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Review

Endocytosis and post-endocytic sorting of connexins ☆

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ABSTRACT

The connexins constitute a family of integral membrane proteins that form intercellular channels, enabling adjacent cells in solid tissues to directly exchange ions and small molecules. These channels assemble into distinct plasma membrane domains known as gap junctions. Gap junction intercellular communication plays critical roles in numerous cellular processes, including control of cell growth and differentiation, maintenance of tissue homeostasis and embryonic development. Gap junctions are dynamic plasma membrane domains, and there is increasing evidence that modulation of endocytosis and post-endocytic trafficking of connexins are important mechanisms for regulating the level of functional gap junctions at the plasma membrane. The emerging picture is that multiple pathways exist for endocytosis and sorting of connexins to lysosomes, and that these pathways are differentially regulated in response to physiological and pathophysiological stimuli. Recent studies suggest that endocytosis and lysosomal degradation of connexins is controlled by a complex interplay between phosphorylation and ubiquitination. This review summarizes recent progress in understanding the molecular mechanisms involved in endocytosis and post-endocytic sorting of connexins, and the relevance of these processes to the regulation of gap junction intercellular communication under normal and pathophysiological conditions. This article is part of a Special Issue entitled: The Communicating junctions, composition, structure and characteristics.

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Abbreviations: CHO, Chinese hamster ovary; CIP75, connexin43-interacting protein of 75-kDa; CIP85, connexin43-interacting protein of 85-kDa; Dab2, disabled-2; EEA1, early endosomal autoantigen 1; EGF, epidermal growth factor; ENaC, amiloride-sensitive epithelial sodium channel; ERAD, endoplasmic reticulum-associated degradation; ESCRT, endosomal sorting complex required for transport; HECT, homologous to E6-AP carboxy terminal; GPCR, G protein-coupled receptor; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; MAPK, mitogen-activated protein kinase; Nedd4, neural precursor cell expressed, developmentally downregulated 4; NRK, normal rat kidney; OCP1, Organ of Corti protein 1; PLC β3, phospholipase Cβ3; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; RING, really interesting new gene; SCF, Skp1/Cul1/F-box complex; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Tsg101, tumor-susceptibility gene product 101; ZO-1, Zonula occludens 1

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1. Introduction

Connexins are tetramembrane spanning proteins that are able to form channels between adjacent cells. These channels assemble into intercellular plasma membrane domains known as gap junctions [1]. The connexin gene family constitutes 20 members in humans [2,3]. Connexins are expressed in nearly all cell types, both during development and in the adult organism [4]. Most tissue types express several connexin isoforms. Gap junctions enable adjacent cells in solid tissues to directly exchange ions, such as K^{+} and Ca^{2+}, second messengers, such as inositol triphosphate, ATP, cAMP, and cGMP, and small metabolites, including glucose and glutamate. Gap junctions have a fundamental role in tissues containing electrically excitable cells. For instance, gap junctions function as electrical synapses between neurons and have important roles in the synchronous contraction of the heart muscle by mediating electrical coupling between cardiomyocytes [5,6]. Gap junctions also have critical roles in non-excitable tissues, by regulating cell growth and differentiation, embryonic development and tissue homeostasis [7,8]. Loss of functional gap junction channels has been implicated as a causative factor in multiple diseases, including heart failure, neuropathology, deafness, skin disorders and cataracts [9]. There is also substantial evidence that loss of connexin expression is important in cancer development, and several members of the connexin gene family act as tumor suppressors [10,11].

Intercellular communication via gap junctions is controlled by multiple mechanisms. The most rapid regulation of gap junction channels is achieved by altering the conductance of single channels or their probability of opening [12]. Slower regulation of gap junctions can be achieved by changing the rate of connexin synthesis, trafficking of connexins to the plasma membrane or assembly of connexins into gap junctions [12]. There is also increasing evidence that modulation of connexin endocytosis and sorting to lysosomes may be critically involved in controlling gap junction intercellular communication [13–16]. Here, we review recent progress in understanding the mechanisms involved in the regulation of connexin endocytosis and post-endocytic sorting to lysosomes. We also discuss how modulation of the connexin endocytosis and degradation rates might play important roles in controlling the level of functional gap junctions under normal and pathological conditions.

2. Synthesis and trafficking of connexins to the plasma membrane

Connexin proteins span the membrane four times and have their amino and carboxy termini localized on the cytosolic side of the membrane [4]. The four transmembrane regions, the two extracellular loops and the amino terminus contain several identical residues among the various connexins. In contrast, the length and amino acid compositions of the carboxyl terminus and the intracellular loop vary extensively between connexins. Connexin proteins are most commonly referred to by their predicted molecular weight in kilodaltons. The best-studied connexin isoform is connexin 43 (Cx43).

Connexins are generally considered to be co-translationally inserted into the endoplasmic reticulum, although cell-free studies indicate that Cx26 can also insert into membranes post-translationally or directly into the plasma membrane [17–19]. Connexins with correct conformation are transported via the Golgi apparatus and the *trans*-Golgi network to the plasma membrane [20–23]. Cx26 may also follow an alternative route to the plasma membrane that bypasses the conventional secretory pathway [24–26]. Along their trafficking from the endoplasmic reticulum to the plasma membrane, connexins oligomerize into hexameric structures that form a cylinder with an aqueous pore extending through the channel, called a connexon. Connexons can consist of six identical or different connexin isotypes, termed homomeric and heteromeric connexons, respectively [4]. In contrast to the oligomerization of most other multisubunit integral membrane proteins, Cx43 and Cx46 oligomerize after they have left the endoplasmic reticulum, probably in the *trans*-Golgi network [20,27,28]. On the other hand, Cx32 oligomerizes within the endoplasmic reticulum, suggesting that the site of oligomerization is isoform specific [29,30]. The trafficking of connexons from the *trans*-Golgi network to the plasma membrane is mediated by microtubules [31–34]. A recent study by del Castillo et al. shows that transport of connexons from the trans-Golgi network to the plasma membrane is regulated by the integral membrane protein consortin [35].

