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Review

Endocytic trafficking of connexins in cancer pathogenesis

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ABSTRACT

Gap junctions are specialized regions of the plasma membrane containing clusters of channels that provide for the diffusion of ions and small molecules between adjacent cells. A fundamental role of gap junctions is to coordinate the functions of cells in tissues. Cancer pathogenesis is usually associated with loss of intercellular communication mediated by gap junctions, which may affect tumor growth and the response to radio- and chemotherapy. Gap junction channels consist of integral membrane proteins termed connexins. In addition to their canonical roles in cell-cell communication, connexins modulate a range of signal transduction pathways via interactions with proteins such as β-catenin, c-Src, and PTEN. Consequently, connexins can regulate cellular processes such as cell growth, migration, and differentiation through both channel-dependent and independent mechanisms. Gap junctions are dynamic plasma membrane entities, and by modulating the rate at which connexins undergo endocytosis and sorting to lysosomes for degradation, cells can rapidly adjust the level of gap junctions in response to alterations in the intracellular or extracellular milieu. Current experimental evidence indicates that aberrant trafficking of connexins in the endocytic system is intrinsically involved in mediating the loss of gap junctions during carcinogenesis. This review highlights the role played by the endocytic system in controlling connexin degradation, and consequently gap junction levels, and discusses how dysregulation of these processes contributes to the loss of gap junctions during cancer development. We also discuss the therapeutic implications of aberrant endocytic trafficking of connexins in cancer cells.

1. Introduction

Gap junctions comprise assemblies of channels that provide for the direct exchange of ions, low-molecular-weight second messengers, and nutritional metabolites between cells [1]. Gap junction channels consist of integral membrane proteins called connexins [2]. Twenty-one connexin proteins are found in humans [3]. Six connexin proteins can assemble to form a so-called connexon, which can dock with a connexon in a neighboring cell to create a complete gap junction channel. In addition to their canonical role in forming intercellular channels, connexins have several noncanonical functions. For instance, connexons that are located in the plasma membrane but not assembled into gap

junctions can open, thereby providing a pathway for the exchange of ions and small molecules between the intracellular and extracellular environments [4]. Such channels, also called hemichannels, play important roles in paracrine signaling [4]. Moreover, through their intracellular domains, connexins bind to a variety of other cellular proteins, thereby modulating processes such as differentiation, cell proliferation, and apoptosis via channel-independent mechanisms [5–7]. Emerging evidence also suggests that connexins can affect the function of other types of cellular structures involved in intercellular communication, such as tunneling nanotubes and extracellular vesicles [8–11].

In most tissues and organs, connexins have half-lives of 1.5 to 5 hours

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[12–14]. Following gap junction endocytosis, connexins can be shuttled to lysosomes for degradation by one of three mechanisms: 1) fusion between endocytosed gap junctions and lysosomes, 2) autophagy, or 3) sorting of connexins along the endocytic pathway. By altering the rate of connexon endocytosis and degradation, cells can adjust the levels of gap junctions in response to cellular cues [15–22]. Thus, accurate regulation of gap junction endocytosis and degradation is pivotal for proper gap junction function [23].

Cancer pathogenesis is frequently associated with loss of functional gap junctions, and several connexin isoforms have tumor suppressor properties [24–33]. However, the tumor suppressor ability of connexins appears to vary by both cancer type and stage. Moreover, under certain conditions, connexins may promote cancer progression [24–33]. Thus, connexins can be regarded as conditional tumor suppressor proteins [30]. The absence of gap junctions in cancer cells may be due to loss of connexin expression at the transcriptional or translational level [32,34,35]. In other situations, connexins are expressed in cancer cells but are subjected to aberrant post-translational regulation that results in their relocalization from the plasma membrane to various types of intracellular compartments [32,34,35]. Mounting evidence suggests that this process may involve dysregulation of the transport of connexins along the endocytic pathway. Moreover, several environmental toxicants that are known or suspected to be human carcinogens inhibit gap junction channels and induce their degradation [36–40]. Aberrant trafficking of connexins in the endocytic system appears to be intrinsically involved in mediating the loss of gap junction channels promoted by such agents.

Here, we first highlight the role played by the endocytic system in the degradation of connexins under normal conditions. We then provide an overview of and discuss the experimental evidence indicating that aberrant trafficking of connexins along the endocytic pathway is important in mediating the gap junction loss during different stages of cancer development. We also discuss the therapeutic implications of the derailed endocytic trafficking of connexins in cancer cells.

2. Formation and regulation of gap junctions

Connexins are tetraspan integral membrane proteins with N- and C-terminal cytoplasmic tails [41] (Fig. 1A). Connexin protein family members are designated with the abbreviation Cx followed by their predicted molecular mass in kilodaltons [1]. Connexin 43 (Cx43) is the most commonly expressed connexin in human tissues and the best studied member of this protein family [1]. Connexins are inserted into and obtain their transmembrane topology in the endoplasmic reticulum during translation [42,43]. A relatively large proportion of the pool of newly synthesized connexins is subjected to endoplasmic reticulum-associated degradation (ERAD) [44–46]. Connexins not undergoing ERAD follow the secretory pathway to the plasma membrane [47,48]. As they are trafficked via the secretory pathway, the connexins oligomerize into connexons, which consist of six connexins and are the precursors to the intercellular channels that constitute the gap junctions [49–51] (Fig. 1B). In which organelle the oligomerization of connexins occurs and how this is regulated is considered to be connexin-isoform specific [47]. In the *trans*-Golgi network, the newly formed connexons are shuttled into vesicles, which traffic the connexons to the plasma membrane via pathways that are dependent on or independent of microtubules [50,52–55]. A gap junction channel is formed when a connexon docks with a connexon in an adjacent cell (Fig. 1B). The gap junction channels assemble to form distinct plaques in the plasma membrane denoted as gap junctions, which may comprise only a few or up to thousands of intercellular channels. Once a newly synthesized connexon enters the plasma membrane, it may diffuse until it encounters the outer edge of a preexisting gap junction plaque, where it docks with a connexon in another cell to establish a new gap junction channel [52,54,56,57]. Alternatively, connexons are sorted directly to preexisting gap junction plaques or to their immediate vicinity [55].

A connexon and a gap junction channel may comprise two or more connexin isoforms, and the gap junction channel permeability is, among other factors, determined by its connexin isoform composition (Fig. 1B) [1]. The steady-state level of gap junctions is determined by numerous factors, such as the rates of connexin synthesis, ERAD, connexon assembly, sorting of connexons to the plasma membrane, gap junction assembly, endocytosis of hemichannels and gap junctions, connexon recycling, and degradation of connexins in lysosomes [1]. The intracellular regions of connexins play major roles in modulating both the intracellular trafficking of connexins and gap junction channel gating by mediating protein-protein interactions and by containing amino acid residues that can be post-translationally modified [47,58]. Among the post-translational modifications that regulate connexins are phosphorylation [59], acetylation [60,61], S-nitrosylation [62], ubiquitination [16,18,63,64], and SUMOylation [65]. In addition, gap junction channel gating is strongly influenced by changes in voltage and the pH and calcium concentration of the intracellular environment [41,66].

