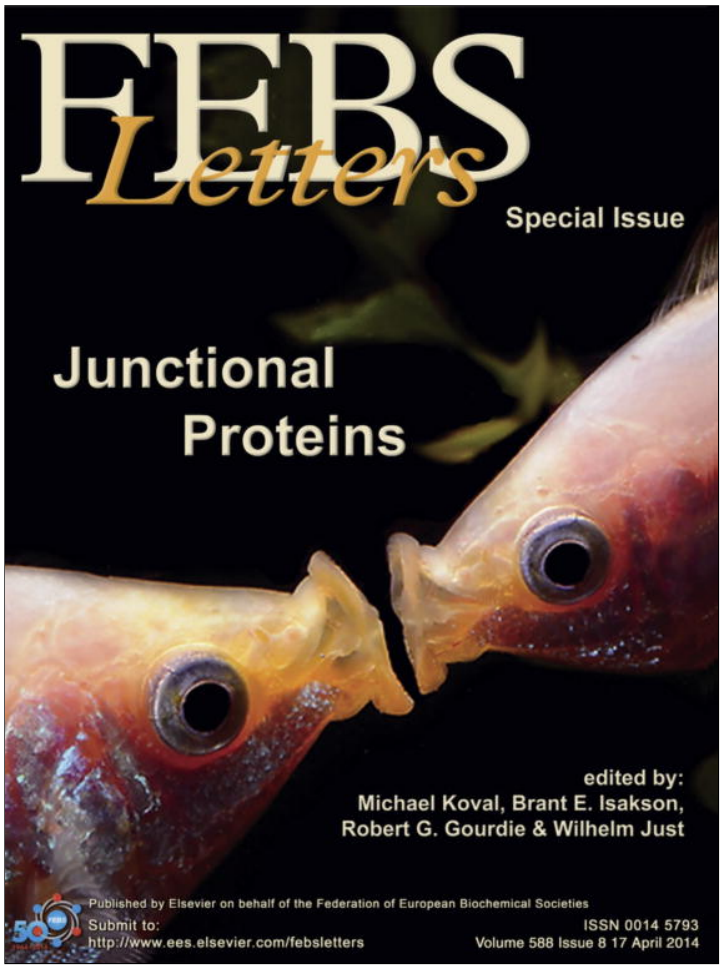

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Review

# Degradation of connexins and gap junctions

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ABSTRACT

**Connexin proteins are short-lived within the cell, whether present in the secretory pathway or in gap junction plaques. Their levels can be modulated by their rate of degradation. Connexins, at different stages of assembly, are degraded through the proteasomal, endo-/lysosomal, and phago-/lysosomal pathways. In this review, we summarize the current knowledge about connexin and gap junction degradation including the signals and protein–protein interactions that participate in their targeting for degradation.**


## 1. Introduction

Connexins have an unexpected short half-life of only 1–5 h even when their half-lives have been determined in tissues [1–5]. It is still puzzling that connexins, either unassembled or assembled into connexons (hemichannels) and especially when assembled and localized at gap junction plaques have – and apparently need – such a fast turnover. The degradation of connexins, connexons, and gap junctions, and alterations of their turnover accompany various physiological and pathological conditions (e.g., cell migration, mitosis, ischemia, etc.). This was discussed at one of the roundtables (themed "Internalization and degradation pathways of connexins and gap junctions") that were entertained at the 2013 International Gap Junction Conference (Charleston, SC, http://academicdepartments.musc.edu/igjc2013) and chaired by the senior authors of this article. Discussed topics, emerging concepts and hypotheses, and other relevant issues related to the turnover of connexins and gap junctions are summarized here.

Gap junction plaques are internalized from the plasma membrane as vesicle-like double-membrane structures termed annular gap junctions. The first electron microscopy images of these structures were published by Bjorkman from granulosa cells of the ovarian follicle [6]. Numerous other ultrastructural studies have shown the presence of annular gap junctions in several cell types and differentiating tissues [7–12]. A few ultrastructural studies reported annular gap junctions enclosed within double-membrane structures, which would be consistent with autophagic degradation of endocytosed gap junctions [9,13,14]. Live-imaging studies have also shown gap junction internalization [15–18]. Their internalization as double-membrane structures is in agreement with studies reporting that docked hemichannels (or connexons) in gap junction plaques appear inseparable under physiological conditions [19,20]. This internalization occurs through a combined endo/exocytic process [21], because one of the cells acts as an acceptor and the other acts as a donor (Fig. 1).

Studies of wild type and mutant connexins have addressed their subcellular localization, channel function, internalization and/or turnover in transfected cells. The turnover of connexins is determined by the rates of synthesis and degradation, which occurs through the proteasomal and the lysosomal pathways. Several reviews on different aspects of connexin and gap junction degradation have been recently published [22–26].

This review will focus on the roles of these pathways on degradation of connexins within the secretory pathway vs. those in gap junction plaques, and the signals and protein–protein interactions that participate in targeting connexins for degradation through the proteasomal vs. the lysosomal pathways. First, we will briefly describe the different pathways and then describe the role of these pathways in the degradation of connexins and gap junctions localized in different cellular compartments.