At least 40% of newly synthesized wild-type Cx43 and Cx32 and up to 100% of some mutant forms of Cx32 have been estimated to undergo endoplasmic reticulum-associated degradation (ERAD) [36,37]. Although Cx32 and Cx43 are the only connexin isoforms known to undergo ERAD, it is anticipated that ERAD is a general mechanism underlying quality control of connexins. The degradation of Cx32 and Cx43 at the endoplasmic reticulum is strongly affected by various cytosolic stressors [37]. Oxidative or thermal stress inhibits dislocation to the cytosol and subsequent proteasomal degradation of Cx32 and Cx43 [37]. The wild-type connexin protein escaping ERAD in response to cytosolic stress remains in a full-length, membrane-integrated form, and is able to undergo trafficking via the secretory pathway and form functional gap junctions at the plasma membrane [37]. Reducing connexin ERAD has been hypothesized to be a mechanism for upregulating gap junctional communication under normal physiological conditions and under pathological conditions, such as ischemia-reperfusion injury and fever [38]. In line with the notion that controlled ERAD of connexins may have important physiological roles, androgen depletion has been shown to induce degradation of a major fraction of Cx32 and Cx43 at the endoplasmic reticulum in human prostate cancer cells [39]. The enhanced degradation of connexins in response to androgen depletion is associated with reduced gap junctional intercellular communication. The degradation of Cx43 at the endoplasmic reticulum is regulated in part by CIP75 (connexin43-interacting protein of 75-kDa) [40]. CIP75 belongs to the UbL (ubiquitin-like)-UBA (ubiquitin-associated) protein family, which is involved in the translocation of proteins across the endoplasmic reticulum membrane, and in mediating the delivery of proteins to proteasomes during ERAD [41]. Recently, Su et al. found that CIP75 stimulates proteasomal degradation of Cx43 at the endoplasmic reticulum in a process independent of Cx43 ubiquitination [42].

3. Assembly of connexons into gap junctions

Connexons are delivered to the plasma membrane at non-gap junctional sites, and diffuse laterally to the periphery of the gap junction plaques where they dock with connexons in neighboring cells to form intercellular channels [23,33]. Docking between connexons occurs by a “lock and key” mechanism involving six protrusions from each connexon [4,43,44]. By electron microscopy, gap junctions can be identified as intercellular areas in which the two plasma membrane domains are tightly apposed to each other. The two membranes of a gap junction appear to be separated by an extracellular gap of 2–4 nm for which the junction is named [45,46]. The conductance and selectivity of a gap junction channel is strongly affected by its connexin composition. Connexons in one cell can dock with connexons consisting of different connexin isoforms in the adjacent cell to form a so-called heterotypic gap junction channel [1]. The connexin composition of the gap junction channels differs significantly between cell and tissue types. Different combinations of connexin isoforms enable the various cell types to form gap junctions with different conductive and selective properties. Moreover, expressing several connexin isoforms might have a compensating role, enabling cells to express functional gap junctions even if one of the isoforms is mutated or lost. Although the best-known function of connexins is to form gap junction channels, substantial evidence indicates that connexons at the plasma membrane that are not assembled in gap junctions may

have important functions by providing exchange of ions and signaling molecules between the extracellular and intracellular environments, and are often referred to as hemichannels [47]. Under normal conditions, most hemichannels are considered to be in a closed state, but stimuli such as mechanical stress or reduced calcium concentration in the extracellular space can provoke channel opening [48–53].

The formation of gap junctions is intimately linked to the formation of adherens junctions. Several studies indicate that assembly of connexons into gap junction plaques is facilitated by adherens junction formation [54–59]. It has been suggested that the close membrane–membrane contact formed by adherens junctions facilitates the assembly of connexons into gap junctions. There is also increasing evidence that assembly of gap junctions and adherens junctions involves a reciprocal relationship between the two junction complexes. For instance, Lo and co-workers have found that Cx43 and N-cadherin may co-assemble in a multiprotein complex, and that expression of Cx43 at the cell surface is dependent on N-cadherin and, vice versa, that N-cadherin expression at the cell surface requires Cx43 [59]. The role of E-cadherin and N-cadherin in gap junction assembly is likely to differ between different cell types. Recently, Mehta and colleagues reported that E-cadherin and N-cadherin may have opposite effects on the assembly of Cx43 into gap junction plaques in rat liver epithelial cells [56]. While E-cadherin was found to facilitate assembly of Cx43 into gap junctions, N-cadherin attenuated the assembly by causing endocytosis of Cx43.