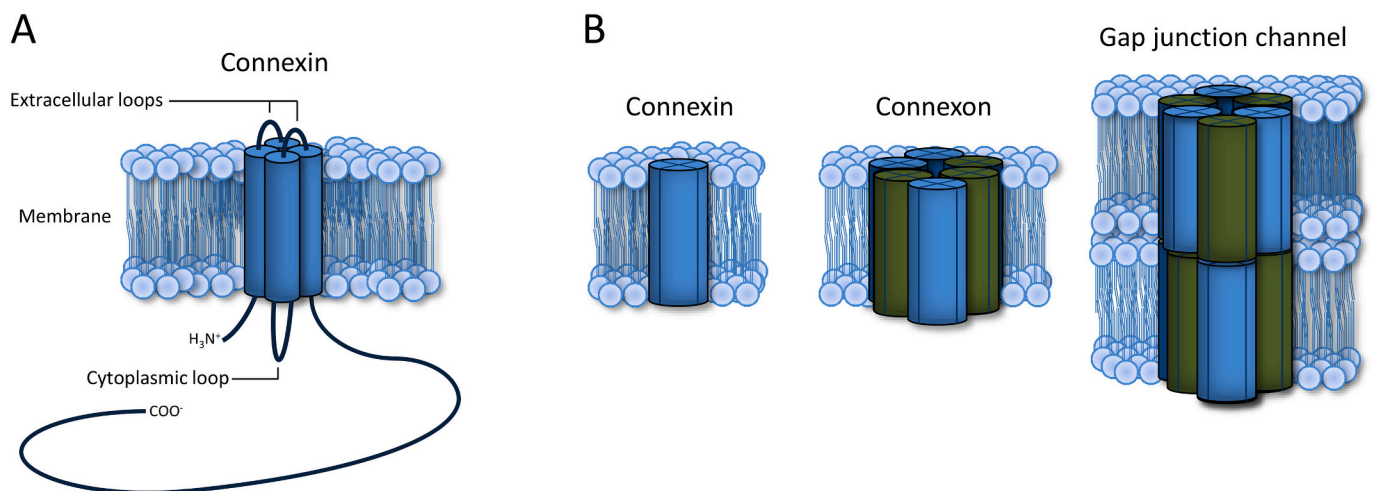Besides full-length Cx43, internal translation initiation can result in

Fig. 1. Depiction of connexins, connexons, and gap junction channels. (A) Connexins are tetra-membrane-spanning proteins whose N- and C-termini are located in the cytoplasm. (B) Six connexins oligomerize to form a connexon. A connexon can comprise six identical connexin isoforms (homomeric connexon) or two or more different connexin isoforms (heteromeric connexon). Two connexons of adjacent cells dock to form a gap junction channel, which may comprise two identical homomeric or heteromeric connexons (homotypic channels) or two different homomeric or heteromeric connexons (heterotypic channels).

the cellular expression of six N-terminally truncated Cx43 isoforms [67–70]. These truncated versions of Cx43 can modulate the trafficking and degradation of full-length Cx43 or they can translocate to the nucleus to affect gene transcription [47,48,71–74].

3. Endocytic trafficking in normal and cancerous cells

Small molecules, including ions, sugars, and amino acids, are transported across the plasma membrane by transmembrane protein pumps and channels [75]. In contrast, macromolecules must be transported into the cell by a process called endocytosis, in which pieces of the plasma membrane invaginate and pinch off to form intracellular membrane-bound vesicles. Endocytosis is important in regulating the levels of receptors, channels, and cell-cell junctions at the plasma membrane [75]. The endocytic system consists of a number of membrane-enclosed, acidic compartments that display a tubulo-vesicular morphology and are highly dynamic in nature [76–78]. It receives cargo from the plasma membrane via endocytosis, from the *trans*-Golgi network and from the cytoplasm via autophagy. After entering the endocytic system, the cargo is either recycled to the plasma membrane or transported to lysosomes for degradation.

The early endosome is the initial destination for cargo molecules internalized from the plasma membrane and is a major sorting station in the endocytic system [76–78]. The early endosomes have a complex structure, containing both tubular and vacuolar domains. The limiting membrane of early endosomes comprises a mosaic of subdomains, each with distinct molecular compositions and functions [79]. Many of these domains are involved in the sorting of cargo and in generating vesicle carriers that shuttle the cargo to other organelles. Some molecules that have entered early endosomes via endocytosis may be recycled to the plasma membrane [80]. Other domains are involved in mediating the sorting of cargo into the intraluminal vesicles of the endosomes. The formation of such vesicles involves the inward budding of parts of the outer membrane of the endosomes into their lumen. The endosomes that contain such intraluminal vesicles are also known as multivesicular bodies [81].

The limiting membrane of early endosomes is characterized by a high concentration of the small GTPase Rab5, which is a master regulator of the endocytic pathway [82]. It is also enriched in the phosphoinositide phosphatidylinositol 3-phosphate (PI3P) [83], and the presence of both GTP-Rab5 and PI3P acts as a signal for the recruitment of several different effector proteins that simultaneously bind to GTP-Rab5 and PI3P [84]. Among these proteins is early endosome antigen 1 (EEA1), which mediates the homotypic fusion of early endosomes [85–88]. Membrane tethering and fusion in the endocytic pathway involves extensive cooperation both between different Rab effectors and between Rab effectors and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) [82].

Membrane-associated cargo located in the endocytic membranes that is destined for degradation in lysosomes is sorted into the intraluminal vesicles of the endosomes [76–78]. This process is orchestrated by the endosomal sorting complex required for transport (ESCRT), which is made up of four cytosolic protein complexes, in addition to several accessory proteins, such as the AAA-type ATPase vacuolar protein sorting-associated protein 4 (VPS4) and ALG-2-interacting protein X (Alix) [89]. Initially, the ESCRT complex is targeted to the outer membrane of early endosomes through binding of one of its components, hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), to PI3P [89]. Cargo proteins destined for lysosomal degradation are tagged by ubiquitin, often K63-linked polyubiquitin chains. In specific microdomains of the limiting membrane of the endosomes, the ubiquitinated cargo proteins are recognized by components of the ESCRT machinery containing ubiquitin-binding domains [89]. After ubiquitinated cargo has been captured by the ESCRT machinery, the cargo is deubiquitinated and sorted into the intraluminal vesicles. Control of cargo ubiquitination by E3 ubiquitin ligases and deubiquitinases is therefore crucial for the

ESCRT-mediated sorting of the cargo into intraluminal vesicles and its subsequent degradation.

The maturation of early endosomes to late endosomes is associated with a gradual exchange of Rab5 for Rab7 and, as a consequence, the recruitment of a different set of effector proteins to the endosomal limiting membrane [82]. Late endosomes subsequently fuse with lysosomes, leading to proteolysis of the endocytosed molecules [90].

In some cases, rather than being targeted to the lysosome for degradation, the intraluminal vesicles of endosomes can be secreted from the cell upon the fusion of endosomes and the plasma membrane and are then known as exosomes [91]. Exosomes have recently been demonstrated to play important roles in intercellular communication [91]. Their formation can involve the ESCRT machinery in a similar manner to the formation of intraluminal vesicles destined for lysosomal degradation or can be independent of ESCRT [91].

Dysregulation of the sorting of cargo in the endocytic system is emerging as a hallmark of cancer cells [92–94]. For instance, by delaying the endocytosis and/or post-endocytic sorting of growth factor receptors to lysosomes, cancer cells may gain prolonged receptor signaling [95]. Derailed endocytosis also has important roles in promoting alterations in cell-cell and cell-extracellular matrix adhesion in cancer cells. For instance, cancer cells may display increased endocytosis and post-endocytic sorting of E-cadherin to lysosomes, which contributes to loss of adherens junctions and consequent loss of epithelial cell polarity and increased migration and invasion potential [96]. Cancer cells also frequently show aberrant regulation of the balance between endocytosis and recycling of integrins, resulting in alterations in the ratio of the level of integrins in the plasma membrane to that in endosomes, which may promote cancer cell migration and invasion [97]. In accordance with the observation that endocytosis and sorting of cargo via the endocytic system are frequently derailed in cancers, the protein components of the molecular machinery that regulates these processes often display altered expression in cancer cells compared with that in normal cells, and some are subjected to mutations [92–94]. In recent years, it has also become clear that exosomes are involved in cancer development and that they have potential as diagnostic biomarkers, therapeutic targets, or anticancer drug carriers [98].