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## 2. Proteasomal and lysosomal degradation pathways

### 2.1. The 26S proteasome

The 26S proteasome is a cytoplasmic protein complex formed by a barrel-shaped 20S core particle, which contains the proteolytic activity, and two 19S regulatory particles, one on each side of the core particle. The regulatory particle is composed of a ring-shaped base made of six ATPases and a lid [27]. Several proteasome inhibitors have been used to study the function of the proteasome including ALLN (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal), lactacystin, $ZL_3VS$ (carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone), epoxomicin and bortezomib [28,29].

Degradation of cellular proteins by the proteasome can be ubiquitin-dependent or ubiquitin-independent. Modification of proteins by ubiquitin, a three-step process, results from the activity of an ubiquitin-activating enzyme (E1, the rate-limiting step), an ubiquitin-conjugating enzyme (E2) and an ubiquitin-protein ligase (E3). This modification can be attached to the protein as one (mono) or several (multiple mono) single ubiquitin molecules, or as multimers of ubiquitin (poly-ubiquitin chain). Although lysine48-conjugated ubiquitin chains have been associated with targeting proteins for proteasomal degradation, more recent studies suggest that these poly-ubiquitin chains can be formed by linkage of ubiquitin molecules between one of the six other lysines available for conjugation. Although it was initially thought that ubiquitination occurred only on lysine residues of the target protein, ubiquitination has been found in threonines, serines or cysteines and the amino group of the N-terminal residue of the protein targeted for proteasomal degradation (reviewed in [30]). It has been hypothesized that the variety of proteasome-targeting ubiquitin motifs may determine the degradation rate of the target protein.

### 2.2. Lysosomal degradation pathways

Cells have evolved two degradation pathways that depend on the activities of lysosomal enzymes, endo-/lysosomal and phago-/lysosomal (termed macroautophagy, or simply, autophagy). The endo-/lysosomal pathway is designed for the uptake of extracellular nutrients and factors via the formation and internalization of vesicles from the plasma membrane. These vesicles then fuse with endosomes and lysosomes to degrade the cargo.

Macroautophagy is a lysosomal degradation pathway essential for cell survival that is known to be activated by nutrient depletion. It is responsible for the degradation of structures already located in the cytoplasm such as non-functional organelles, protein aggregates, and invading pathogens [31–34]. During autophagy, materials targeted for degradation are first sequestered by an isolation membrane (phagophore) forming a double-membrane vesicle (autophagosome), which then fuses with a lysosome where the cargo is degraded.

Because lysosomes are involved in both the endo-/lysosomal and auto-/phagosomal degradation pathways, the manipulation of specific components allows these pathways to be distinguished. The identification of autophagy-related genes and experimental manipulation of their levels have allowed evaluation of the contribution of autophagy to connexin/gap junction degradation. The microtubule-associated protein 1 light chain 3 (LC3) is considered the most specific autophagosomal marker [35]. LC3 is proteolytically processed shortly after translation and converted into LC3-I. LC3-I is converted to LC3-II after being recruited to developing phagophores and covalently conjugated to phosphatidyl-ethanolamine of the phagophore membrane. LC3-II remains on autophagosomes for most of their lifetime [35,36]. Other autophagosome-related genes include Atg5, Atg6 (Beclin) and Atg7.

## 3. Signals involved in degradation of connexins

Several modifications including phosphorylation, ubiquitination, SUMOylation, methylation, acetylation, nitrosylation, and glutamate γ-carboxylation have been found in connexins (reviewed recently in [37,38]). Of these, phosphorylation and ubiquitination are known to be involved in protein degradation. Evidence that phosphorylation and ubiquitination also play a role in the internalization of gap junction plaques and degradation of connexins has been obtained. The role of phosphorylation in internalization and degradation of gap junctions has been reviewed [39].

### 3.1. Ubiquitination

Cx43 and avian Cx45.6 are polyubiquitinated [40,41]. In NRK cells and TPA-treated rat liver epithelial IAR20 cells, Cx43 is multiply mono-ubiquitinated [42,43]. Several E3 ubiquitin ligases are involved in ubiquitination of connexins. Nedd4, Smurf2 and TRIM21 are among the E3 ubiquitin ligases that interact with Cx43 [44–46]. All of them are associated with gap junction plaques (Fig. 2). Ubiquitination of connexins at gap junction plaques appears to have a role in endocytosis and/or intracellular vesicle trafficking [42,47]. An interaction of E3 ubiquitin ligases with connexins that are either rapidly degraded by endoplasmic reticulum-associated degradation or in their transit to the plasma membrane may not be revealed by immunofluorescence, because the connexins are not concentrated in a particular area (as they are in gap junction plaques).

Nedd4 interacts via its WW domains with the carboxyl-terminus of Cx43 and ubiquitinates Cx43 in gap junctions [42,46]. It has been proposed that the resulting ubiquitinated Cx43 recruits Eps15 (a member of the clathrin-coat-associated sorting proteins [CLASPs] (reviewed in [48]) to gap junction plaques and mediates their internalization [42]. CLASPs encode conserved peptide sequences that contact ubiquitin-moieties across their surface (termed ubiquitin interacting motifs [UIMs]) and thus ensure ubiquitin signal-specificity. Interestingly, alternative CLASPs such as Eps15/Eps15R and Epsin1/2 may replace the canonical CLASP, AP-2, allowing clathrin-mediated endocytosis to occur independent of AP-2.

