation of the Src-EGFR-ERK1/2 cascade by OUA regulates cell adhesion in a concentration-dependent manner: 10 nM OUA, a concentration near the hormonal level, increases the degree of sealing of the TJs, inducing the transcription, translation, and expression at the TJs of CLDNs [139], and 300 nM or higher concentrations of OUA promote cell detachment resulting from TJ, AJ, De, GAPJ, and FA disassembly, endocytosis,

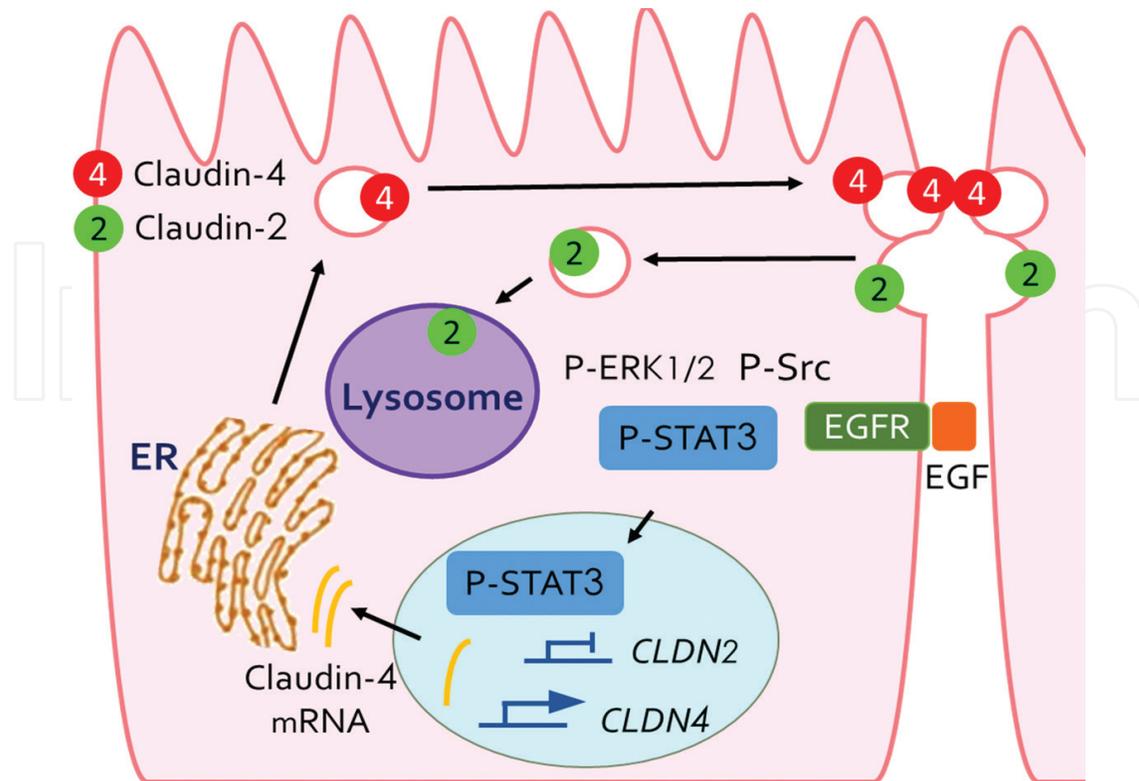


Figure 4. EGF triggers a Src-ERK1/ERK2-STAT3 cascade to induce the degradation of CLDN-2 in the lysosomes. Occupancy of EGFR by its ligand induces the simultaneous phosphorylation of the kinases Src, ERK1/ERK2, as well as the phosphorylation of the transcription factor STAT3. The phosphorylated STAT3 is translocated into the nucleus, where it upregulates the transcription of CLDN-4 at the same time downregulates that of CLDN-2. In the cytoplasm, the same cascade plays a role in the induction of CLDN-2 endocytosis and CLDN-4 exocytic fusion, events that result in the lysosomal degradation of CLDN-2, an insertion of CLDN-4 at the TJs, and an increment of the degree of sealing of the TJs.

and posterior degradation of their cell adhesion molecules [140]. Occludin, CLDN-2, and CLDN-4 endocytoses are clathrin-dependent [141]. 300 nM but not 10 nM OUA increases p62 signal and its colocalization with CLDN-2 in MDCK cells; degradation of CLDNs at 300 nM OUA is inhibited with NH_4Cl and bafilomycin A1 [141], suggesting that ouabain activates CLDN-2 degradation through autophagy (**Figure 5**). OUA increments the size of intracellular structures that bind an antibody against Rab11, a recycling endosome marker, indicating that OUA is not inducing recycling of CLDN-2 (**Figure 6**). The mechanism of OUA action is shown in **Figure 7**.