4. Endocytosis of gap junctions

During the endocytosis of a gap junction, the region of the plasma membranes in which the gap junction is located is invaginated into one of the neighboring cells. The resulting double-membrane vacuole is termed a connexosome, also known as an annular gap junction [99,100] (Fig. 2). Thus, the outer membrane of the connexosome stems from the plasma membrane of the cell in which it has been internalized, whereas its inner membrane originates from the plasma membrane of the cell with which the gap junction was formed before it was internalized. Sometimes, entire gap junction plaques can undergo endocytosis, whereas in other situations only a small portion of the gap junction is internalized to form a connexosome. The molecular mechanisms involved in gap junction internalization have been reviewed previously [99] and will therefore only be briefly summarized here. The available experimental data suggest that gap junction internalization involves clathrin [101–104]. Clathrin is recruited to Cx43-based gap junctions by the adaptor AP-2 via two tyrosine-based motifs in the Cx43 C-terminal tail [105,106]. Clathrin also mediates the internalization of gap junctions formed by other connexins, such as Cx31.1 and Cx32 [107,108]. Among other proteins that participate in gap junction internalization are 14-3-3 and myosin-VI [104,109–111]. The fission of the connexosomes from the plasma membrane is mediated by dynamin [110].

Increasing evidence points toward an important role of connexin phosphorylation in the regulation of gap junction internalization. Kinases that have been implicated in promoting gap junction endocytosis by phosphorylating Cx43 include SRC, protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and proline-rich tyrosine kinase 2
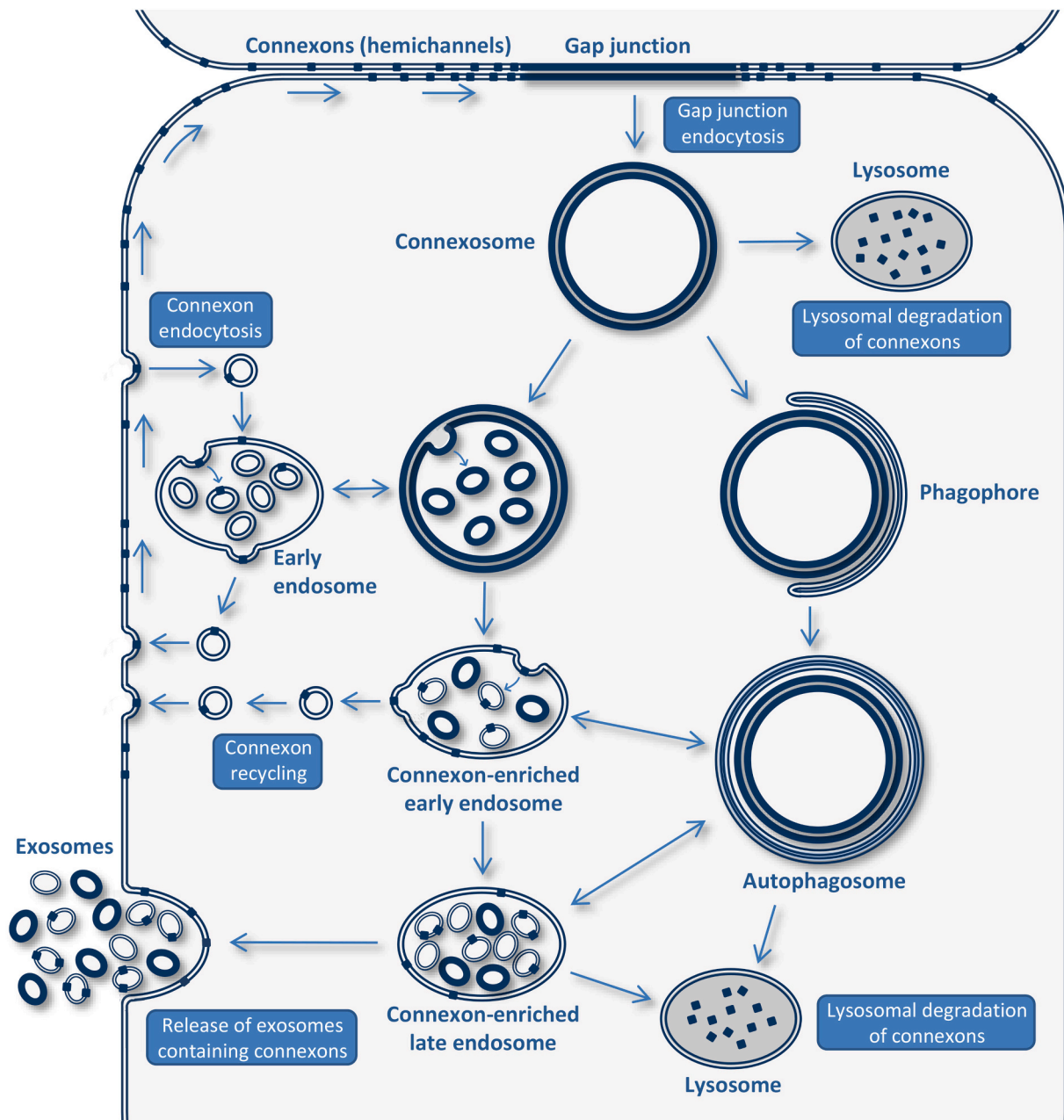
Fig. 2. Pathways for the trafficking of connexons to lysosomes after gap junction endocytosis. The figure depicts the three pathways that have been suggested to be involved in mediating the delivery of connexons to lysosomes after gap junction endocytosis. This is a highly simplified depiction of these three pathways, and the reader is referred to the original articles cited in the main text for a more accurate description of the various steps of the three pathways. In the first pathway, connexosomes fuse directly with lysosomes. In the second pathway, connexosomes are degraded by autophagy. In this process, a phagophore is formed around the connexosome, which eventually closes to form an autophagosome. The autophagosome then fuses with the lysosome to form the autolysosome, where the connexons are degraded. In the third pathway, the connexosome is considered to be transformed into a connexon-enriched multivesicular endosome or a vacuole that resembles a multivesicular endosome. This process is thought to involve undocking of the connexons and the concomitant physical separation of the two membranes of the connexosome, as well as fusion events between the connexosome and early endosomes or lysosomes. The undocked connexons are subsequently thought to be sorted along the endocytic pathway to lysosomes for degradation or possibly recycled from early endosomes to the plasma membrane. There may be cross-talk between the endocytic and autophagosomal pathways to mediate the degradation of connexosomes. As an alternative to being sorted to lysosomes for degradation, the intra-luminal vesicles of endosomes containing connexons can be secreted as exosomes if the endosomes fuse with the plasma membrane. Undocked connexons at the plasma membrane, also known as hemichannels, may also be sorted to early endosomes following endocytosis, after which they may recycle to the plasma membrane or be sorted to lysosomes for degradation.

(PYK2) [18,112–117]. The emerging picture is that endocytosis of Cx43-based gap junctions is controlled by interplay between phosphorylation and ubiquitination of Cx43 [118]. A PY (XPPXY) motif in the Cx43 C-terminus mediates the binding between Cx43 and the E3 ubiquitin ligase NEDD4 [63,119,120]. Studies by Leykauf *et al.* and Spagnol *et al.* have shown that phosphorylation of S279 and S282, located N-terminally to

the PY motif (^{279}pSPMpSPPGY^{286} where pS is phosphoserine), increases the binding affinity with the WW domains of NEDD4 *in vitro* [119,120]. NEDD4 promotes Cx43 ubiquitination and negatively regulates the size of Cx43-based gap junctions by promoting their endocytosis and the subsequent lysosomal degradation of Cx43 [63,121]. Mechanistically, the NEDD4-induced ubiquitination of Cx43 results in increased binding

between Cx43 and the ubiquitin-binding protein EPS15, which has been suggested to mediate gap junction internalization [63,122].