The Nedd4-interacting protein 2 (NDFIP2) is a short-lived protein that is degraded in the lysosome. siRNA silencing of NDFIP2 decreased the number of gap junction plaques associated with a small increase in Cx43 half-life, whereas overexpression of NDFIP2 increased the number and size of gap junction plaques and decreased the half-life of Cx43 [49]. Thus, it is possible that Nedd4-interacting proteins like NDFIP2 may regulate internalization of Nedd4-ubiquitinated Cx43 (and other connexins) from the plasma membrane.

Smurf2 modulates Cx43 endocytosis and degradation [45]. Treatment of IAR20 cells with the tumor promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA), a protein kinase C activator, promotes Cx43 phosphorylation and ubiquitination, recruitment of Smurf2 to gap junction plaques and increases interaction between these proteins [43,45,50,51]. Treatment with a siRNA against Smurf2 leads to an increase in the size of gap junction plaques and in gap junction intercellular communication, and to prevention of the TPA-induced decrease in Cx43 at gap junctions [45]. The ubiquitin-binding proteins, Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and Tsg101 (tumor susceptibility gene 101), components of the endosomal sorting complex required for transport (ESCRT) have been identified as important for targeting of annular gap junction-endosome intermediates for lysosomal fusion and degradation [47] (Fig. 2).