The final outcome of the treatment with high concentrations of OUA is the detachment and death of OUA-sensitive cells [132, 137, 140, 142]. Ionic imbalance that results from the inhibition of the enzyme has been considered the prime cause of cell death given the fame of the $\text{Na}^+\text{K}^+\text{-ATPase}$ as an ion transporter. However, cells do not detach when they are cultured in low K^+ medium [137], which mimics the diminished $[\text{K}^+]_i$ content induced by OUA. The cytotoxic action of OUA in humans and rodent cells depends on the features of the type α subunit expressed, rather than by any downstream components of the cell

death machinery [142]. In this respect, epithelial cells expressing a OUA-resistant isoform of Na^+, K^+ -ATPase do not detach when they are incubated in low K^+ - or K^+ -free medium [137, 143]. Therefore, ionic imbalance by itself is not sufficient to detach cells; OUA and the activation of kinases (p38 tyrosine kinases, Src, and ERK1/2) are necessary (**Figure 7**), a finding that agrees with the triple role of the Na^+, K^+ -ATPase: transporter, signaling receptor, and cell-cell adhesion molecule [144].

4.4. Autophagy plays a role in claudin-16 degradation in lysosomes

Renal hypomagnesemia with hypercalciuria and nephrocalcinosis is an autosomal recessive disease characterized by abundant renal Mg^{2+} and Ca^{2+} wasting that causes renal