The rate of aggregation of undocked Cx43 connexons into gap junctions is regulated by the scaffold protein ZO-1 (zonula occludens-1). ZO-1 interacts directly with various connexin isoforms, including Cx43 [60,61]. Several lines of evidence indicate that disruption of the interaction between Cx43 and ZO-1 results in increased gap junction size. For instance, fusion of a green fluorescent protein (GFP) tag to the carboxyl terminus of Cx43 results in increased gap junction size due to masking of the carboxyl terminal amino acids of Cx43 that comprise the ZO-1 binding site [62,63]. A similar effect on gap junction size is observed when the Cx43–ZO-1 interaction is disrupted using a peptide inhibitor [63]. The notion that ZO-1 regulates the size of gap junctions is supported by studies demonstrating that mice expressing Cx43 with a truncated carboxyl-terminal domain, unable to interact with ZO-1, form enlarged gap junctions at cardiac intercalated disks [64]. A recent study by Rhett et al. indicates that ZO-1 interacts with Cx43 in a region around gap junction plaques termed the perinexus, and limits the rate at which undocked Cx43 connexons incorporate into gap junction plaques [65].

4. Gap junction endocytosis

In contrast to many integral membrane proteins, connexins have high turnover rates in most tissue types, with half-lifes of 1.5–5 h [66,67]. The turnover kinetics of connexins in various tissue types and cell lines in comparison with other integral membrane proteins was recently reviewed by Hervé et al. [14]. The observation that connexins have a high turnover rate implies that modulation of the connexin degradation rate might be an important mechanism for controlling the level of gap junctional intercellular communication under physiological and pathopysiological conditions, as discussed in previous reviews [13–15,68–70]. There is growing evidence that the connexin degradation rate can be modulated both at the level of gap junction endocytosis as well as at the level of post-endocytic sorting of connexins to lysosomes. Gap junctions are highly dynamic structures. Under normal physiological conditions, connexons are continually added to the edges of existing gap junctions and removed from the center of the gap junction by endocytosis [33,71]. Under some conditions, entire gap junction plaques may be internalized [72]. Electron microscopy studies suggest that during endocytosis of gap junctions, the two apposed membranes of the junction are internalized into one of the adjacent cells, forming a double-membrane vacuole called an annular gap junction or connexosome [15,72–83]. This model for gap junction endocytosis has been supported by studies involving live imaging of GFP-tagged Cx43 and Cx32 [74,84–86], as well as by correlative fluorescence and electron microscopy of Cx43 containing a tetracysteine tag [71]. In addition to the endocytosis of large gap junction portions, multiple small domains of the plaque can be internalized [87].

Clathrin is recruited to gap junctions during endocytosis and is required for gap junction internalization [76,79,80,88,89]. Clathrin is distributed in distinct patches rather than coating the entire surface of the gap junction plaque [80]. Recruitment of clathrin to gap junctions during endocytosis has been suggested to be mediated by the clathrin-adaptors AP-2 and Disabled-2 (Dab2) [80]. Several studies also indicate that gap junction endocytosis involves the GTPase dynamin [80,88,90]. Cx43 has also been found to interact with the rab GAP-like protein CIP85 (Cx43-interacting protein of 85-kDa) [91]. CIP85 colocalizes with Cx43 gap junctions at the plasma membrane and is involved in regulating the rate of lysosomal degradation of Cx43 [91]. By electron microscopy, internalized gap junctions are found to be coated with actin filaments [76]. Studies by Falk and colleagues indicate that endocytosis of gap junctions is dependent on intact actin filaments and on the unconventional, retrograde actin motor myosin-VI [80].

As described above, ZO-1 regulates the rate of aggregation of undocked Cx43 connexons into gap junction plaques, but may also play a role in gap junction internalization. For instance, significant increase in the association between Cx43 and ZO-1 has been reported during remodeling of myocyte intercellular contacts, raising the possibility that ZO-1 might be involved in gap junction endocytosis and degradation during cardiac development and disease [92,93]. Increased association is also observed between Cx43 and ZO-1 in Sertoli cells under conditions where gap junction endocytosis is induced by the non-genotoxic carcinogen lindane (γ-hexachlorocyclohexane) [94]. ZO-1 localizes on both sides of the gap junction plaques in untreated cells, but appears to be mainly localized to one side during endocytosis of the gap junctions in response to lindane treatment [95]. The lindane-induced endocytosis of Cx43 gap junctions is dependent on c-Src, and dissociation of ZO-1 from gap junctions appears to occur at the side of the plaque where c-Src is recruited [95].

Intercellular communication via Cx43 gap junctions is rapidly disrupted after stimulation of certain G protein-coupled receptors (GPCR) [96–98]. Studies by Moolenaar and co-workers indicate that loss of phosphatidylinositol 4,5-bisphosphate (PtdIns[4,5]P_2) at the plasma membrane is necessary and sufficient for the inhibition of Cx43 gap junction intercellular communication in response to GPCR stimulation [99]. Moreover, ZO-1 was found to bind to phospholipase Cβ3 (PLC β3) and to be required for the GPCR-induced inhibition of Cx43 gap junction channels, possibly by organizing Cx43 and PLC β3 into a complex that regulates the local levels of PtdIns(4,5)P_2. In subsequent studies, Baker et al. found that activation of GPCR by thrombin and endothelin-1 results in rapid internalization of Cx43 gap junctions, in a process involving clathrin and ZO-1 [100].

Majoul and colleagues have found that drebrin (developmentally regulated brain protein) has an important role in stabilizing Cx43 gap junctions at the plasma membrane [101]. Drebrin is an actin-binding protein that is involved in morphogenesis, patterning and maintenance of dendritic spines in neurons [102]. Drebrin binds to the C-terminal tail of Cx43 and colocalizes with Cx43 at gap junction plaques. Depletion of drebrin by siRNA results in destabilization of gap junctions and relocalization of Cx43 to intracellular vesicles, which is associated with impaired intercellular communication via gap junctions [101]. Possibly, drebrin may stabilize Cx43 gap junctions by acting as a link between Cx43 and the actin cytoskeleton [101].