In line with a model in which ubiquitination of Cx43 promotes gap junction internalization and Cx43 degradation, mimicking mono-ubiquitination of Cx43 by fusing ubiquitin to the C-terminal tail of Cx43 results in reduced levels of gap junctions and increased Cx43 turnover [147]. Moreover, two lysine residues in the Cx43 C-terminal tail, K264 and K303, have been suggested to act as ubiquitination sites, and their mutations were found to result in decreased endocytosis and degradation of Cx43, which correlated with larger gap junctions [123]. However, another study in which all lysines of Cx43 were mutated to arginines concluded that Cx43 ubiquitination does not participate in regulating Cx43 degradation [124]. Thus, further studies are required to obtain a more complete understanding of the role of ubiquitination in the regulation of gap junction endocytosis and Cx43 degradation. As well as being subjected to ubiquitination, Cx43 can be modified by small ubiquitin-related modifier (SUMO), which appears to stabilize Cx43 and thereby increase the level of gap junctions [252].

Increasing evidence suggests that, after endocytosis of gap junctions or undocked connexons, the connexons can, under certain conditions, be trafficked back to the plasma membrane [22,125–129]. For instance, after the endocytosis of undocked connexons, certain types of cytosolic stress can impede their post-endocytic sorting to lysosomes and promote their recycling [22]. Once sorted back to the plasma membrane, they can function as hemichannels or form gap junctions [22]. Other studies have reported that entire connexosomes can undergo recycling [126].

The physiological importance of accurate regulation of gap junction endocytosis was recently investigated by Hyland *et al.* with a zebrafish model [23]. The zebrafish were subjected to homozygous deletion of the amino acids 256–289 in the C-terminal tail of Cx43, a region that encompasses both the binding sites for AP-2 and NEDD4, as well as the proposed ubiquitination site K264. These zebrafish were found to have abnormally large gap junctions and an excessive Cx43 protein level in the heart due to impaired Cx43 endocytosis, which was associated with cardiac malformation and dysfunction [23]. They also displayed several other developmental defects, such as aberrant structure and function of the vasculature [23].

Norris has demonstrated that endocytosis of gap junctions may also participate in the transfer of organelles between adjacent cells [130]. By combining three-dimensional electron microscopy and immunogold labeling of Cx43 in ovarian follicles, whole organelles such as endosomes and mitochondria were found to be incorporated into connexosomes as they are formed during gap junction internalization.

5. Endocytic trafficking of connexins under normal conditions

The available experimental evidence suggests that, after gap junction internalization and formation of connexosomes, connexins can follow three distinct pathways en route to lysosomes [131–133] (Fig. 2). In the first pathway, connexosomes directly fuse with lysosomes [20,134–136]. In the second pathway, connexosomes are degraded by autophagy [122,126,137–139]. In this process, a phagophore is formed around the connexosome, eventually creating an autophagosome that encloses the connexosome. Subsequently, the autophagosome fuses with the lysosome, forming an autolysosome in which the connexins are degraded along with other cargo. Notably, connexins are not merely degraded by autophagy but are also modulators of the biogenesis of autophagosomes [140]. In the third pathway involved in the sorting of connexins to lysosomes after gap junction endocytosis, the endocytic system is considered to have a key role [126,128,141–143]. The first step in this pathway involves a morphological conversion of the connexosome into a multivesicular endosome, or a vacuole that resembles a multivesicular endosome, that is rich in connexons. This process involves the physical separation of the inner and outer membranes of the connexosome, which must imply that the connexons undergo undocking [126,142,143]. The process is also associated with fusion of the

connexosome with early endosomes [126,128,141–143] or lysosomes [143]. A possible scenario is that these fusion events result in a lower pH in the lumen of the connexosome, which may facilitate the undocking of the connexons and the concomitant splitting of the two membranes of the connexosome. Subsequently, the undocked connexons are thought to be sorted from early endosomes along the endocytic pathway to lysosomes for degradation, or, alternatively, recycled from the early endosomes to the plasma membrane [126,128,141–143] (Fig. 2). In this model, the early endosome is considered to play a key role in determining whether endocytosed connexons are either subjected to sorting to lysosomes for degradation or recycled to be reutilized in the formation of new gap junctions [128,142]. Thus, this model implies that the molecular components that are involved in the connexon sorting decisions made in the early endosomes are important parts of the molecular machinery that maintains the dynamic nature of gap junction size [128].

Several lines of experimental evidence are in accordance with a model in which the endocytic system plays important roles in the constitutive degradation of connexins. In our laboratory, we have studied the subcellular localization of Cx43 at the ultrastructural level by performing immunoelectron microscopy analyses of IAR20 rat liver epithelial cells, which endogenously express Cx43, and of HeLa cells stably transfected with Cx43 [142,144]. These studies used a technique for immunolabeling in which the cells are subjected to relatively mild chemical fixation prior to freezing, cryosectioning, and immunostaining [145–147]. This method does not involve denaturing steps such as dehydration in organic solvents. Moreover, because the cells are subjected to only mild chemical fixation and are not embedded in resins, most antigens retain their antigenicity [145–147]. By using this technique, we found that Cx43 frequently localizes to endosomes under basal conditions, both in their outer membrane and in their intraluminal vesicles [142,144].

The notion that the constitutive degradation of Cx43 involves the endocytic pathway is further supported by super-resolution microscopy analyses. In these studies, Cx43 was frequently found to localize to the lumen of intracellular vesicular compartments positive for EEA1, an early endosomal marker, or for LAMP-1, a marker of late endosomes and lysosomes [144].

Further evidence indicating that the endocytic pathway is involved in the constitutive degradation of Cx43 has been provided through the analysis of cells that are experimentally induced to form enlarged endosomes. Ectopic expression of a Rab5 mutant that is GTPase-defective, Rab5-Q79L, increases homotypic fusion between early endosomes, causing unusually large early endosomes [148]. Such large endosomes have been extensively used to analyze the localization of proteins and lipids in the different microdomains of early endosomes. Cx43 is localized to the lumen of such large early endosomes, as determined by confocal fluorescence microscopy [149].

The extent of the localization of connexins to endocytic pathway compartments may vary by cell cycle stage. For instance, during mitosis, gap junctional communication is lost, and this is correlated with accelerated endocytosis of Cx43-based gap junctions and sorting of Cx43 to early endosomes [125,127]. At late stages of mitosis, Cx43 undergoes recycling from early endosomes, and once it has returned to the plasma membrane, it may participate in forming *de novo* gap junctions after cytokinesis [125,127]. In addition to the cellular pool of Cx43 that is assembled into gap junctions, Cx43-based hemichannels can also be sorted to early endosomes in a process mediated by Rab5 and clathrin [15].

The role of the endocytic pathway in the degradation of other connexin isoforms besides Cx43 is less well characterized. However, Spray and colleagues have shown that a considerable fraction of Cx32-containing vesicles isolated from rat hepatocytes are positive for EEA1, suggesting that they are early endosomes, whereas a smaller fraction of the vesicles were positive for LAMP-1 [150]. In accordance with this observation, Cx32 has been found to localize to the lumen of

Rab5-Q79L-induced large endosomes [107]. Other connexin isoforms, such as Cx37, have also been shown to partly colocalize with EEA1 and LAMP-1 or -2 under constitutive conditions, as determined by confocal fluorescence microscopy [151].

Pointis and colleagues [152] have shown that Cx33 can sequester Cx43 in early endosomes in Sertoli cells to inhibit gap junction intercellular communication. Sertoli cells are located in the seminiferous tubules of the testis and control germ cell proliferation and differentiation locally through paracrine signaling and direct intercellular junctions [153]. As determined by live-cell imaging of a mouse Sertoli cell line ectopically expressing Cx33 and Cx43 with fluorescent tags, heteromeric Cx33/Cx43 gap junctions exhibit an increased rate of endocytosis compared with gap junctions formed by Cx43 alone [154]. Moreover, after the endocytosis of Cx33-/Cx43-based gap junctions, both Cx33 and Cx43 colocalize with Rab5, a marker for early endosomes [152]. The ability of Cx33 to sequester Cx43 in early endosomes may be connexin isoform specific because it does not inhibit gap junctions formed by Cx26 or Cx32 [155]. The extent to which other connexin isoforms can inhibit Cx43-based gap junctions by sequestering Cx43 in early endosomes remains to be determined.