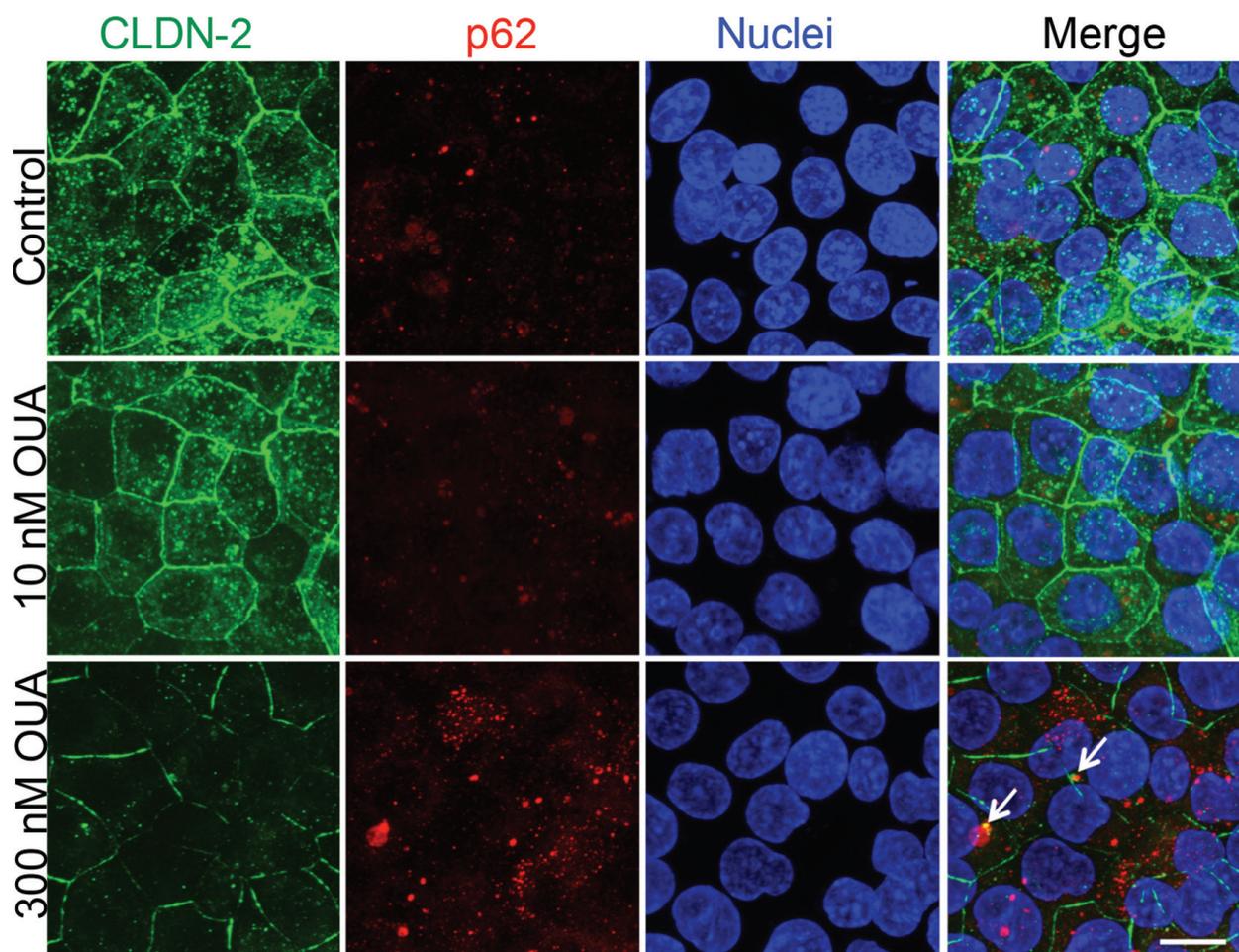


Figure 5. High ouabain concentrations increase autophagy. Control MDCK cells have their CLDN-2 localized at the TJs, in a normal quantity, and in the cytoplasm in numerous spots (green); p62 shows no colocalization with CLDN-2. Upon incubation in media containing ouabain (OUA) 10 nM images remains unchanged, indicating that low OUA concentration does not activate autophagy. On the contrary, the incubation with OUA 300 nM decreases CLDN-2 all around the cell and increases p62 that colocalizes with internalized CLDN-2 (white arrows). This result supports the observation that 300 nM OUA increases autophagy of CLDN-2. Confluent monolayers of MDCK cells were grown on coverslips overnight and then incubated with control media, ouabain 10 nM or 300 nM for 20 h. Barr corresponds to 10 μm .

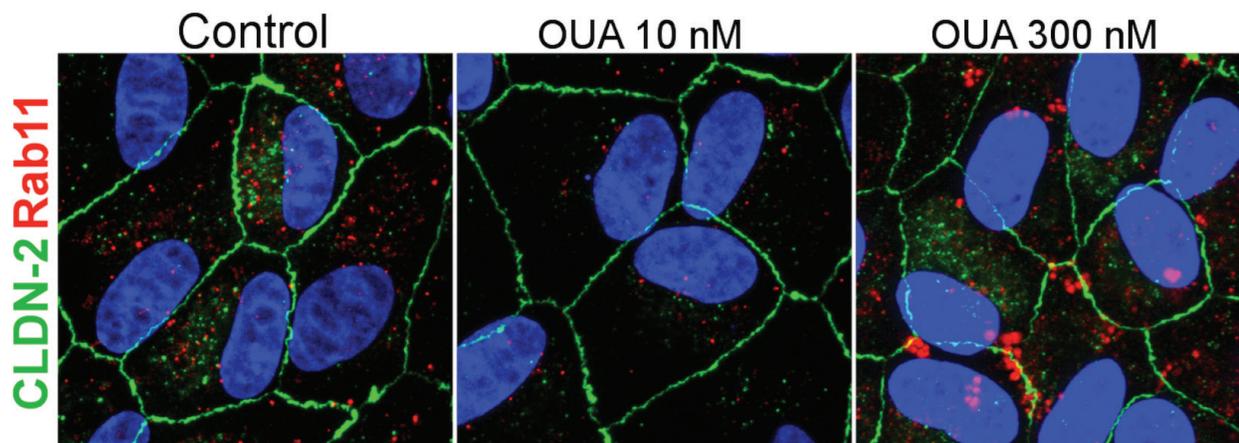


Figure 6. Ouabain does not induce recycling of CLDN-2. MDCK cells monolayers were plated on glass coverslips overnight and incubated in control conditions (control) or in media with OUA 10 or 300 nM for 6 h. Cells were stained with antibodies against CLDN-2 and Rab11, a small GTP-binding protein of the recycling endosome. At this time, CLDN-2 has not been degraded yet and does not colocalize with Rab11 at any condition, suggesting that CLDN-2 is not internalized through the recycling endosome. Nevertheless, ouabain 10 nM decreases the intensity of the signal and the number of spots observed in the cytoplasm, implying that low OUA concentrations decrease recycling, while 300 nM increases the signal as it corresponds to cells with very active endocytosis. These results indicate that under OUA stimuli there seems to be a very active endocytic pathway, but CLDN-2 is not being recycled nor internalized through it.

parenchymal calcification and renal failure. It can only be cured through renal transplantation. The illness results of the lack of stable expression of CLDN-16 and/or CLDN-19 caused by mutations in *CLDN-16* and *CLDN-19* genes [145, 146]. Patients with a mutation in *CLDN-19* show hypomagnesemia, yet this symptom is accompanied by malfunction of several glands, as well as visual impairment [146, 147]. Claudin-16 protein forms paracellular Ca^{2+} pores, while CLDN-19 forms anion impermeable TJs [148, 149]. Both CLDNs form stable membrane dimers able to conduce cations which are bound to the dimers of the neighboring cell. Dimer formation depends on the lateral interaction of their third and fourth transmembrane domains of both CLDNs. Mutations in these domains, which impair dimerization, decrease transepithelial permeability [150]. Most known disease-related mutations of CLDN-16 provoke protein misfolding and induce the accumulation of the defective protein in the endoplasmic reticulum and its degradation in the proteasome [151, 152]; other CLDN-16 mutants reach the plasma membrane but are unable to bind the ZO-1 scaffold; consequently, they are efficiently endocytosed, delivered to the lysosome, and degraded there [151, 153]. Inhibitors of endocytosis may provide novel therapeutic strategies [152]. CLDN-16 phosphorylation of serine 217 stabilizes it in TJ, but when this phosphorylation is inhibited, CLDN-16 is dissociated from ZO-1 and sent to degradation in lysosome, which decreases TER and increases the transport of Mg^{2+} from the apical to the basolateral domain [154]. However, phosphorylation can also induce degradation in lysosomes or proteasomes [151]. Thus, in salivary glandular epithelial cell, SMG-C6, the activation of M3 muscarinic receptor with carbachol switches on ERK1/2, which in turn phosphorylates CLDN-4 at the serine 195. The phosphorylated CLDN-4 is endocytosed in clathrin-coated vesicles and subsequently degraded in the proteasome, a phenomenon that decreases TER [155].