5. Post-endocytic sorting of connexins

Following internalization of gap junctions, connexins are degraded in lysosomes [10,36,103–105]. There is increasing evidence that

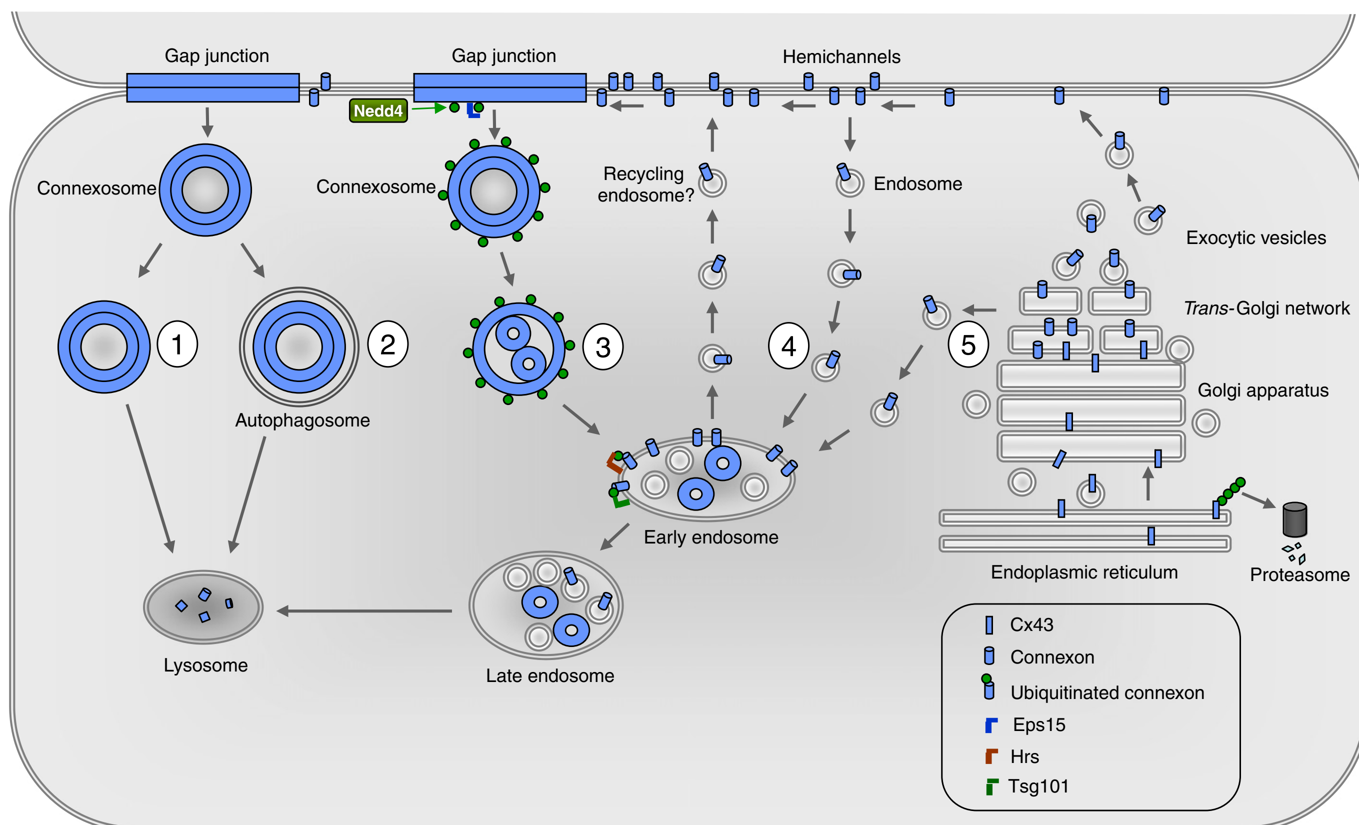

Fig. 1. Pathways for lysosomal delivery of Cx43. Cx43 is co-translationally inserted into the endoplasmic reticulum and transported via the Golgi apparatus and the *trans*-Golgi network to the plasma membrane. A sub-population of newly synthesized Cx43 in the endoplasmic reticulum undergoes ERAD. Oligomerization of Cx43 into connexons occurs after the protein has left the endoplasmic reticulum. Newly synthesized connexons are continually added to the edges of existing gap junctions and dock with connexons in neighboring cells to form gap junction intercellular channels. During endocytosis of a gap junction, both membranes of the junction are internalized into one of the adjacent cells, to form a connexosome. The figure depicts five possible pathways for delivery of Cx43 to lysosomes: 1) Fusion between connexosomes and lysosomes, 2) sequestration of connexosomes by autophagosomes and subsequent fusion between the autophagosomes and lysosome, 3) transformation of the connexosome into a Cx43-enriched, multivesicular endosome with a single limiting membrane and subsequent trafficking of Cx43 to early and late endosomes prior to degradation of Cx43 in lysosomes, 4) endocytosis of connexons at the plasma membrane not assembled in gap junctions, and 5) delivery of Cx43 to the endo-lysosomal system directly from the secretory pathway. Note that although depicted in the figure, it is currently unknown whether early and late endosomes are involved in the pathways 4 and 5. The molecular mechanisms involved in the separation of the gap junction double membrane in pathway 3 are currently unknown. Ubiquitination of Cx43 is mediated by the E3 ubiquitin ligase Nedd4. Ubiquitination of Cx43 has been shown to be involved in pathway 3, and has been suggested to regulate internalization of Cx43 gap junctions as well as sorting of Cx43 from early endosomes to lysosomes. Eps15 binds to ubiquitinated Cx43 at the plasma membrane and participates in gap junction endocytosis. Ubiquitinated Cx43 is thought to be recognized and retained at the limiting membrane of the early endosome by the ubiquitin-binding proteins Hrs and Tsg101. In addition to a ubiquitin-dependent pathway for Cx43 endocytosis, Cx43 may also follow a ubiquitin-independent pathway, in which the YXXϕ motif in the Cx43 C-terminal tail has a key role. It is currently unknown if Cx43 ubiquitination is involved in pathway 1, 2, 4 or 5.