Carette *et al.* have demonstrated by transmission electron microscopy that the endocytic pathway and autophagy can simultaneously participate in mediating the degradation of a connexosome [126]. This observation is in accordance with the emerging concept that there is a tight interplay between the endocytic pathway and autophagy in mediating the degradation of cellular proteins internalized from the plasma membrane [156].

6. Aberrant endocytic trafficking of connexins in cancer development

6.1. Alterations in endocytic trafficking of connexins in response to exposure to non-genotoxic carcinogens

6.1.1. Environmental toxicants that are known or suspected to be carcinogenic to humans cause dysregulation of endocytosis and post-endocytic trafficking of Cx43

Many chemical carcinogens are genotoxic [157,158]. However, a large fraction of chemical carcinogens induce cancer via non-genotoxic mechanisms; i.e., they increase the incidence of cancer without directly interacting with DNA and inducing mutations [157,158]. These types of carcinogens have numerous modes of action [157,158]. Inhibition of cell-cell communication via gap junctions is among those cellular processes that have been suggested to be involved in cancer induction by non-genotoxic carcinogens [159]. Several pesticides and other types of environmental toxicants that are known or suspected human carcinogens inhibit gap junctional communication and increase the endocytosis and degradation rate of Cx43 across different human cell types. These include lindane (γ-hexachlorocyclohexane), DDT (1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane), ioxynil (4-hydroxy-3,5-diiodobenzonitrile), and polychlorinated biphenyls (PCBs) [36–40].

In general, the molecular basis of the loss of gap junctions in response to exposure to non-genotoxic carcinogens is still poorly understood. However, studies investigating the pesticide lindane suggest that aberrant trafficking of connexins in the endocytic system is involved in this process. Lindane, which was classified as "carcinogenic to humans" by the World Health Organization in 2015, is lipophilic and upon exposure it distributes widely in the body [160]. Exposure to lindane of a mouse Sertoli cell line expressing Cx43 endogenously has been found to result in increased endocytosis of gap junctions followed by the sequestration of Cx43 in early endosomes [161,162]. The lindane-induced endocytosis of Cx43-based gap junctions in these cells is mediated by the MAPK pathway [162]. Exposing WB-F344 rat liver epithelial cells to lindane has also been demonstrated to stimulate the endocytosis of gap junctions, which is associated with accentuated degradation of Cx43 in lysosomes [36]. Thus, the fate of Cx43 after gap junction endocytosis in

response to lindane exposure appears to vary between cell types; in some, Cx43 accumulates in early endosomes, whereas in others, Cx43 is sorted to lysosomes for degradation.

The human carcinogen cadmium has numerous molecular and cellular effects, which may affect all stages of carcinogenesis [163]. It was recently reported that exposure of BRL 3A rat liver fibroblast cells to cadmium results in reduced levels of Cx43, which was attributable to increased degradation of Cx43 via the endolysosomal pathway [164].

A recent study by Sovadinová and colleagues found that treatment of murine Leydig cells with any of three chemicals classified as endocrine disruptors – methoxychlor, triclocarban, or triclosan – resulted in loss of functional Cx43-based gap junctions, which was suggested to be due to increased gap junction internalization [40]. These authors also demonstrated that the occupational toxicant fluorene, a polycyclic aromatic hydrocarbon, induces internalization of Cx43-based gap junctions in murine Leydig cells and that this is associated with colocalization between Cx43 and ubiquitin [165]. These findings raise the possibility that the ubiquitin system may be involved in mediating the loss of gap junctions under these conditions.

6.1.2. The tumor promoter TPA induces endolysosomal degradation of Cx43 in a process that involves complex interplay between Cx43 phosphorylation and ubiquitination

A wide variety of non-genotoxic carcinogens are classified as tumor promoters in humans [158,166]. Tumor promoters are agents that share an ability to induce clonal expansion of initiated cells, i.e., cells harboring mutations in cancer-critical genes [167,168]. Several of these agents inhibit intercellular communication via gap junctions, which is often associated with induction of the internalization and degradation of gap junctions [159,169]. 12-*O*-tetradecanoylphorbol-13-acetate (TPA), also known as phorbol 12-myristate 13-acetate (PMA), has been used extensively in studies aimed at elucidating the molecular mechanisms by which tumor promoters inhibit gap junctional communication and induce connexin degradation. TPA is a potent skin irritant prepared from the seed of the croton plant [170], and it has been demonstrated to be a strong tumor promoter in experiments involving mouse models of skin tumor initiation and promotion [167,168]. Mechanistically, TPA acts as a functional mimic of diacylglycerol (DAG), which is generated endogenously by cells as a means of activating PKC-α [170]. Because of its ability to activate PKC-α, TPA is a powerful stimulator of cell proliferation, although it does not directly induce mutations in the cell genome [168].

TPA is a strong inhibitor of gap junction intercellular communication and inducer of Cx43 endocytosis and degradation in different cell types [114,171,172]. Several lines of evidence suggest that TPA promotes Cx43 degradation by accelerating its sorting to lysosomes along the endocytic pathway. For instance, via live-cell imaging analysis, TPA has been shown to induce the endocytosis of subdomains of Cx43-based gap junctions, which is followed by fusion of the endocytosed Cx43-enriched vesicles and early endosomes, resulting in a gradual relocalization of Cx43 from the plasma membrane to early endosomes [141]. In accordance with this finding, TPA treatment increases Cx43 staining in multivesicular endosomes, as determined by immunoelectron microscopy [142,144]. Immunoelectron microscopy analyses of TPA-treated cells have also revealed that, after the endocytosis of Cx43-based gap junctions, the two membranes of the connexosome are physically separated and the connexosome appears to be subjected to a morphological transformation into a multivesicular endosome enriched in Cx43 [141,142,144]. In accordance with the notion that TPA-induced endocytosis of gap junctions involves the separation of the two membranes of the connexosome, this process has been shown to correlate with loss of the insolubility of Cx43 in 1% Triton X-100 at 4 °C [173]. A similar morphological conversion of the connexosome and loss of the insolubility of Cx43 in Triton X-100 is also associated with gap junction endocytosis induced by epidermal growth factor (EGF) [142,173].

The ubiquitin system has been suggested to have a pivotal role in

TPA-induced endocytosis of Cx43-based gap junctions and subsequent lysosomal degradation of Cx43. The TPA-induced phosphorylation of Cx43 is accompanied by excessive Cx43 ubiquitination [114]. A possible scenario is that the enhanced level of Cx43 ubiquitination under these conditions invokes the recruitment of diverse types of ubiquitin-binding proteins that collectively mediate the internalization of gap junctions and the sorting of Cx43 to lysosomes [174]. In accordance with this model, two ubiquitin-binding proteins that are part of the ESCRT machinery, Hrs and tumor susceptibility gene 101 protein (Tsg101), have been found to participate in the sorting of Cx43 from early endosomes to lysosomes following TPA treatment [128]. Tsg101 binds to the Cx43 C-terminal tail, and the binding is increased upon TPA treatment [175]. Simultaneous depletion of Hrs and Tsg101 inhibits the TPA-induced degradation of Cx43 and results in the accumulation of hyper-ubiquitinated Cx43 at the outer membrane of early endosomes [128]. From these observations, we have previously proposed a model for the trafficking of Cx43 along the endocytic pathway in which ubiquitinated Cx43 is recognized by Hrs and Tsg101, which, together with other components of the ESCRT complex, execute the deubiquitination of Cx43 at the endosomal membrane and subsequently its sorting into the endosomal lumen [128]. In this model, the Cx43 pool that is sorted into the endosomal lumen following TPA treatment is destined for lysosomal degradation.