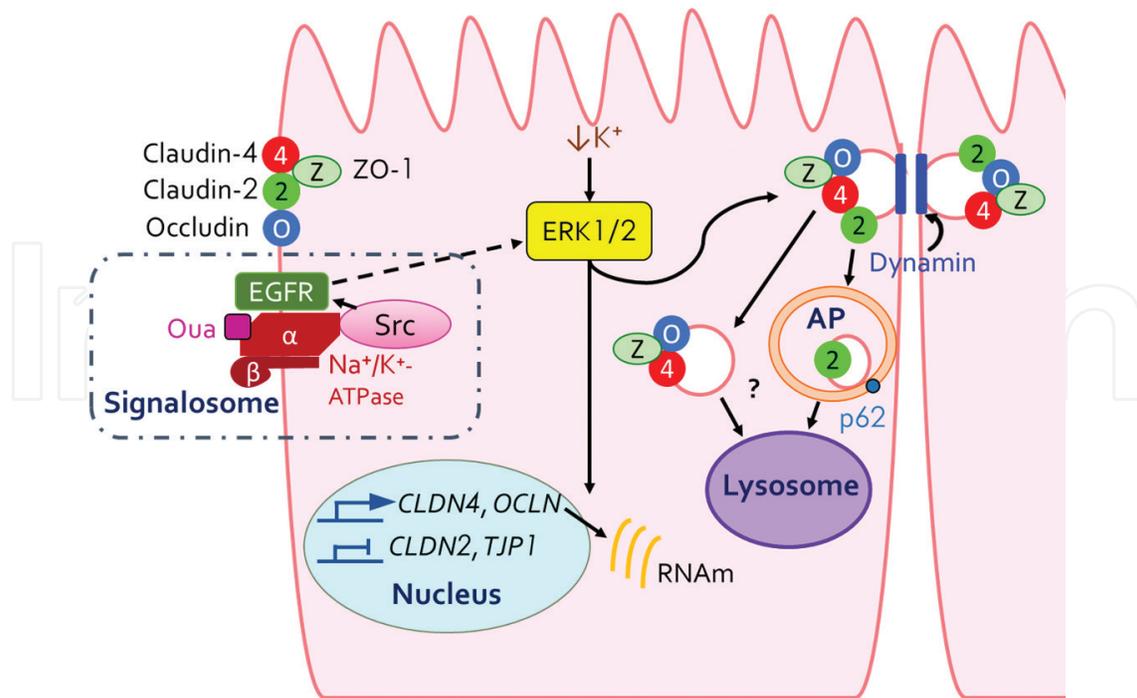


Figure 7. A high concentration of ouabain induces endocytosis and lysosomal degradation of claudins. OUA induces the formation of the signalosome (structure enclosed by the interrupted line), a caveolar complex including some Na⁺,K⁺-ATPases, and their associated Src and EGF receptors (EGFR). OUA activates the Src-ERK1/ERK2 pathway, which induces the clathrin- and dynamin-dependent endocytosis of TJ components. Our results indicate that there are two types of endocytic vesicles: one containing a core complex with essential TJ proteins, such as ZO-1, OCLN, and CLDN-4, and a second one consisting of components that confer a differentiated functional characteristic to TJs, such as CLDN-2, that makes TJs permeable to water and Na⁺. Src-ERK1/ERK2 pathway is also required to reduce CLDN-2 and ZO-1 mRNA levels. Surprisingly, during the OUA-induced aperture of the TJs, the cellular content of CLDN-4 and OCLN mRNAs increases. *TJP1* is the gene that codes for ZO-1, and EGFR is the receptor of EGF. Ionic imbalance, indicated by the lowering of K⁺, may also activate ERK1/2.

5. Role of lysosomes in the degradation of adherens junctions

5.1. Biology of adherens junctions

In chordates, AJs are Ca²⁺-dependent cell-cell adhesions between neighboring epithelial cells at the lateral domain, immediately below the TJs (Figure 1, red). In prechordates, AJs present an inverted localization with respect to the TJs: AJs are the most apical junction of the lateral membrane, placed over the septated junctions. AJs consist of the nectin-afadine and the cadherin-catenin complexes. The nectin's complex forms a scaffold necessary for the assembly of the AJs [156–159], whereas the cadherins serve as homotypic adhesion receptors [160, 161]. The associated plaque proteins catenins and afadins, in turn, bind the receptors to the cytoskeleton of actin [162, 163]. The homotypic adhesion of cadherin plays an important morphogenetic role because it underlays the selection and association of cells of the same type to form specific tissues, a process denominated “cell sorting” [164]. Based on the fact that there are numerous cadherins in the unicellular choanoflagellate *Monosiga brevicollis*, and that some of these proteins are expressed on the collar, a structure rich in sticking microvilli that traps and endocytose bacteria from the media to get nutrients, it has been

suggested that cadherin-based intercellular adhesion may be originated from the co-option of the “trapping food” function of ancestral cadherins of unicellular organisms [165].