connexins can follow different post-endocytic pathways *en route* to their final destination in lysosomes (Fig. 1). Several studies suggest that connexosomes are able to directly fuse with lysosomes [89,105–108]. Cx43 may also be delivered to lysosomes directly from early secretory compartments [105]. In addition, connexons at the plasma membrane not assembled in gap junctions can be endocytosed and degraded in lysosomes [109]. VanSlyke and Musil have shown that cytosolic stress reduces degradation of Cx43 pools not assembled in gap junctions [109]. Cytosolic stress did not affect Cx43 endocytosis, but appeared to counteract the post-endocytic trafficking of Cx43 to lysosomes. Under these conditions, Cx43 was able to recycle to the plasma membrane and assemble into functional gap junctions. The finding that degradation of Cx43 is reduced by cytosolic stress is likely to have significant physiological relevance. There is also evidence suggesting that Cx43 may undergo recycling in unstressed cells. For instance, a recent study by Boassa et al. indicates that Cx43 undergoes recycling to the plasma membrane upon mitotic exit, which is associated with reestablishment of gap junctions [110]. Moreover, Gilleron et al. have found that Cx43 partly colocalizes with Rab11, a small GTPase involved in the recycling pathway [90].

Importantly, recent studies indicate that connexins can be degraded by autophagy. Berthoud and colleagues found that starvation induces loss of Cx43 gap junctions and increased degradation of Cx43 in NRK (normal rat kidney) cells [111]. Starvation had similar effects on Cx50 transfected into HeLa cells. Both Cx43 and Cx50 were found to colocalize with autophagosomal markers in starved cells. As determined by electron microscopy, starved mouse embryonic fibroblasts endogenously expressing Cx43 were found to contain internalized gap junctions enclosed by double-membrane structures resembling autophagosomes. Autophagy was also found to be involved in degradation of a cataract-associated mutant, Cx50-P88S, which accumulates in the cytoplasm. A recent study by Hesketh et al. indicates that autophagy may play an important role in degradation of Cx43 in cardiomyocytes [112]. As determined by electron microscopy, internalized gap junctions in the failing canine ventricular myocardium were found to be sequestered by multilamellar membrane structures with characteristics of autophagosomes.

Several lines of evidence indicate that Cx43 can be trafficked to early and late endosomes prior to its degradation in lysosomes. For instance, Cx43 has been shown to co-localize with the early endosome markers EEA1 (early endosomal autoantigen 1) or Rab5 in response to exposure to tumor-promoting chemicals, such as TPA and lindane [77,113,114]. Moreover, Cx43 has been reported to co-localize with EEA1 in mitotic cells [110]. Trafficking of Cx43 to early

endosomes also appears to be a common feature of cancer cells. For instance, Segretain et al. have found that Cx43 is sequestrated in early endosomes during Leydig cell tumor progression [115]. Endosomal Cx43 sequestration was found to be an early event in Leydig cell tumorigenesis and was associated with uncontrolled Leydig cell proliferation *in situ*.

After entering the early endosome, endocytosed proteins can be trafficked further downstream the degradation pathway to the lysosome, undergo recycling to the plasma membrane, or be transported to the *trans*-Golgi network [116]. Early endosomes have important roles in regulating the levels of integral membrane proteins at the plasma membrane, and participate in a range of processes, including cell signaling, cytokinesis, polarity and migration. There is also growing evidence that early endosomes, in addition to being involved in sorting of endocytosed proteins, function in signal propagation by facilitating the recruitment of signaling molecules and integration of signaling cascades at their surface [117]. Endocytosed proteins destined for lysosomal degradation are transported into intraluminal vesicles in early endosomes that bud from the limiting membrane of the endosome [118]. As determined by immuno-electron microscopy, Cx43 localizes both at the limiting membrane of the early endosome, as well as in its intraluminal vesicles [77,113]. The observation that Cx43 is transported to early endosomes following endocytosis of Cx43 gap junctions raises the question of how Cx43 is trafficked from connexosomes, which are double-membrane structures, to early endosomes, which consist of a single limiting membrane. Based on immuno-electron microscopy studies, we have proposed that internalized gap junctions can undergo a maturation process from double-membrane vacuoles to multivesicular, single-membrane vacuoles in response to protein kinase C (PKC) activation [77,113,119]. This process is associated with loss of the detergent resistance of Cx43 [119]. The transformation of the double-membrane structure of the connexosome is accompanied by trafficking of Cx43 to early endosomes, possibly by fusion between connexosomes and early endosomes [77,113]. A scenario in which the gap junction double membrane is transformed into two single membranes during or after its internalization implies that the gap junction channels undock during or shortly after internalization. Thus, in this model for gap junction endocytosis, most Cx43 localized in early endosomes is expected to exist as undocked connexons [113]. The precise molecular mechanisms involved in the separation of the gap junction double membrane in response to PKC activation remains to be determined. Possibly, the PKC-induced phosphorylation of Cx43 might cause a conformational shift of the gap junction channel that triggers undocking of the connexons.