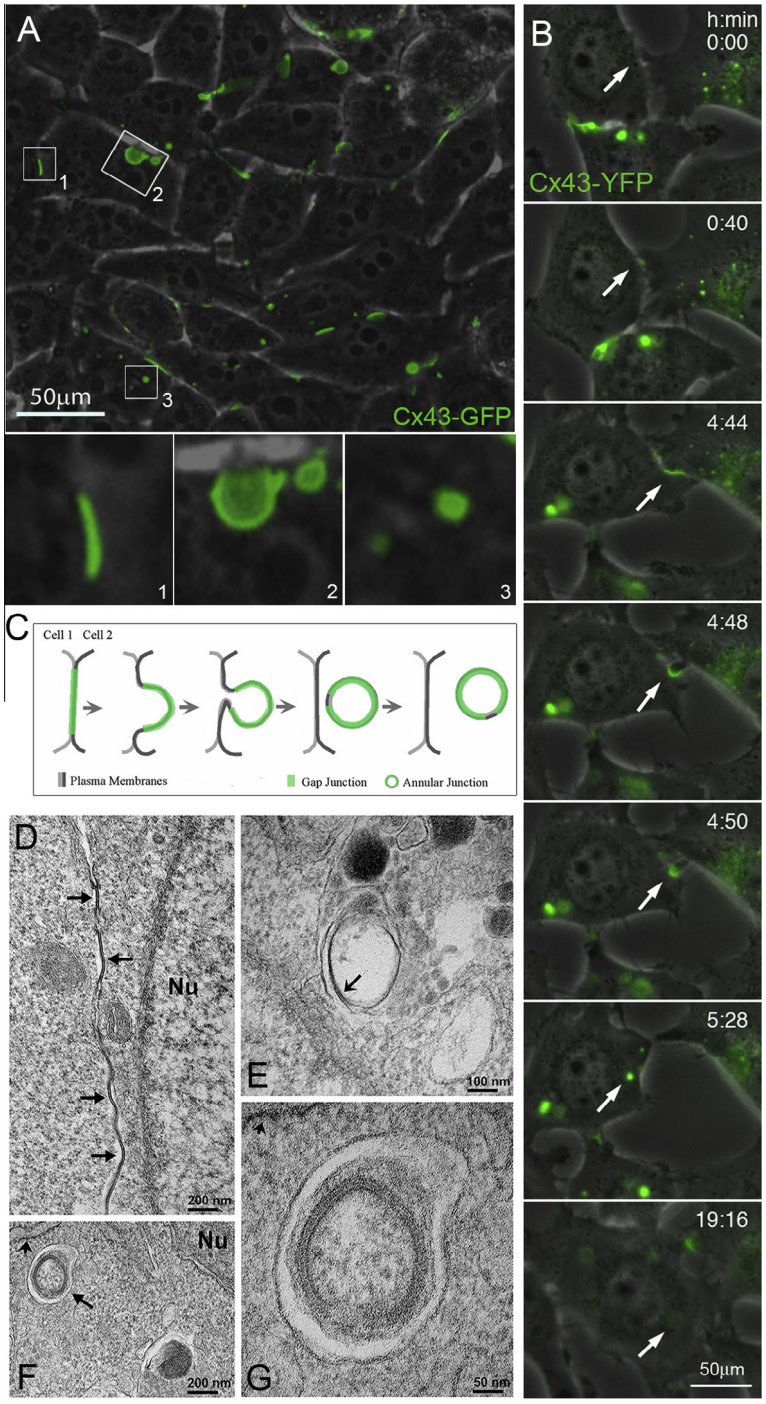


**Fig. 1.** Internalization of gap junction plaques. (A) Combined phase-contrast and fluorescence images of HeLa cells transfected with Cx43-GFP reveal efficient expression and assembly of gap junctions at appositional membranes of transfected cells (visible as green fluorescent lines and puncta such as the one shown in insert (1). Over time, gap junction plaques invaginate into the cytoplasm of one of the two cells (insert 2), detach from the plasma membrane and form endocytosed cytoplasmic annular gap junctions or connexosomes (insert 3). (B) Selected still-images of a time-lapse recording of stably transfected Cx43-YFP expressing HeLa cells showing the formation of a gap junction plaque, its internalization into the cytoplasm of one of the adjacent cells, and subsequent degradation of the generated annular gap junction, indicated by the loss of its fluorescence (marked with arrows). (C) Schematic representation depicting the progressive stages of gap junction internalization shown in A and B. (D) Electron micrograph showing gap junction plaques (arrows) at the appositional membranes of mouse embryonic fibroblasts. (E) Electron micrograph showing an internalized gap junction structure within a membrane-surrounded compartment, which probably corresponds to a secondary lysosome or autolysosome. Note that the pentalaminar pattern characteristic of gap junctions is being lost (arrow) in the internalized structure, probably because it is undergoing degradation. (F) Electron micrograph obtained from mouse embryonic fibroblasts starved by incubation in Hank's balanced salt solution for 1 h showing a double membrane structure, probably an autophagosome (arrow) that encloses an internalized gap junction in close proximity to rough endoplasmic reticulum (short arrow). (G) Electron micrograph showing the internalized gap junction enclosed within the autophagosome at higher magnification; the rough endoplasmic reticulum membrane is indicated by the short arrow. Nu: nucleus (A–C reproduced with permission from Landes Bioscience from Ref. [90], D–G reproduced with permission from The Company of Biologists from Ref. [91]).