5.2. Endocytic route and selective autophagy degrade key proteins of adherens junctions

In a normal epithelium, β -catenin is mostly associated to E-cadherin at the plasma membrane, and the cytosolic pool of β -catenin is kept low by degradation in the proteasome. However, a proliferation signal, triggered by a Wnt ligand, impedes the β -catenin degradation and induces its accumulation in the nucleus to activate proliferation (**Figure 8B**) [27]. E-cadherin is degraded by lysosomes through an endocytic route [166]. The cytoplasmic domain of E-cadherin has an endocytosis signal that is normally masked by α , β and p120 catenins [167]. The induction of E-cadherin endocytosis and degradation starts with the tyrosine phosphorylation of E-cadherin by the kinase Src that promotes the disassembly of the cadherin-catenin complex. Then, the phosphorylated E-cadherin is ubiquitinated on the cytosolic domain by the ubiquitin ligase Hakai. The adhesion protein is then endocytosed and delivered to the lysosomes via early and late endosomes (**Figure 8A**) [168]. β -Catenin may be degraded through an alternative mechanism. Thus, when there are nutrients available, a low normal level of autophagy results from the fact that most β -catenin is repressing the transcription of p62. Under starvation, β -catenin interacts with LC3II and is itself targeted for autophagic degradation (**Figure 8B**) [169]. During embryonic development, cadherin-6 restrains autophagy and drives an epithelial-mesenchymal transition (EMT) to allow a mesenchymal migratory phenotype, which is exacerbated in thyroid cancer [170]. As it happens with other junctions, the role of β -catenin is context dependent. The EMT induced by the transforming growth factor β 1 in mouse kidney proximal tubular epithelial C1.1 cells is accompanied by induction of autophagy and by the nuclear translocation of β -catenin [171].

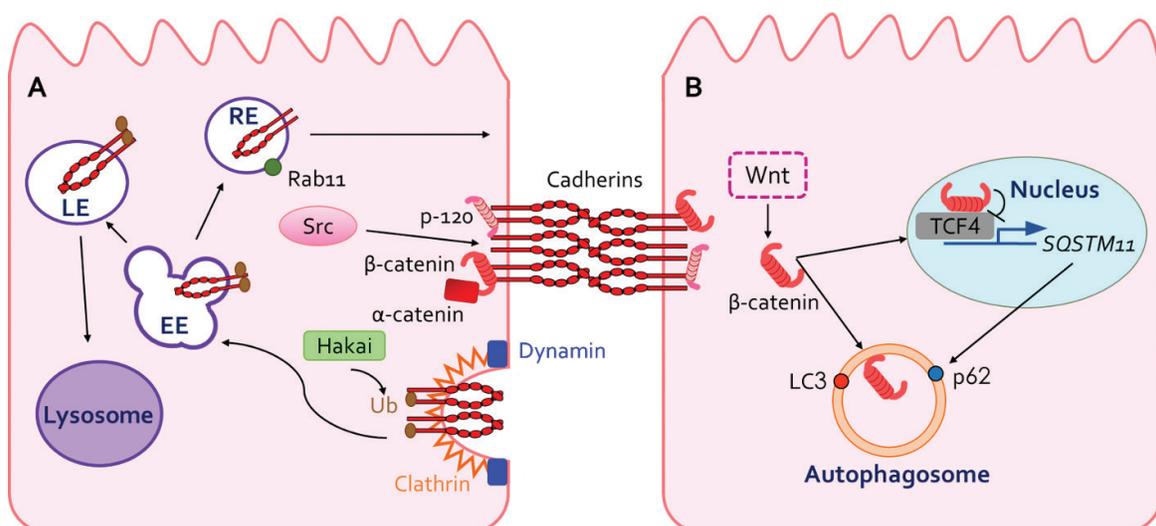


Figure 8. Degradation of E-cadherin and β -catenin involves endosomal lysosomal and autophagic routes. (A) Normal conditions. When nutrients are abundant, E-cadherin and β -catenin are degraded through the endocytic-lysosomal and proteasomal routes, respectively. (B) Starvation. Under nutrient shortage, β -catenin switches to a selective macroautophagy for degradation. Wnt represents the WNT signaling cascade, TCF4 is the transcription factor 1.