Ubiquitination of integral membrane proteins has a key role in determining their fate at early endosomes. As described below, recent studies suggest that ubiquitination of Cx43 may be important in modulating both gap junction endocytosis, as well as in the sorting of Cx43 at early endosomes.

6. Regulation of Cx43 ubiquitination

Increasing evidence indicates that connexin turnover is regulated by a complex interplay between connexin phosphorylation and ubiquitination. Many connexin isoforms, including Cx43, are phosphoproteins [120]. Among the protein kinases involved in regulating Cx43 phosphorylation are mitogen-activated protein kinase (MAPK), PKC, protein kinase A, casein kinase 1, Src and cdc2 [120]. Cx43 is mainly phosphorylated on serines, but may also be phosphorylated on tyrosines and threonines. Phosphorylation of Cx43 is thought to occur exclusively in its C-terminal tail. Other connexin isoforms, for instance Cx36 and Cx56, have been shown to also be phosphorylated within their cytoplasmic loop [120]. Phosphorylation of connexins can regulate gap junctions through a variety of mechanisms, for instance by affecting connexin synthesis and trafficking, assembly of connexons into gap junctions or gating of gap junction channels [121]. There is also substantial evidence that phosphorylation of Cx43 is important in regulating gap junction internalization and degradation, as previously reviewed by Laird [68]. Studies carried out the recent years suggesting that Cx43 phosphorylation is tightly linked to Cx43 ubiquitination, might have important implications for our understanding of how endocytosis and degradation of connexins are regulated.

Ubiquitin is a globular protein consisting of 76 amino acids that is able to covalently conjugate to other proteins [122]. The best-known function of ubiquitination is to regulate protein degradation, but has also important roles in regulating the activity or localization of proteins. Ubiquitin is involved in a variety of cellular processes, including endocytosis and post-endocytic trafficking of integral membrane proteins. Ubiquitin is covalently conjugated to proteins by forming a bond between the carboxy-terminal glycine of ubiquitin and the ε-NH_2 group of a lysine residue in the substrate protein. Alternatively, ubiquitin can be conjugated to the α-NH_2 group of the N-terminal amino acid of the substrate [123,124]. In 1995, Laing and Beyer provided the first evidence that ubiquitin is involved in degradation of Cx43 [103,125]. By using CHO (Chinese hamster ovary)-ts20 cells, which express a thermolabile E1 ubiquitin-activating enzyme, the Cx43 protein level was found to increase under conditions where the ubiquitin-activating enzyme was defective [125]. The increased levels of Cx43 expression observed under these conditions was accompanied by enlarged Cx43 gap junctions at the plasma membrane as well as more diffuse Cx43 localization in the cytoplasm. The authors also demonstrated that Cx43 can be conjugated to ubiquitin [103,125]. Subsequent studies by Ruts and Hülser indicated that ubiquitin is localized at gap junction plaques, as determined by immuno-gold electron microscopy [126].

Ubiquitination of Cx43 has been found to be regulated by epidermal growth factor (EGF) [127]. In many cell types, EGF causes inhibition of gap junction channel permeability by inducing MAPK-mediated phosphorylation of Cx43 [128,129]. Warn-Cramer et al. have demonstrated that the serines at positions 255, 279 and 282, located in the carboxyl-terminal tail of Cx43, act as major phosphorylation sites during MAPK-induced inhibition of gap junctions [130,131]. In some cell types, such as the rat liver epithelial cell line IAR20, EGF is not only able to block Cx43 gap junction channel permeability, but also stimulates gap junction endocytosis and Cx43 degradation [127]. The EGF-induced endocytosis of Cx43 is accompanied by increased Cx43 ubiquitination [127]. There appears to be a close association between EGF-induced Cx43 phosphorylation and ubiquitination. For instance, both the phosphorylation and ubiquitination levels of Cx43 are increased few minutes after EGF exposure, and correlate during endocytosis and post-endocytic trafficking of Cx43 [113,127]. Moreover, both EGF-induced ubiquitination and endocytosis of Cx43 is counteracted by inhibitors of MEK1 [127]. There is increasing evidence that the EGF receptor regulates endocytosis and degradation of Cx43 in multiple tissue types. For instance, activation of EGF receptor by heparin-binding EGF (HB-EGF) causes increased endocytosis and degradation of Cx43 in mouse cardiomyocytes [132].

Ubiquitination of Cx43 is also induced by the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [104,113]. TPA is a potent PKC activator which induces inhibition of gap junction channels in multiple cell types [133–137]. The TPA-induced phosphorylation of Cx43 and inhibition of Cx43 gap junction channels involve the MAPK pathway [138–140]. In addition to inducing inhibition of gap junction channel permeability, TPA exposure also results in increased endocytosis of Cx43 gap junctions and enhanced Cx43 degradation in many cell types [136,141–143]. TPA-induced endocytosis and degradation of Cx43 correlate with increased Cx43 ubiquitination [104,113]. PKC has a major role in the TPA-induced ubiquitination of Cx43. The TPA-induced Cx43 ubiquitination, endocytosis and degradation are strongly counteracted by inhibitors of PKC and partly counteracted by inhibitors of MEK1 [104]. Based on studies using antibodies that differentiate between mono- and polyubiquitinated proteins, Cx43 has been suggested to be modified by multiple monoubiquitins

rather than a polyubiquitin chain in response to TPA treatment [104,144]. In future studies it will be important to elucidate in further detail the type of ubiquitin modification Cx43 is subjected to, as well as to determine the Cx43 ubiquitin conjugation sites, by using mass spectrometry. It will also be interesting to elucidate whether ubiquitination of distinct sites on Cx43 may affect gap junctions differentially.