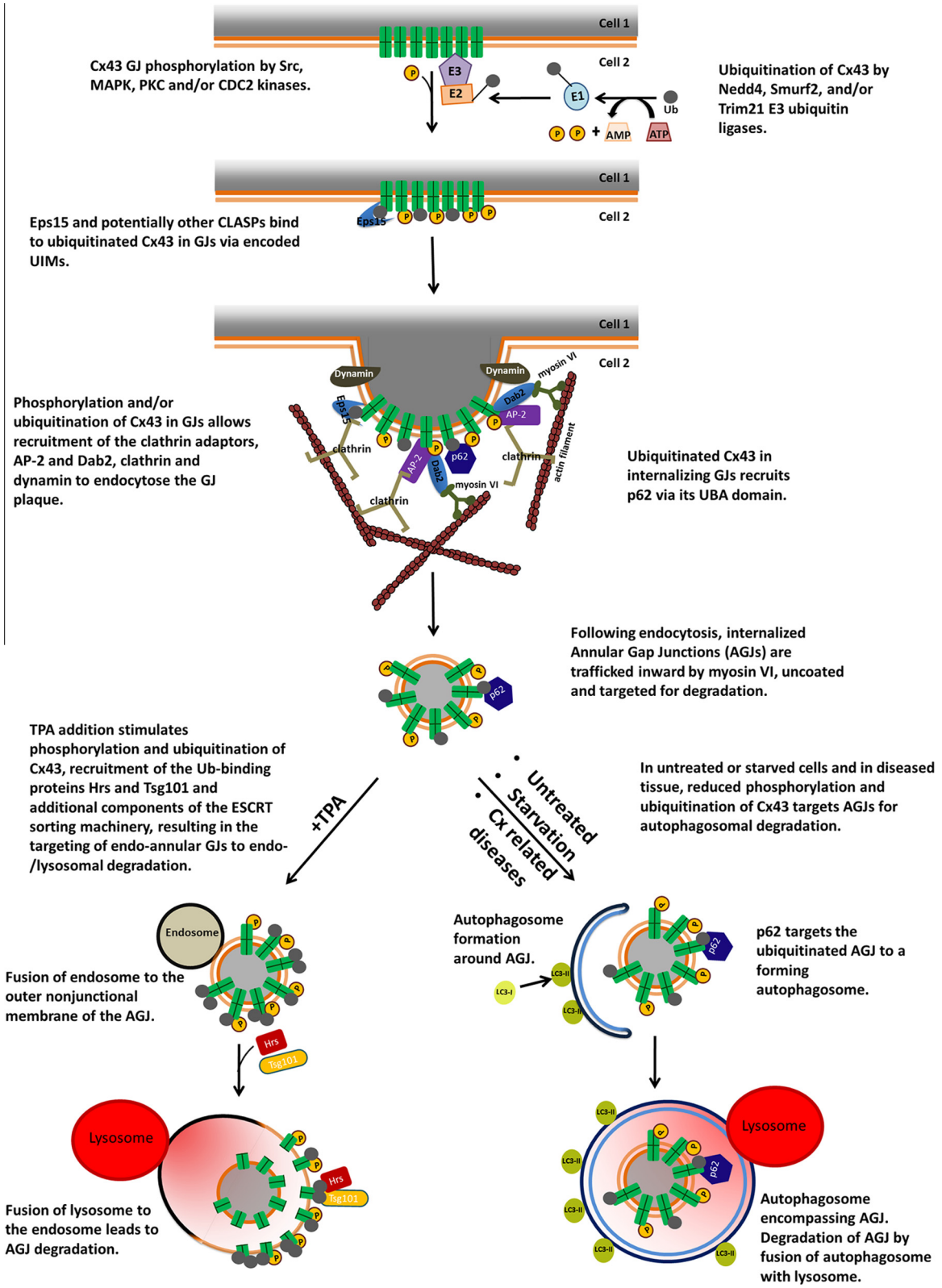


**Fig. 2.** Schematic representation of the signals participating in the proposed steps that lead to gap junction internalization, formation of annular gap junctions in the cytoplasm of the acceptor cell, and annular gap junction degradation through the phago-/lysosomal or the endo-/lysosomal pathway based on studies published in the literature. (Abbreviations are: AGJ, annular gap junction; CLASPs, clathrin-coat-associated proteins; ESCRT, endosomal sorting complexes required for transport; GJ, gap junction; UBA, ubiquitin-associated domain; UIMs, ubiquitin-interacting motifs.)

Proteomic analyses have identified lysyl residues modified by ubiquitin in several connexins. Mass spectrometric analyses of endoproteinase Lys-C/trypsin-digested HEK293T cell homogenates immunoprecipitated with anti-di-glycine-lysine antibodies show the presence of a di-glycine motif attached to lysines 9 and 303, suggesting that Cx43 is ubiquitinated at these amino acid residues in these cells [52]. Using a similar strategy in mouse tissues, this di-glycine-lysine motif has been found in several lysyl residues from different connexins [53]. Interestingly, 12 of the 23 lysines from mouse Cx43, predicted to localize in the cytoplasm, have this modification [53]. However, ubiquitination at these sites needs to be experimentally validated, since some cautionary statements about the interpretation of mass spectrometry results have been made [30].

### 3.2. Interaction with components of the clathrin-mediated endocytic machinery

Internalization of gap junctions occurs preferentially into one of two coupled cells [3,17] suggesting the presence of markers that determine the sidedness of the internalization. This directionality may be aided by several proteins. Several components of the clathrin-mediated endocytosis machinery (e.g., clathrin, the clathrin-adaptors AP-2 and Dab2, the GTPase dynamin2), the retrograde actin motor myosin VI, and filamentous actin have been found in close-association with annular gap junctions [8,17,54–58] (Fig. 2). By analyzing Cx43 C-terminal deletion mutants, Wayakanon et al. obtained evidence that a region including the clathrin/AP-2 binding sites may be important in determining the directionality of internalization [59].

Two of the three canonical tyrosine-based AP-2 binding sites (sites S2 and S3) of the YXXΦ type (where X stands for any amino acid and Φ is a hydrophobic amino acid with a bulky side chain) present in the C-terminus of Cx43 cooperate to mediate clathrin-mediated gap junction endocytosis [15]. Based on the limited availability of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) in gap junction plaques, a signaling lipid required for AP-2 binding and successful clathrin-mediated endocytosis [60,61], it is tempting to speculate that cooperation between the tyrosine-based AP-2 binding sites may compensate the decreased level of $PIP_2$ present in gap junctions. Whether these AP-2 binding sites play a role in constitutive and acutely induced gap junction endocytosis, as occurs for example in G-protein coupled receptor endocytosis [62] remains to be determined.

### 3.3. Interaction with ZO-1

Disaggregation of ventricular muscle leads to internalization of gap junctions [10] and causes an increase in the amount of the scaffolding protein, zonula occludens-1 (ZO-1) associated with Cx43 [63]. In vascular endothelial cells treated with thrombin or endothelin-1 and in Cx43-GFP transfected 42GPA9 Sertoli cells treated with lindane (a carcinogenic pesticide), ZO-1 is displaced from gap junction plaques on the side of plaque invagination [64,65]. These results imply that the increased Cx43/ZO-1 interaction occurs in the donor cell, and that there should be a decrease in the Cx43/ZO-1 interaction in the acceptor cell. It remains to be determined whether the interaction of ZO-1 with actin plays a role in this process.

### 3.4. Interaction with p62

Sequestosome-1 (also known as p62) is a bifunctional protein that binds LC3 and ubiquitinated proteins and serves as a cargo receptor to deliver polyubiquitinated (lysine63-linked) and potentially monoubiquitinated oligomeric protein complexes to the autophagic degradation pathway [31,66–68]. Since connexins are modified by ubiquitin, their interaction with p62 is likely to target them for autophagic degradation (Fig. 2).

## 4. Degradation of connexins/connexons in their transit to the plasma membrane

### 4.1. Proteasomal pathway

The participation of the proteasome in connexin degradation was first reported for Cx43 [40]. Degradation of Cx43 in CHO cells containing a temperature sensitive mutant E1 was impaired at the restrictive temperature, and ubiquitinated proteins were detected in Cx43 immunoprecipitates from normal CHO cells re-precipitated with anti-ubiquitin antibodies [40]. Treatment of different Cx43-expressing cell lines with proteasomal inhibitors increases Cx43 levels and decreases its rate of degradation [40,69–72].