6. The peculiar nonselective autophagy mechanisms of degradation of large portions of communicating junctions and desmosomes

6.1. Biology of communicating junctions and desmosomes

GAPJs are molecular ducts that communicate the cytoplasm of contiguous cells and allow the epithelium to respond coordinately to various stimuli or extracellular signals (**Figure 1**, yellow). These junctions are made up of tetraspan proteins: connexins in chordates and innexins in prechordates [172]. Six connexins polymerize to form a hemichannel or connexon in a cell, which attaches to a connexon in the neighboring cell, forming in this manner an intercellular channel that can be opened by diverse stimuli. The dense clustering of tens to thousands of intercellular channels originates a GAPJ [173–175]. Connexins are associated with a scaffold of ZO-1 or ZO-3, vinculin, Src, and tubulin [176]. This association is important for the localization of connexons, the formation of the multimolecular clusters of intercellular channels in the plasma membrane, and the regulation of intercellular communication [177].

Des are cell-to-cell adhesion structures that confer mechanical strength to epithelia and cardiomyocytes. These junctions are composed of five main proteins: the desmosomal cadherins, desmogleins, and desmocollins are the receptors for adhesion. Their cytoplasmic tails bind to plaque proteins of the armadillo family, plakoglobin and plakophilin (**Figure 1**) [178]. The armadillo proteins attach to another plaque protein, desmoplakin, which, in turn, links the protein cluster to the cytoskeleton made of intermediary filaments of cytokeratin [179]. Observations in tissues and cultured cells have shown that Des can adopt a Ca^{2+} -dependent adhesion state that progresses to a Ca^{2+} -independent hyper-adhesion state, a process that requires PKC activation [22, 180–182].

6.2. Macromolecular assemblies of communicating junctions and desmosomes are degraded by autophagy

GAPJs are extremely stable junctional structures: as soon as they are formed, they become indestructible [183, 184]. Nevertheless, they are very dynamic due to the fact that connexins have a very short half-life of only 1–5 h [185]. Consequently, there is a permanent turnover that involves the closure of the intercellular conduction by several stimuli, for example, the binding of EGF to its receptor. The central portion of the GAPJ is then internalized, including the bound hemichannels and membrane of the neighboring cell, forming a peculiar structure named annular GAPJ (**Figure 9**) [186]. In some conditions, annular GAPJ may be recycled back to the plasma membrane [187] although, usually, they are degraded through autophagy; yet, the precise mechanism, the kind of autophagy involved, and the fate of the cells depend on the trigger and/or the cellular context [185, 187–189]. A mechanism that stops autophagy implicates the hijacking of components of the initiation of autophagy, for example, Atg16, by the connexins themselves. On nutrient starvation, connexins release Atg16, the blockade is lost and autophagy proceeds [190].

On liver cells of BRL 3A expressing connexin-43, cadmium inhibits GAPJ intercellular communications and induces the degradation by autophagy of connexin-43 as well as apoptosis.

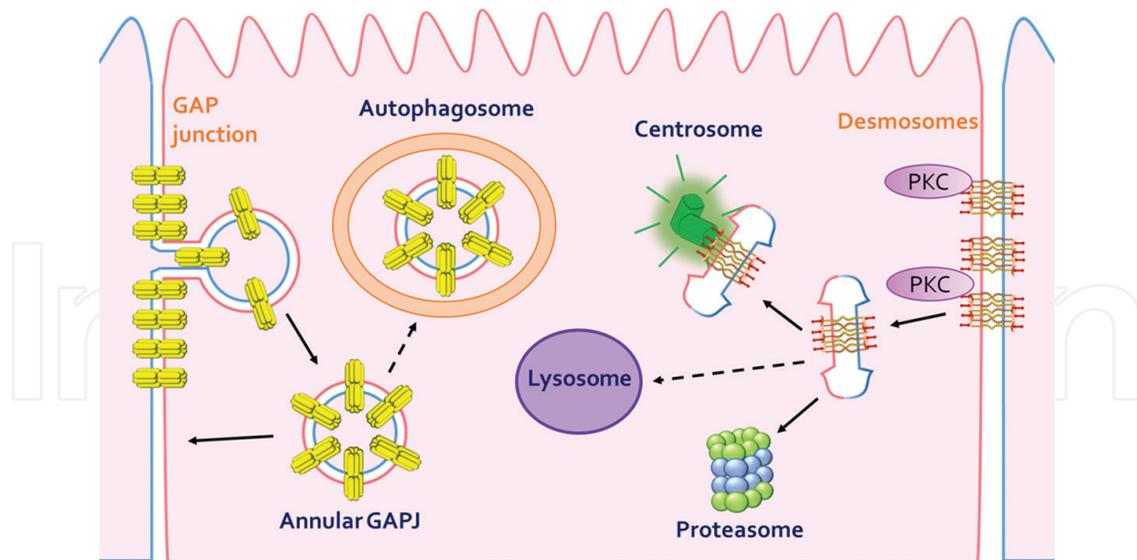


Figure 9. Big portions of GAPJ and complete desmosomes (Des) are degraded by nonselective autophagy. The central portion of the GAPJs is internalized and degraded by autophagy. Dependent on the cell type and condition, complete Des are internalized and degraded by autophagy, and halves of Des are degraded by autophagy and in the proteasome.