Cx43 has been shown to be present in exosomes derived from various cell types, where it forms connexons that participate in the physical interaction and communication between the exosomes and their acceptor cells [8,9,176,177]. Cx43 is also present in exosomes derived from cancer cells, which may have important clinical implications. For instance, exosomal Cx43 enhances the resistance of glioma cells to temozolomide and their migration potential [178]. Recently, Martins-Marques et al. demonstrated that, in addition to being involved in targeting Cx43 for lysosomal degradation, the TPA-induced ubiquitination of Cx43 correlates with enhanced sorting of Cx43 into exosomes in a process that is mediated by Tsg101 [179]. The molecular mechanisms that determine whether, in response to TPA treatment, the Cx43-positive intraluminal vesicles within endosomes are sorted to lysosomes for degradation or released to the extracellular space as exosomes are currently poorly understood and represent an important subject for future studies.

Studies conducted in recent years have started to shed light on the molecular mechanisms underlying TPA-induced Cx43 ubiquitination. The E3 ubiquitin ligases NEDD4 and SMURF2 bind to Cx43 and regulate Cx43 degradation under basal conditions [63,119,141,144]. They have also been shown to mediate the endocytosis of Cx43-based gap junctions and the subsequent sorting of Cx43 to lysosomes via the endocytic pathway in response to TPA exposure [141,144]. However, which lysine residues in Cx43 that are ubiquitinated by NEDD4 and SMURF2 under normal physiological conditions or in response to TPA treatment have not been identified. It is also important to take into consideration the possibility that these two E3 ubiquitin ligases may partly regulate the TPA-induced degradation of Cx43 indirectly by catalyzing the ubiquitination of proteins other than Cx43. In addition to NEDD4 and SMURF2, the TPA-induced internalization and degradation of Cx43-based gap junctions has been shown to be regulated by a deubiquitinating enzyme called associated molecule with the SH3 domain of STAM (AMSH) [180].

Smyth et al. have provided further insights into how gap junction levels are regulated by Cx43 phosphorylation and ubiquitination under normal conditions and following TPA treatment [111]. Introducing a point mutation in S373 in the C-terminal tail of Cx43 caused strongly reduced Cx43 ubiquitination and heightened levels of Cx43 at the plasma membrane, and this mutant did not undergo increased ubiquitination or degradation after TPA treatment [111]. Thus, Cx43 ubiquitination status and degradation are positively regulated by phosphorylation of S373.

Recently, Cx43 was reported to be post-translationally modified by ubiquitin-related modifier 1 (URM-1) [181]. It was suggested that TPA treatment results in increased conjugation of URM-1 to Cx43, but the possible implications of this finding for the TPA-induced endocytosis and degradation of Cx43 remains to be determined.

Notably, although several studies have shown that Cx43 ubiquitination is involved in TPA-induced endocytosis and sorting of Cx43 to lysosomes, the role of ubiquitination in Cx43 endocytosis and degradation in response to exposure to other non-genotoxic carcinogens, such as lindane and the other environmental and occupational toxicants described in Section 6.1.1, remains to be determined.

6.2. Dysregulation of the endocytic trafficking of connexins in cancer cells

The loss of gap junctions in cancer cells can be due to aberrant regulation of connexins at multiple levels, from the transcriptional to the post-translational [24–33]. Connexins are frequently observed to fail to form gap junctions in cancer cells and instead show cytoplasmic localizations [32,34,35]. The molecular mechanisms underlying such dysregulation of the intracellular trafficking of connexins in tumors are poorly characterized. However, emerging evidence suggests that this process may involve aberrant regulation of the trafficking of connexins along the endocytic pathway (Table 1). For instance, whereas Cx43 is expressed and thought to form gap junctions in the normal colonic epithelium, the cancer cells of colon tumors are found to be either negative for Cx43 or to display Cx43 in intracellular vesicular compartments instead of in the plasma membrane, as determined by immunohistochemistry [182]. The loss of Cx43 expression in the cancer cells of colon tumors is associated with poorer patient prognosis [183]. In accordance with these observations, many colon cancer cell lines do not express Cx43, and those colon cancer cells that do express Cx43 are unable to form gap junctions, Cx43 instead localizing in intracellular vesicular compartments [183]. In HT29 human colon cancer cells stably transfected with Cx43, these compartments were found to represent multivesicular endosomes as well as other, smaller vesicles, as determined by immunoelectron microscopy [183]. In another study in which the subcellular localization of Cx43 was examined in two human glioblastoma cell lines by confocal fluorescence microscopy, Cx43 was shown to predominantly localize in lysosomes and late endosomes, and only a small fraction of the cellular pool of Cx43 was assembled into gap junctions [184]. These observations suggest that aberrant trafficking of Cx43 in the endocytic system may be involved in mediating the loss of Cx43-based gap junctions in these types of cancer cells. One possible scenario is that such accumulation of Cx43 in the compartments of the endocytic system in cancer cells may be due to an increased rate of endocytosis of Cx43-based gap junctions compared with that in normal cells. In other situations, cancer cells may exhibit increased endocytosis and lysosomal trafficking of hemichannels. For instance, in BxPC3 and Capan-1 human pancreatic cancer cells, assembly of Cx43 into gap junctions is counteracted because it undergoes internalization prior to gap junction formation [15]. In these cells, Cx43 therefore fails to form gap junctions and localizes instead in cytoplasmic vesicular compartments, many of which are LAMP-1 positive [15]. Another possible reason for the observation that Cx43 often localizes in compartments of the endocytic system in cancer cells may be that, rather than being trafficked to the plasma membrane as under normal conditions, newly synthesized Cx43 is sorted directly from the secretory pathway to the compartments of the endocytic system. In accordance with this scenario, it has been shown that in MDA-MB-231 human breast cancer cells, Cx43 is delivered directly from compartments of the secretory pathway to lysosomes, without being trafficked to the plasma membrane [20]. Supporting the notion that connexins under certain conditions may be trafficked directly from the secretory pathway to lysosomes, a recent study demonstrated that mutation of two cysteine residues in the C-terminal tail of Cx32, C280 and C283, counteracts its trafficking from the trans-Golgi network to the plasma membrane, which is associated with strongly increased lysosomal degradation of Cx32 [185].

Table 1

Overview of studies reporting moderate or extensive localization of Cx43 in compartments of the endocytic system in cancer cells and its possible association with loss of Cx43-based gap junctions.

Cancer type	Cellular model system	Observation	Ref.
Breast cancer	MDA-MB-231 human breast cancer cells	Do not form gap junctions. Cx43 trafficked directly from early secretory compartments to lysosomes.	[20]
Cervical cancer	C33A human cervical cancer cells and HeLa human cervical cancer cells stably transfected with Cx43	Form gap junctions. Moderate levels of Cx43 localizes in multivesicular endosomes, as determined by IEM.	[144]
	HPV16-immortalized but non-tumorigenic cervical keratinocytes (W12) and W12 keratinocytes that are transformed and invasive (W12GPXY)	W12 cells form gap junctions. W12GPXY cells do not form gap junctions; Cx43 localizes in cytoplasmic vesicular compartments, partly colocalizing with markers for early and late endosomes, as determined by CFM.	[190,191]
Colon cancer	HT29 human colon cancer cells stably transfected with Cx43	Do not form gap junctions. Cx43 locates predominantly in multivesicular endosomes, as determined by IEM.	[182]
Pancreatic cancer	BxPC3 and Capan-1 human pancreatic cancer cells	Gap junction assembly impaired due to excessive endocytosis of Cx43 hemichannels. Cx43 localizes in cytoplasmic vesicular compartments, displaying considerable colocalization with LAMP-1, as determined by CFM.	[15]
Glioblastoma	Human glioblastoma cell lines SKI-1 and U251	Few gap junctions present. Cx43 localizes in cytoplasmic vesicular structures, partly colocalizing with markers for late endosomes and lysosomes, as determined by CFM.	[184]
Leydig cell tumor	Transgenic IT6-F mouse model of Leydig cell tumorigenesis	Early stages of Leydig cell tumorigenesis is associated with loss of gap junctions and concomitant sequestration of Cx43 in cytoplasmic vesicular structures, partly colocalizing with markers for early endosomes, as determined by DFM.	[187]
	BLT-1 mouse Leydig tumor cell line	Few gap junctions present. Cx43 localizes in cytoplasmic vesicular structures, partly colocalizing with markers for early endosomes, as determined by DFM.	[187]

Abbreviations: CFM, confocal fluorescence microscopy; DFM, deconvolution fluorescence microscopy; IEM, immuno-electron microscopy.