Both wild type Cx43 and Cx32 undergo endoplasmic reticulum associated proteasome-mediated degradation (ERAD) [73] during or after protein biosynthesis. In several cell types that endogenously express Cx43 or Cx32, degradation of newly synthesized connexins was inhibited by ALLN (a proteasomal inhibitor) to a similar extent in the presence or absence of brefeldin A [73]. Proteasomal degradation of connexins retained in the endoplasmic reticulum requires their dislocation from this subcellular compartment into the cytosol. Several cytosolic stresses including changes in redox state and thermal stress can modulate connexin dislocation and endoplasmic reticulum-associated proteasomal degradation [71,74]. It has been estimated that a significant proportion ($\geqslant$40%) of newly synthesized wild type Cx32 and Cx43 may undergo endoplasmic reticulum-associated degradation [74], an unusually high rate from a metabolic point of view. It is not clear yet why connexin protein biosynthesis, translocation into the endoplasmic reticulum-membrane, and/or connexon oligomerization may occur at such an inefficient rate, requiring degradation of almost one-half of newly synthesized connexin polypeptides.

CIP75 interacts with Cx43 and stimulates its degradation [75–77]. This protein (which is 100% identical in sequence to the mouse UBIN protein [25,78]) contains an ubiquitin-like (UBL) and an ubiquitin-associated (UBA) domain that interacts with a region in the carboxyl terminus of Cx43 [75]. Although CIP75 interacts with ubiquitinated proteins and endoplasmic reticulum-retained Cx43 (Cx43-HKKSL, a genetically engineered Cx43-fusion protein encoding an HKKSL-endoplasmic reticulum retention signal [79]), the Cx43-HKKSL that co-precipitates with CIP75 is not ubiquitinated in lactacystin-treated cells expressing HA-tagged ubiquitin [77]. Co-immunoprecipitation experiments suggest that Cx43-HKKSL is in a complex with CIP75 and the proteasome subunits of the regulatory particle, Rpn1 and Rpn10. Treatment with DTT and bortezomib (which would lead to accumulation of dislocated Cx43-HKKSL in the cytosol) increased the amount of CIP75 and Rpn1 interacting with Cx43-HKKSL [76]. Recently published findings suggest that CIP75 plays a role in dislocating Cx43 from the endoplasmic reticulum and that it acts as an adaptor mediating the interaction of Cx43 with the proteasome [76].

### 4.2. Lysosomal pathway

Cx43 accumulates in lysosomes in Cx43-infected MDA-MB-231 cells (a human breast tumor cell line that is communication-deficient even after infection). Since treatment with BFA for 6 h did not affect the lysosomal localization of Cx43 and inhibition of protein synthesis decreased the intensity of Cx43 immuno-reactivity, Qin et al. proposed that Cx43 could be targeted to the lysosome from an early secretory compartment [72]. A similar mechanism may be responsible for the accumulation of Cx43 in a lysosomal

compartment in bovine aortic endothelial cells treated with TGF-β1 [80].

## 5. Degradation of gap junctions

### 5.1. Proteasomal pathway

In some cell types, treatment with proteasomal inhibitors leads to an increase in Cx43 immuno-reactive gap junction plaques [1,71], and can also prevent (at least in part) the brefeldin A-induced decrease in immuno-reactive Cx43 at gap junction plaques [70,72]. Yet, the proteasome inhibition-induced increase in Cx43 immuno-reactivity at gap junction plaques implicates that the proteasome is somehow involved in the degradation of gap junctional plaques. A high proportion of Cx43 gap junctional plaques in BICR/$M1R_k$ cells also bind anti-ubiquitin antibodies [81], however modification by ubiquitin could be signaling either for proteasomal, or lysosomal degradation depending on its type. The type of ubiquitin modification was not specified. Most likely, the role of the proteasome in degradation of gap junction plaques is indirect, a conclusion that is supported by the highly oligomeric organization of gap junctions, and the proteasome being designed to degrade single unfolded polypeptides that need to be "spooled" into the central proteasome core (see Section 2.1). Several mechanisms have been proposed to explain its participation. (1) A short-lived protein may be required for targeting correctly folded connexins for proteasomal degradation [71]. (2) The proteasome regulates internalization of gap junctions by modulating the interaction of Cx43 with ZO-1 [82]. (3) Treatment with proteasome inhibitors leads to an increase in the activity of the Akt protein kinase, which in turn, phosphorylates Cx43 leading to increased stability of the protein at gap junction plaques without requiring ubiquitination of Cx43 [83]. Mounting evidence now strongly suggests that the role of ubiquitination of connexins at gap junction plaques plays a critical role in the internalization of gap junctions [42,47] (Fig. 2).

### 5.2. Endo-/lysosomal pathway

Membrane vesicles containing intact endocytosed gap junctions, or gap junctions apparently in the process of degradation have been observed in several ultrastructural studies [7,57,84–86], suggesting that annular gap junctions may fuse with lysosomes [57,84,86]. These vesicular structures suggest involvement of the endo-/lysosomal pathway in the degradation of annular gap junctions.