Inhibition of autophagy exacerbates Cd^{2+} -induced inhibition of the intercellular communication and apoptotic cell death [188] revealing the protective role that autophagy plays on cell fate.

Des are also very stable structures which can reach a hyper-adhesion state insensitive to Ca^{2+} depletion [191]. It has been shown that a half of Des is internalized after extracellular Ca^{2+} depletion in a PKC- and actin-dependent process [182, 192]. Internalized half desmosome is then transported by kinesins and microtubules toward the centrosome and remains there without recycle to the plasma membrane. Degradation proceeds in lysosomes and proteasomes [193]. In mouse epidermis, the complete Des are engulfed and internalized [192]. Nevertheless, the degradation mechanism is different when disassembly is triggered with autoantibodies from pemphigus vulgaris patients; in this case, Des disassemble in smaller complexes made of the autoantibody, desmoglein-3, and plakoglobin that are endocytosed and delivered to the lysosomes through the endocytic route [194].

7. Continuous recycling in focal adhesion requires autophagy

7.1. Biology of focal adhesion and hemidesmosomes

FAs, also known as focal contacts, and HDes are the cellular junctions that attach cells to the extracellular matrix. HDes are common in stratified epithelia and bind epithelial cells to the underlying extracellular matrix (**Figure 1**, blue) [6]. The adhesion receptors of both, FAs and HDes, are transmembrane proteins of the family of integrins, which exist as heterodimers of α and β subunits form. There are 19 α -integrins and 8 β -integrins that combine to form 25 existing heterodimers in mammals [195, 196]. HDes provide stable adhesion and mechanical resistance to epithelial tissues by anchoring the extracellular

matrix to the cytokeratin cytoskeleton, through a protein complex that includes the adhesion receptors $\alpha6\beta4$ integrin, BP180, and the tetraspanin CD151, and the intracellular adapter proteins plectin and BP230 [6]. The expression of several HDes proteins depends on the transcription factor SOXF [197]. While the extracellular region of integrins of FAs binds the extracellular matrix, the cytosolic portion contacts specific plaque proteins such as focal adhesion kinase (FAK) and paxillin, which are important signaling proteins. Other protein components of the FA plaque, such as talin, vinculin, and α -actinin, bind the adhesion receptors to the actin microfilaments [5, 198, 199].

7.2. Role of lysosomes in the regulation of focal adhesions

FAs are essential in cell migration and, therefore, for embryogenesis, wound healing, immune cell function, cancer progression, and promoting metastasis [200]. Cell migration requires endocytosis and recycling of integrins given by endocytic signals in its cytoplasmic tail. These signals bind either clathrin or caveolin-1 to induce integrin endocytosis. Once inside the cell, integrins anchored to protein complexes are sent to the early endosomes, where they can be sorted either to late endosomes and lysosomes for degradation (**Figure 10, 1**) or to recycling endosomes and plasmatic membrane for the assembly of new FAs. A short loop for recycling requires Rab4 proteins and is generally activated in response to growth factors (**Figure 10, 2**); the long loop is Rab11 and Arf6 dependent and delivers integrins to the perinuclear recycling compartments (PNRCs) and, from there, to the cell membrane (**Figure 10, 3**) [190]. The actin cytoskeleton is essential to the recycling pathway; in fact, depletion of the actin-related protein (Arp) 2/3 or the nucleating-promoting factors such as the members of the Wiskott-Aldrich syndrome protein (WASP) blocks recycling and induces delivery to the lysosomes [5, 198].

FA disassembly is linked to autophagy in two ways: a nonselective autophagy triggered by extreme stress condition, such as starvation or hypoxia (**Figure 10, 4**), and a selective autophagy for housekeeping and quality control that includes ubiquitin-tagged substrate association of them with an autophagic cargo receptor (ACR) attached to LC3II. This autophagy provokes the disassembly of FA Under starvation, $\beta1$ integrin is degraded in autophagosomes in cervix adenocarcinoma epithelial HeLa cells. This autophagy is inhibited by high mTOR activity at the leading edge during migration, which promotes increased motility [201], whereas the activation of selective autophagy promotes FA disassembly in metastatic mammary epithelial cells (4T1) [202]. Thus, the inhibition of autophagosome reduces cancer cell's malignancy, indicating that selective autophagy is also a cell migration regulator (**Figure 10**).

Besides migration, autophagy is linked to anoikis, a type of cell death due to detachment from the substrate. Loss of integrin-mediated adhesion initiates autophagy, which delays anoikis and downregulates apoptotic signals. This process affords cells time to reattach; however, in cancer cells, high autophagic activity after detachment provides resistance and promotes malignancy, allowing the cell to support stress condition, increase motility, and resist anoikis [203, 204].

Although there are several illnesses produced by the lack of HDes protein expression, little is known about HDes degradation.