Segretain *et al.* have demonstrated that aberrant trafficking of Cx43 along the endocytic pathway correlates with the loss of gap junctions in cancer cells *in situ* during Leydig cell tumorigenesis. Leydig cells are located in the connective tissue surrounding the seminiferous tubules in the testis, and Cx43 is the only connexin isoform expressed in these cells [186]. Leydig cell tumors are rare, comprising 1%-3% of all testicular neoplasms. By using a transgenic mouse model in which mice develop testicular tumors originating from Leydig cells by the age of 5–6 months, it was found that early stages of Leydig cell tumorigenesis are associated with the sequestration of Cx43 in early endosomes *in situ* [187]. This was linked to loss of gap junction intercellular communication. At advanced stages of Leydig cell tumorigenesis (6–7 months), the Cx43 protein level was dramatically reduced, although the Cx43 mRNA level was unaffected [187]. These observations suggest that the loss of Cx43-based gap junctions in Leydig cell tumors is due to alterations in the post-translational regulation of Cx43 and that it involves aberrant trafficking of Cx43 in the endocytic system.

Studies that use cervical cancer cell lines as model systems have started to shed light on the molecular mechanisms underlying the delocalization of Cx43 from the plasma membrane to intracellular compartments in cancer cells. In the normal cervical epithelium, Cx43 is strongly expressed in the suprabasal layers, where it forms gap junctions [188,189]. In accordance with the observation that Cx43 is expressed in the normal cervical epithelium, Cx43 is expressed in the immortal but untransformed cervical epithelial cell line W12G, where it forms gap junctions and provides for extensive gap junctional communication [190]. In contrast, in cells derived from W12G cells that have undergone transformation and are invasive, Cx43 is unable to form gap junctions but localizes instead to the cytoplasm [190]. In these cells, Cx43 partly localizes to compartments of the endocytic system [191]. Similarly, in C33A cervical cancer cells, which also endogenously express Cx43, a considerable fraction of Cx43 localizes to various compartments of the endocytic system, whereas other fractions of Cx43 are assembled into gap junctions [144,190]. Graham and colleagues have demonstrated that the cytoplasmic pool of Cx43 in cervical cancer cells is maintained by the tumor suppressor protein human discs large (hDlg), a member of the MAGUK (membrane-associated guanylate kinase) family [191,192]. Mechanistically, hDlg binds to Cx43 via its C-terminal tail and appears to maintain the cytoplasmic localization of Cx43 by antagonizing its lysosomal degradation [191,192].

Studies from our laboratory have shown that ectopic overexpression of NEDD4 in cervical cancer cells causes enhanced Cx43 ubiquitination, which is associated with elevated endocytosis of gap junctions followed by the endolysosomal degradation of Cx43 [144]. More studies are needed to determine whether this process contributes to the loss of Cx43-based gap junctions during cervical cancer development. However, NEDD4 is overexpressed and acts as an oncogene in multiple cancer types [193–195], raising the possibility that NEDD4 over-activation or overexpression may participate in promoting the loss of Cx43-based gap junctions in cancer cells by inducing Cx43 ubiquitination.

7. Therapeutic implications of derailed endocytic trafficking of connexins in cancer cells

Since connexins may act as tumor suppressor proteins, restoring functional gap junctions between cancer cells represents a potential therapeutical strategy for cancer, as previously reviewed [24–33]. Intercellular communication via gap junctions can have a major impact on how cancer cells respond to chemotherapy. For instance, restoring functional gap junctions in cancer cells has been shown to cause increased sensitivity to the platinum-based drug cisplatin [196–200]. Cisplatin is used as first-line therapy for a number of cancer types, including testicular, cervical, bladder, ovarian, head, neck, and non-small-cell lung cancer, but many patients ultimately develop cisplatin-resistant disease [201]. The molecular basis underpinning the

increased toxicity of cisplatin caused by intercellular communication via gap junctions is incompletely understood, but may involve signals produced by the DNA-dependent protein kinase complex in response to cisplatin-induced DNA damage [199]. These signals, known as "death signals," are conveyed via gap junctions and induce cell death in adjacent cells. Cell-cell communication via gap junctions is also an important determinant of the response of cancer cells to radiation by mediating the transfer of death signals from irradiated to nonirradiated cells [202]. Thus, pharmacological strategies aimed at restoring gap junction intercellular communication between cancer cells, in combination with radio- or chemotherapy, could provide a new approach to increasing the clinical response to these treatment modalities [199,202].

The observation that the loss of gap junctions during cancer development can be due to the delocalization of connexins from the plasma membrane to endocytic compartments opens up the possibility of developing drugs to counteract this delocalization, with the aim of restoring gap junction functionality. Deciphering the molecular basis of gap junction endocytosis and aberrant trafficking of connexons in the endocytic system in cancer cells could therefore reveal new opportunities for reestablishing gap junction intercellular communication in a therapeutic setting. In this context, it would be important to identify potentially unique molecular mechanisms involved in mediating gap junction endocytosis and post-endocytic sorting, which possibly could be targeted in cancer cells without disrupting the endocytosis of other types of cargo. It would also be important to further outline the molecular mechanisms that determine whether connexons trafficked to early endosomes are to be sorted into intraluminal vesicles that are destined for lysosomal degradation, intraluminal vesicles that are to be released as exosomes, or vesicles that are to be recycled to the plasma membrane.

Gap junctions may have different roles during different stages of cancer development, depending on the type of cancer and the connexin isoform(s) constituting the gap junctions. For instance, although intercellular communication via gap junctions may display tumor-suppressive functions during the early stages of carcinogenesis, this form of cell-cell communication may be involved in facilitating invasion and metastasis [24–27,30]. Increasing evidence suggests that aberrant regulation of endocytosis and trafficking of proteins along the endolysosomal system has an important role in invasion and metastasis, and several metastasis suppressor genes function in part through the regulation of these processes [203]. A possible scenario is that the endocytic system may have distinct roles in modulating the level of gap junctions during invasion and metastasis compared with earlier stages of tumorigenesis. Thus, when considering the therapeutic potential of targeting the endocytic trafficking of connexins in cancer cells, it is important to recognize that the mechanisms involved in the aberrant endocytic trafficking of connexins may vary at different stages of tumorigenesis and that restoring functional gap junctions in cancer cells may, under some conditions, promote invasion and metastasis.

8. Conclusions and perspectives

The endocytic system is intrinsically involved in mediating the sorting of connexins to lysosomes for degradation under physiological conditions. Mounting evidence also suggests that aberrant regulation of the trafficking of connexins along the endocytic pathway contributes to loss of gap junctions during cancer development. Strategies to manipulate the endocytic trafficking of connexins in conjunction with radio- or chemotherapy could provide new approaches to increasing therapy efficacy in the future.