Implicit in the degradation of annular gap junctions through the endo-/lysosomal pathway is the fusion of a double-membrane vesicle containing densely packed gap junction channels with a single-membrane organelle. The mechanism by which this occurs is unclear. It has been suggested that the inner membrane of the annular gap junction splits from the outer membrane, generating a single-membrane cytoplasmic annular hemi-gap junction vesicle that can then fuse with a single-membrane endosome [23,24,47,87]. However, the signals that would drive such a gap junction channel splitting shortly after internalization are not clear. It is possible that the small membrane separations devoid of gap junction channels (observed by ultrastructural studies in annular gap junctions [10,17,22]), likely corresponding to the "neck" of the plasma membrane invagination before forming the annular gap junction, are the sites for fusion with endosomes (shown schematically in Fig. 2). In this case, the outer membrane of this small region could fuse with the single-membrane endosome generating a larger vesicle (part endosome on one side, part gap junction plaque on the other side). The inner layer of the gap junction could then split away from the outer layer, for example based on increasing acidification as is typical for cargo/cargo-receptor separation, forming an internal vesicle (complementing the outer hemi-gap junction plaque) that then can be degraded via the lysosome, or resulting in the formation of multi-vesicular bodies, a vesicular structure known to proceed to lysosomal fusion [87,89] (Fig. 2, left bottom portion). Analysis of cells incubated in media containing fluorescently labeled wheat germ agglutinin (WGA), a plasma membrane impermeant lectin that binds sialic acid and N-acetylglucosamine demonstrated that about 50% of annular gap junctions (probably the ones that were internalized during the WGA-labeling period) contained a punctum of fluorescently labeled WGA [22]. Sialic acid and N-acetylglucosamine are carbohydrate moieties that are common on the surface of glycosylated plasma membrane proteins. Since connexins are not glycosylated and are densely packed within gap junction plaques, these data support the idea that the small membrane separation observed in annular gap junctions corresponds to non-junctional membranes where fusion with endosomes may take place.

### 5.3. Autophagy

Several recent studies in different tissues, primary cultured cells and cell lines have demonstrated that gap junctions are subject to constitutive and induced autophagy (Figs. 1 and Fig. 2). Autophagosomes exhibit a highly characteristic, clearly recognizable double-membrane structure on ultra-thin sections, making conventional electron microscopy a highly reliable technique for the characterization of autophagosomes [36]. In all studies, cytoplasmic annular gap junctions were detected inside phagophores by ultrastructural analyses (see Fig. 1E–G as examples). Also, all of these studies have shown co-localization of endogenously or exogenously expressed LC3 with several connexins including Cx26, Cx32, Cx43 and Cx50 under control conditions or after induction of autophagy and in the absence and/or the presence of lysosomal inhibitors [89–93], indicating the presence of these gap junction components in autophagosomes. Additionally, expression of a conjugation-deficient LC3 prevented its co-localization with Cx43 annular gap junctions [90].

Inhibition of autophagy by pharmacological agents (e.g., wortmannin or bafilomycin A1) resulted in accumulation of annular gap junctions [90]. Treatment of serum-deprived COS-7 cells exogenously expressing Cx43 with the PI3K inhibitor, 3-methyladenine, decreased the degradation of the Triton X-100-insoluble fraction of Cx43 [89]. Genetic silencing of autophagy-associated genes (e.g., LC3, Atg6, LAMP-2 or p62) resulted in accumulation of annular gap junctions and reduced co-localization of LC3 with Cx43 [90]. Genetic deletion of Atg5 or siRNA-knockdown of Atg7 reduced the starvation-induced decrease in connexin levels [90,93]. The starvation-induced decrease in connexins was sensitive to lysosomal inhibitors [90,93], but not to proteasomal inhibitors [89].

In the heart, cytoplasmic LC3-positive vesicles were observed in starved mice expressing GFP–LC3 [36]. Starvation also decreased Cx43 immuno-reactivity at appositional membranes and increased intracellular staining in mouse hearts [92]. In a canine model of heart failure, gap junctions became lateralized and appeared to be internalizing. In this model, double membranes that were suggested to be putative isolation membranes were detected in close association with internalized gap junctions [92]. Levels of LC3-II in whole heart homogenates and of Cx43 in lipid rafts were increased in failing, compared with normal heart [92]. In the liver, levels of Cx26, Cx32 and Cx43 were higher in the autophagosome subcellular fraction from starved compared with fed wild type mice [89]. Additionally, levels of Cx43 were increased in livers from macroautophagy-compromised compared with wild type mice [89].

The structural organization of annular gap junction vesicles representing cytoplasmically localized multi-protein complexes, that in addition are para-crystalline packed, renders autophagy as the most apparent annular gap junction degradation pathway.

However, the level of constitutive and induced autophagy contributing to gap junction turnover may vary and depend on the cell type [90,93], and possibly also on the connexin subtype [89]. Bejarano et al. estimated that in transfected COS-7 cells, ~20% of the gap junction plaque Cx43 (Triton X-100-insoluble fraction) was degraded by autophagy under normal conditions [89].

Based on results obtained by silencing expression of Eps15, Nedd4 or Atg7 and changes in their interaction with Cx43, Bejarano et al. have proposed a mechanism for the autophagic degradation of Cx43 at gap junction plaques: Nedd4-mediated ubiquitination of Cx43 in gap junction plaques recruits Eps15 to gap junctions, which would initiate their autophagy-dependent internalization and degradation [89] (see Fig. 2, bottom right portion).