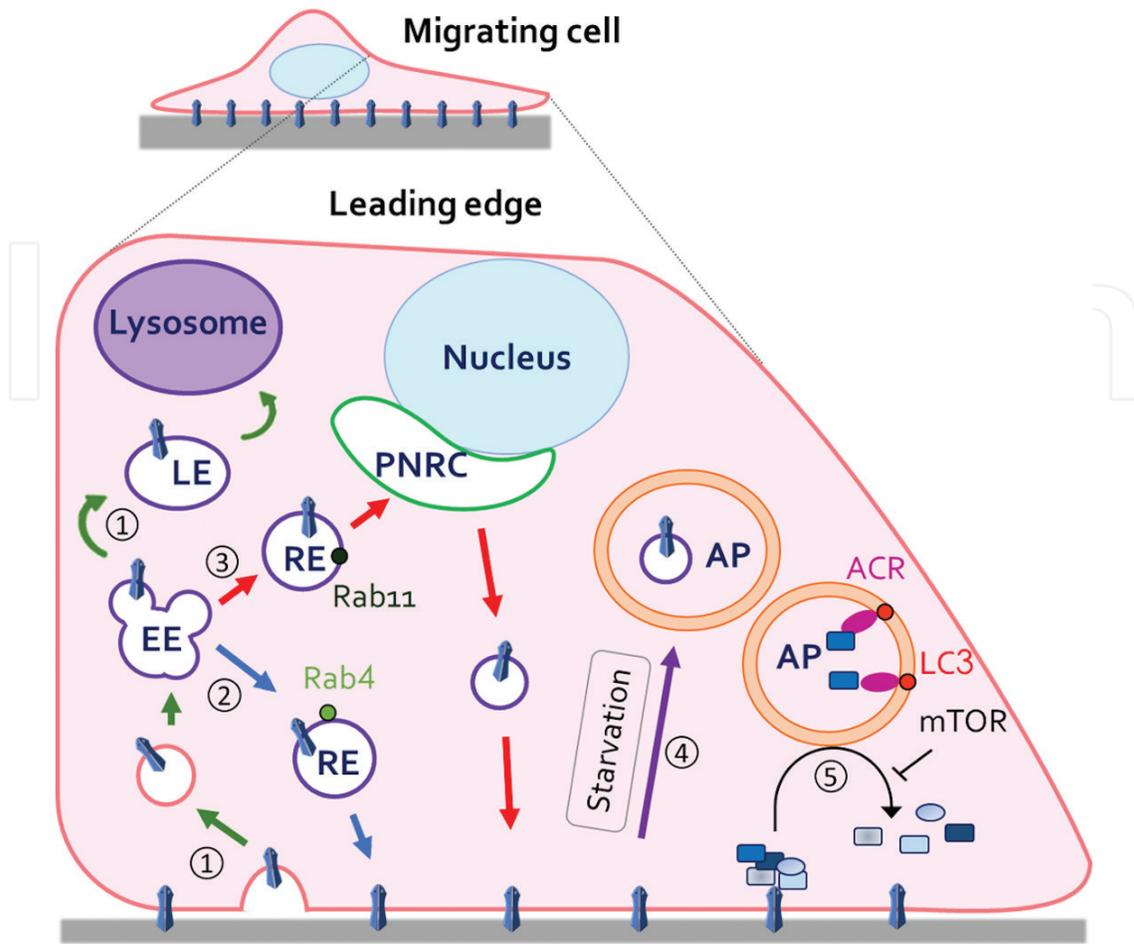


Figure 10. Autophagy is crucial for the recycling of integrins in focal adhesions during migration. Cell migration requires continuous recycling of integrins. (1) Integrin endocytic pathway degradation. (2) Integrin short loop recycling; vesicular transport of integrins from the EE to the Rab4 containing RE, and from there back to the plasma membrane. (3) Integrin long loop recycling; vesicles transport integrins from EE to Rab11 containing RE, later on, to a perinuclear recycling compartment (PNRC) and then to the plasma membrane. (4) During starvation, integrins are endocytosed and directed to the AP. (5) Cell migration leading edge. FA's protein paxillin is recognized by autophagic cargo receptors (ACR) and degraded by selective macroautophagy, which induces FAs disassembly through a mTOR dependent pathway. In the leading edge, FAs must be first formed and then degraded to allow motility. Autophagy plays a crucial role in this process.

8. Concluding remarks

Lysosomal degradation mechanisms are crucial for the formation, differentiation, and degradation of epithelial cell junctions. Epithelial cells use selective autophagy to degrade claudin-2, in response to the stimulation with the epidermal growth factor. Ouabain, at a concentration close to the hormonal, does not induce autophagy of tight junction proteins and, at high concentrations though, induces lysosomal degradation that can involve autophagy. The precise sequence of events and outcome of each lysosomal degradation mechanism is context dependent; nevertheless, it is clear that the degradation through macroautophagy of large plaque of complete communicating junctions and desmosomes, as well as of desmosomal halves, takes place either in natural tissues or in cultured cells. It is also clear that the

desmosomal transition from weak to strong adhesion stages requires lysosomal activity, that β -catenin undergoes selective autophagy in some conditions and that E-cadherin degradation is performed in lysosomes through an endocytic route.

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