The E3 ubiquitin ligases specify timing and substrate selection of the ubiquitination reaction and are key regulatory determinants in the ubiquitination process. The E3 ubiquitin ligases can be classified into three major families: (1) the HECT (homologous to E6-AP carboxy terminal) E3s; (2) the RING (really interesting new gene) E3s; and (3) the U-box E3s. Members of the HECT family of E3 ubiquitin ligase family contain an approximately 350-residue region at their carboxyl termini, termed the HECT domain [145]. Within the HECT domain is a strictly conserved cysteine residue that acts as a site for thiol ester formation with ubiquitin transferred from an E2-conjugating enzyme. This cysteine residue is essential for substrate ubiquitination. In contrast to the RING and U-box E3 ubiquitin ligases, HECT E3s participate directly in catalyzing ubiquitination by forming a bond with ubiquitin prior to the transfer of ubiquitin to the substrate protein. Another feature shared by many HECT E3s is the WW domain, a short motif that can bind phosphoserine and phosphothreonine or PY (XPPXY, where P is proline, X is any amino acid and Y is tyrosine) motifs in the target protein and thereby mediate substrate recognition [146]. The prototype member of the HECT E3 ubiquitin ligase family is Nedd4 (neural precursor cell expressed, developmentally downregulated 4). Nedd4 is ubiquitously expressed in human tissues and catalyzes ubiquitination of both soluble and trans-membrane proteins, including various channel proteins such as ENaC (amiloride-sensitive epithelial sodium channel) [147–149]. Leykauf et al. have demonstrated that Nedd4 binds to the C-terminal tail of Cx43 [150]. The C-terminal tail of Cx43 harbors a proline-rich region corresponding to the consensus of a PY motif, which is known to act as a binding site for proteins containing WW domains, including the Nedd4/Nedd4-like family of E3 ubiquitin ligases [151]. The rat Nedd4 protein contains three WW domains, all of which are able to bind to rat Cx43 [150]. Phosphorylation of Cx43 is not necessary for Nedd4 binding. However, treating cells with EGF causes a significant increase in the binding of the WW3 domain to phosphorylated Cx43, suggesting that phosphorylation of Cx43 might modulate the binding of Nedd4 [150]. Depletion of Nedd4 by small interfering RNA (siRNA) has been shown to result in accumulation of Cx43 gap junctions at the plasma membrane [150]. Girão et al. have found that mutation of the Cx43 PY motif reduces its interaction with Nedd4, which is associated with reduced Cx43 ubiquitination [144]. The authors suggested that Nedd4 directly catalyzes ubiquitination of Cx43 by interacting with its PY motif.

The PY motif of Cx43 is overlapped by a tyrosine-based sorting signal conforming to the consensus YXXϕ (where Y is tyrosine, X is any amino acid and ϕ is an amino acid with a bulky hydrophobic side chain) [151]. This motif is found in the cytosolic domains of multiple plasma membrane proteins [152]. The motif has an important role in mediating protein internalization from the plasma membrane and in targeting proteins to lysosomes [153,154]. For instance, the YXXϕ signal can interact with AP-2, an adaptor protein found in clathrin coats at the plasma membrane [155,156]. Both the PY and the YXXϕ motifs are important in regulating the level of Cx43 gap junctions at the plasma membrane, possibly by controlling Cx43 endocytosis [151].

Whether connexins other than Cx43 undergo ubiquitination during endocytosis, and what role Nedd4 might play in these processes is currently unknown. Interestingly, Cx26 binds to OCP1 (Organ of Corti protein 1), a protein that is a subunit of an SCF (Skp1/Cul1/F-box complex) E3 ubiquitin ligase [157,158]. OCP1 is abundantly expressed in the Organ of Corti [157–159]. Given that Cx26 is one of the major gap junction proteins in the epithelial support complex of the Organ of Corti, these findings raise the possibility that OCP1 could be involved in regulating ubiquitination of Cx26 in this organ [157,158].

7. Role of ubiquitin in endocytosis and post-endocytic sorting of Cx43

Cx43 is thought to undergo ubiquitination at the plasma membrane [127]. This notion is supported by the observation that ubiquitin is recruited to Cx43 gap junction plaques in response to TPA treatment, as determined by confocal microscopy [113], and by the observation that Nedd4 partly colocalizes with Cx43 gap junctions at the plasma membrane [150]. Studies by Girão et al. indicate that ubiquitination of Cx43 is involved in regulating gap junction endocytosis, and have identified Eps15 as a Cx43 binding partner. Eps15 contains a clathrin-binding domain, as well as ubiquitin-interacting motifs that bind directly to ubiquitinated proteins [160]. Eps15 is thought to function as an adaptor that links ubiquitinated plasma membrane proteins to clathrin-coated pits at the plasma membrane, and may also be involved in mediating clathrin-independent endocytosis of ubiquitinated proteins [161]. The binding between Eps15 and Cx43 requires an intact ubiquitin-interacting motif in Eps15 [144]. The interaction between Eps15 and Cx43 is thought to require Cx43 ubiquitination, since depletion of Nedd4 by siRNA counteracts the interaction between Eps15 and Cx43 [144]. The binding between Eps15 and Cx43 is also reduced when the PY motif of Cx43 is mutated. In a recent study by Catarino et al., fusion of a ubiquitin moiety to the Cx43 carboxyl-terminal tail to mimic Cx43 ubiquitination was found to increase the association between Cx43 and Eps15 [162]. Fusion of ubiquitin to Cx43 resulted in increased endocytosis and degradation of Cx43, in a process that was independent of the YXXϕ motif [162]. Depletion of Eps15 by siRNA results in enlarged gap junctions, suggesting that Eps15 might be involved in mediating endocytosis of Cx43, possibly by acting as a link between ubiquitinated Cx43 and components of the endocytic machinery [144]. However, depletion of Eps15 has no effect on the Cx43 protein level [144].