## 6. Degradation of disease-linked mutant connexins

Several disease-associated connexin mutants do not traffic properly and localize in compartments of the secretory pathway (e.g., endoplasmic reticulum [ER], endoplasmic reticulum Golgi intermediate compartment [ERGIC], and Golgi) [93,96]. Some of the Charcot-Marie Tooth disease (CMTX)-linked Cx32 mutants of this type are dislocated from the ER (after retrograde transport to the ER if they reach post-ER compartments) and are degraded by the proteasome [73]. One of these mutants, Cx32E208K, localizes to the ER and its ER-associated degradation is reduced by cellular stress before its dislocation from the ER, a phenomenon associated with decreased poly-ubiquitination of the mutant protein compared with that of proteasome-inhibitor stabilized mutant protein [95]. Proteasomal inhibition increased the levels of mutant Cx32 protein, but did not alter their cytoplasmic distribution [73]. In contrast, trafficking to the plasma membrane and function of an autosomal recessive cataract-linked Cx50 mutant, Cx50fs, which undergoes proteasomal degradation, were rescued by proteasomal inhibition [96]. These effects were independent of Akt protein kinase activity [96].

Some of the erythrokeratoderma variabilis-linked Cx31 mutants (expressed as eGFP fusion proteins) formed large aggregates in the cytoplasm that did not co-localize with proteins residing in the ER, Golgi or lysosomes. These mutants increased 20S core proteasome immunoreactivity, and co-localization between the proteasomal marker and the mutants was observed [97].

Some connexin mutants that do not traffic properly are not degraded by the proteasome. Chloroquine treatment of HeLa cells transfected with CMTX-associated Cx32 mutants that are retained in the Golgi led to intracellular accumulation of Cx32-positive granules that partially co-localized with a lysosomal-associated membrane protein [98]. Thus, at least some of the mutants may be routed directly to the lysosome for degradation resembling the by-pass pathway described by Qin et al. [72]. The cataract associated Cx50 mutant, Cx50P88S, forms ER-derived intracellular accumulations [99,102]. The Cx50P88S accumulations co-localized with markers of the autophagosome suggesting that these accumulations formed because of an overload of the autophagosomal capacity of the cells [91].

## 7. Conclusions and perspectives

It is likely that *in vivo* all the mechanisms described above participate in connexin, connexon (hemichannel), and gap junction degradation, and that the contribution of each of these pathways is modified depending on environmental conditions. Having multiple proteolytic pathways to control connexin levels, hemichannel function, and gap junctional intercellular communication would allow cells to compensate for a malfunctioning or compromised pathway, and to adapt to changing conditions.

Wild type connexins can be degraded by the proteasome, but ubiquitination of endoplasmic reticulum-retained connexins may not be an absolute requirement. Similarly, the molecular mechanisms triggering gap junction plaque internalization and degradation are still not well defined, but phosphorylation and ubiquitination emerge as likely events. Even less is known about hemichannel degradation.

Several questions remain to be answered regarding modification of connexins by ubiquitin. What are the types of ubiquitin chains modifying connexins? Do modifications by different ubiquitin chains or at different amino acid residues in the connexin polypeptide have different roles (degradation vs. intracellular trafficking)? Do they target connexins for degradation through different pathways? Are different E3 ubiquitin ligases responsible for ubiquitination at different amino acid residues in connexins in different subcellular compartments? Are there other modifications that affect the rate of connexin and gap junction degradation? It would appear plausible that Cx43 in gap junction plaques would become lysine63-polyubiquitinated, since Nedd4 is known to preferentially add lysine63-polyubiquitin chains to target proteins [101], lysine63-polyubiquitinated target proteins are recognized by CLASPs, specifically Epsin1/2 and Eps15/Eps15R [48], and this type of ubiquitin modification can act as an internalization signal for clathrin-mediated endocytosis (CME) [102,105]. However, so far multiple mono-ubiquitination of Cx43 has been described and associated with the internalization of gap junctions. Future research will clearly be required to determine the potentially numerous types and functions of ubiquitination that occur on connexins in different cellular locations (e.g. intracellular membranes versus plasma membranes).

Connexin phosphorylation can decrease intercellular communication by gating channels closed or by targeting gap junctions for internalization and subsequent degradation [26,37,39,106,107]. As shown for Cx43, phosphorylation and/or dephosphorylation events can lead to potentially significant conformational changes in the protein [108,109]. Such structural alterations may allow interaction of components of the endocytic machinery with connexins destined for internalization. The phosphorylation events crucial for triggering gap junction internalization and which of these events are cell-type specific remain to be determined.

Recycling of internalized connexins at the plasma membrane has been demonstrated [110]. It is unknown whether recycled connexins have lost their modifications or whether there is a hierarchy among the different connexin modifications that targets them definitely for degradation or allows them to be recycled to the plasma membrane. The advanced state of research technology available today and the increased interest in gap junction biology promise that future studies on these topics will lead to a better understanding of the diverse and multifaceted aspects of gap junction internalization and degradation.

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