The relative importance of the endocytic pathway and autophagy in the degradation of connexins is likely to be tissue and cell type dependent. An important subject for future studies will be to further delineate the molecular mechanisms that are involved in determining whether internalized connexins are degraded via either of these pathways. It would also be valuable to investigate whether the relative importance of

these pathways in mediating connexin degradation may be altered during the various steps of cancer development. It is becoming increasingly clear that the endocytic pathway and autophagy intersect at multiple levels and that many of the components of their molecular machinery are shared [156]. It is noteworthy in this regard that electron microscopy studies have suggested that these two degradation pathways can simultaneously participate in mediating the degradation of connexins internalized from the plasma membrane [126]. It would be important to gain further insight into how the endocytic and autophagosomal pathways cross-talk in the degradation of connexins and to define the molecular basis of this interplay.

Environmental toxicants comprise substances from numerous different chemical groups and display a variety of different mechanisms of action. For some of these chemicals, the mechanisms of action and their potential hazard for human health is relatively well known, whereas many others are much less studied [157,158]. Non-genotoxic carcinogens have numerous different mechanisms of action, which may be tissue and species specific [157,158]. Thus, it is considerably more challenging to detect non-genotoxic carcinogens in the environment than it is to detect genotoxic carcinogens. A central step toward more reliably predicting the possible human health effects of non-genotoxic carcinogens is to provide a better understanding of the molecular basis underlying their mode of action. Clarifying the molecular mechanisms through which non-genotoxic carcinogens inhibit gap junctional communication and target connexins for endocytosis and degradation may be an important contribution to the risk assessment of these chemicals [159,169]. Moreover, since many chemicals accumulate in the tissues of the body over time, it is important to better understand how mixtures of such chemicals affect gap junctions.

A possible scenario is that the endocytic system not only acts as a conduit for the trafficking of connexins to lysosomes for degradation, but also that the pool of connexins located in the endocytic system has important functions that are distinct from those of the connexin pool located at the plasma membrane. In accordance with this hypothesis, a recent study showed that in lymphokine-activated killer cells, Cx43 is involved in transporting nicotinamide adenine dinucleotide phosphate (NADP$^+$) into the lumen of endolysosomes to mobilize Ca^{2+} and drive cell migration [204]. Furthermore, it is known that, after their internalization, many types of receptors still interact with downstream effectors while located in endosomes, which allows them to continue signaling after internalization [205]. As an analogy, it is conceivable that, after gap junction endocytosis, connexins located at the outer membrane of endosomes may still bind to proteins such as β-catenin, cyclin E, and protein phosphatase and tensin homolog (PTEN) and, through these protein-protein interactions, influence cellular processes such as cell growth, migration, and differentiation in a similar manner to the connexin pool located in gap junctions. In this scenario, endosomes would act as physical platforms that enable connexins to continue to affect various downstream effectors after gap junction endocytosis.

For some cancer types, there is an association between the level of connexins located in cytoplasmic compartments and poor differentiation, invasion potential, and/or metastasis, as previously reviewed by Omori *et al.* [32]. Such observations raise the intriguing possibility that the connexin pool that localizes in cytoplasmic compartments in cancer cells may have certain properties that are selected for during cancer development. In accordance with the notion that connexins located in cytoplasmic compartments may display pro-tumorigenic functions, induced overexpression of Cx32 in the cytoplasm of HuH7 human hepatoma cells has been shown to enhance their proliferation, motility, and metastatic ability [206]. Under these conditions, Cx32 was found to be located in the Golgi apparatus [206]. In future studies, it will be important to learn more about the molecular basis underlying such pro-tumorigenic properties of connexins located in the cytoplasm of cancer cells. It will also be interesting to provide a more comprehensive overview of what types of cytoplasmic compartments connexins are located in under these conditions, and whether connexins located in

compartments of the endocytic system may act as oncogenes in certain cancer types.

In future studies, it will also be important to explore in further detail whether dysregulation of the endocytic trafficking of connexins may participate in mediating the loss of gap junctions in diseases other than cancer. Interestingly, a recent study by Girão and colleagues suggested that remodeling of Cx43-based gap junctions during myocardial ischemia may be associated with aberrant trafficking of Cx43 along the endocytic pathway in a process that involves EHD1 (Eps15 [endocytic adaptor epidermal growth factor receptor substrate 15] homology domain-containing protein 1) [207]. Cx43 was found to bind to EHD1 in the heart, and the interaction between these two proteins increased following ischemia [207]. Moreover, ectopic overexpression of EHD1 in adult rat cardiomyocytes was associated with increased Cx43 endocytosis and colocalization between Cx43 and EEA1 when these cells were subjected to *in vitro* ischemia [207]. Emerging evidence also suggests that aberrant endocytic trafficking of connexins could be involved in diseases associated with connexin mutations. For instance, Beach et al. recently reported that the loss of gap junction function associated with two mutations in Cx26 linked to sensorineural hearing loss may involve aberrant trafficking of Cx26 via the endocytic system and premature degradation of Cx26 in lysosomes [208]. Moreover, Minogue et al. have shown that cataract-linked mutations in two serine residues in the C-terminal tail of Cx50 impairs gap junction function by exposing a tyrosine-based sorting signal in Cx50 that promotes its internalization and lysosomal degradation [209]. Both these serine residues are known phosphorylation sites, and the study suggested that under normal conditions, their phosphorylation stabilizes Cx50 in gap junctions by masking this sorting signal [209]. In another study, a Cx37 variant associated with primary ovarian insufficiency, a disorder that often leads to infertility and affects ~1% of women under the age of 40 years, was suggested to cause a decrease in gap junctions by promoting Cx37 internalization and endo-lysosomal degradation [151]. The variant was found to have a partial dominant-negative effect on the ability of Cx37-wild type to form functional gap junction channels.

A limitation of most previous studies examining the trafficking of connexins along the endocytic pathway is that they have been mostly conducted by using 2D cell culture systems. A key step toward improved insight into the physiological and pathophysiological importance of the trafficking of connexins along the endocytic system will be to study these processes *in vivo* with appropriate model organisms. Moreover, in exploring cell behavior, the use of multicellular compositions such as spheroids and organoids has emerged as a promising experimental model that bridges *in vitro* and *in vivo* conditions. Organoids are self-organizing, three-dimensional tissue cultures that derive from stem cells and resemble the genomics, histology, and functional properties of the originating tissues [210]. Furthermore, organoids can be fashioned to replicate much of the complexity of an organ or a tumor, or to express selected miniature aspects of them. Holistic cultures of tumor organoids with their microenvironment are increasingly being used to study the complexity of cellular processes, as they are more physiologically relevant than 2D cell culture systems. The use of such state-of-the-art model systems is likely to give new insights into the mechanisms involved in the dysregulation of intercellular communication via gap junctions during cancer development.

Abbreviations

Alix	ALG-2-interacting protein X
AMSH	associated molecule with the SH3 domain of STAM
CFM	confocal fluorescence microscopy
Cx43	connexin 43
DAG	diacylglycerol
DFM	deconvolution fluorescence microscopy
EEA1	early endosome antigen 1
ERAD	endoplasmic reticulum-associated degradation
ESCRT	endosomal sorting complex required for transport
EGF	epidermal growth factor
hDlg	human discs large
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
IEM	immuno-electron microscopy
LAMP-1 and -2	lysosome-associated membrane protein-1 and -2
MAGUK	membrane-associated guanylate kinase
MAPK	mitogen-activated protein kinase
PI3P	phosphatidylinositol 3-phosphate
PKC	protein kinase C
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
PCB	polychlorinated biphenyl
PMA	phorbol 12-myristate 13-acetate
PTEN	protein phosphatase and tensin homolog
PYK2	proline-rich tyrosine kinase 2
SUMO	small ubiquitin-related modifier
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Tsg101	tumor susceptibility gene 101 protein
VPS4	vacuolar protein sorting-associated protein 4

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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