In addition to being involved in internalization of Cx43 at the plasma membrane, ubiquitin may also be involved in the post-endocytic sorting of Cx43 to lysosomes. The formation of intraluminal vesicles in early endosomes is preceded by accumulation of endocytosed proteins on flat clathrin-coated membrane areas on the endosomal limiting membrane [118]. Ubiquitination of endocytosed proteins is a signal for their accumulation on such flat clathrin coats, and hence for their subsequent degradation in lysosomes. Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) contains a clathrin-binding domain and localizes in clathrin-coated domains on early endosomes [163]. Hrs binds to ubiquitinated proteins via a ubiquitin-interacting motif, and sorts ubiquitinated membrane proteins to the clathrin-coated domains and counteract their recycling to the cell surface [163]. Hrs also has a key role in the formation of intralumenal vesicles in early endosomes by interacting with Tsg101 (tumor-susceptibility gene product 101), a subunit of the endosomal sorting complex required for transport (ESCRT) I [164]. ESCRT I is a protein complex which, together with ESCRT II and III, regulate protein sorting and formation of intraluminal vesicles in early endosomes. Both Hrs and Tsg101 have been shown to be involved in regulating the trafficking of Cx43 from early endosomes to lysosomes in response to TPA treatment [113]. When Hrs and Tsg101 were simultaneously depleted by siRNA, a sub-pool of ubiquitinated Cx43 was found to remain localized in early endosomes, even after prolonged TPA treatment [113].

Auth et al. have demonstrated that Tsg101 interacts directly with the Cx43 carboxyl-terminal tail [165]. The interaction between Cx43 and Tsg101 was increased in response to TPA treatment. Tsg101 was also shown to interact with the carboxyl-terminal regions of Cx30.2, Cx36 and Cx45 [165]. Tsg101 and other components of the ESCRT machinery have important roles in the regulation of cell

proliferation, polarity and migration [166]. The finding that the post-endocytic sorting of Cx43 is regulated by the ESCRT complex may have important implications for understanding the mechanisms underlying the dysregulation of Cx43 trafficking during cancer development and other diseases.

Recent studies indicate that ubiquitination of integral plasma membrane proteins not only is involved in mediating their trafficking from early endosomes to lysosomes, but may also have important roles in targeting proteins for degradation by autophagy [167]. An important subject for future studies will be to elucidate whether ubiquitination of Cx43 is involved in targeting connexosomes for degradation by autophagy.

8. Role of the proteasome in endocytosis of Cx43 gap junctions

In addition to playing a key role during ERAD, the proteasome is also involved in the endocytosis of plasma membrane proteins [152]. Degradation of Cx43 gap junctions is counteracted by proteasomal inhibitors [16,103,105,125]. The proteasome appears to play an indirect role in degradation of Cx43 gap junctions, by affecting Cx43 internalization [13,103,105,109]. The precise mechanisms by which proteasomal inhibitors interfere with Cx43 endocytosis are unclear. However, since treatment of cells with proteasomal inhibitors causes accumulation of polyubiquitinated proteins and, as a consequence, depletion of the pool of unconjugated ubiquitin, proteasomal inhibitors might counteract Cx43 endocytosis by reducing Cx43 ubiquitination [104,113]. Proteasomal inhibitors may also counteract endocytosis of Cx43 by affecting the stability of various Cx43-interacting proteins. For instance, proteasomal inhibitors have been shown to reduce the interaction between Cx43 and ZO-1, which is associated with the formation of enlarged gap junctions [168].

9. Concluding remarks and future perspectives

It has become clear that connexins may follow multiple pathways *en route* to their degradation in lysosomes. Moreover, the various pathways appear to be differentially regulated in response to physiological and pathophysiological stimuli. There is growing evidence that endosomes may have important roles in controlling the level of connexins at the plasma membrane. This notion is supported by recent studies indicating that connexins are able to undergo recycling to the plasma membrane after endocytosis. In future studies it will be important to obtain a better molecular understanding of how the balance between degradation and recycling of connexins is regulated, and how these processes are affected by various types of cytosolic stress. Post-translational modifications of connexins are likely to have a key role in the regulation of gap junction endocytosis and lysosomal sorting of connexins. Research carried out the recent years suggests that endocytosis and degradation of Cx43 are regulated by a complex interplay between Cx43 phosphorylation and ubiquitination. An important subject for future studies will be to obtain a clearer understanding of how ubiquitination of Cx43 and other connexin isoforms is regulated under normal physiological conditions, and whether dysregulation of these processes contributes to loss of functional gap junctions under various disease states. There is increasing evidence that aberrant regulation of connexin endocytosis and post-endocytic trafficking may be involved in various pathological conditions, including cardiac diseases and cancer. Understanding the molecular basis underlying these processes may have important therapeutic implications.

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