FEBS Letters 588 (2014) 1212–1220

journal homepage: www.FEBSLetters.org

Review

Connexins: Mechanisms regulating protein levels and intercellular communication

Vivian Su [a,b,*], Alan F. Lau [a,b,c]

[a] Pacific Biosciences Research Center, University of Hawai'i at Mānoa, Honolulu, HI 96822, United States
[b] Cancer Biology Program, University of Hawai'i Cancer Center, Honolulu, HI 96813, United States
[c] Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawai'i at Mānoa, Honolulu, HI 96822, United States

ARTICLE INFO

Article history:
Received 12 December 2013
Revised 10 January 2014
Accepted 13 January 2014
Available online 20 January 2014

Edited by Michael Koval, Brant E. Isakson, Robert G. Gourdie and Wilhelm Just

Keywords:
Connexin
Proteasome
Lysosome
Autophagy
Degradation
Trafficking

ABSTRACT

Intercellular communication can occur through gap junction channels, which are comprised of connexin proteins. Therefore, levels of connexins can directly correlate with gap junctional intercellular communication. Because gap junctions have a critical role in maintaining cellular homeostasis, the regulation of connexin protein levels is important. In the connexin life cycle, connexin protein levels can be modified through differential gene transcription or altered through trafficking and degradation mechanisms. More recently, significant attention has been directed to the pathways that cells utilize to increase or decrease connexin levels and thus indirectly, gap junctional communication. Here, we review the studies revealing the mechanisms that affect connexin protein levels and gap junctional intercellular communication.

1. Introduction

Cellular processes are regulated, in part, by the interaction between cells. One important mode of interaction between neighboring cells is mediated by gap junction channels, which provide a direct conduit between the cytoplasms of adjacent cells. Gap junctions allow the transfer of small molecules up to ~1000 daltons in size. Transferred molecules include secondary messengers, small metabolites, and ions [1,2]. Gap junctional intercellular communication (GJIC) is critical during both development and adult stages in maintaining homeostasis [3–5]. Alterations in GJIC can result in a host of human diseases, such as cancer [6], heart disease [7–9], and developmental diseases [10].

Gap junction channels are comprised of integral multi-pass transmembrane proteins called connexins [1,2]. Connexins have four transmembrane domains that are linked by one intracellular loop, two extracellular loops, and have N- and C-terminal cytoplasmic domains. Connexin hexamers, called connexons or hemichannels, are formed in the secretory pathway. The hemichannels are then trafficked to the cell surface where they can act as stand-alone channels, or dock with hemichannels in appositional membranes to form intact gap junctions. There are 21 members of the human connexin protein family and 20 mouse connexins. Of these, connexin43 (Cx43) is the most ubiquitously expressed and the most widely studied.

Connexins have a short half-life of 1.5–5 h, compared to other transmembrane proteins [11–15]. For this reason, the regulation of connexin protein levels and connexin trafficking can potentially have a critical effect on the level of GJIC. Connexin levels can also have an effect on hemichannel functions, which have more recently been demonstrated to have important roles in cell death and tissue remodeling [16]. This review will focus on recent findings on the pathways and mechanisms mediating Cx43 trafficking and degradation.

2. Secretory pathway and proteasomal degradation

Connexins are thought to be co-translationally inserted into the endoplasmic reticulum (ER) membrane, and properly folded in the

Abbreviations: GJIC, gap junctional intercellular communication; Cx, connexin; Cx43, connexin43; ER, endoplasmic reticulum; ERAD, ER-associated degradation; UbL, ubiquitin-like domain; UBA, ubiquitin-associated domain; AGJ, annular gap junctions; CME, clathrin-mediated endocytosis; EGF, epidermal growth factor; UIM, ubiquitin interacting motif; TPA, 12-O-tetradecanoylphorbol 13-acetate; ESCRT, endosomal sorting complex required for transport

* Corresponding author at: Pacific Biosciences Research Center, 1993 East-West Road, Honolulu, HI 96822, United States. Fax: +1 (808) 956 4768.
E-mail address: barlos@hawaii.edu (V. Su).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2014.01.013

ER with assistance from molecular chaperones. In the event of connexin protein misfolding, connexins will undergo degradation by the proteasome [15,17], the cellular organelle generally responsible for the "clean-up" of misfolded proteins. The proteasome is a 26S holoenzyme complex comprised of 2 major subunits, the 20S core and the 19S cap or regulatory particles which are each made up of multiple individual protein subunits [18]. ER-associated degradation (ERAD) is the process responsible for the removal and proteasomal degradation of misfolded proteins localized to the ER. During ERAD, misfolded proteins in the ER are recognized by a molecular chaperone such as Bip, transported out of the ER through the retrotranslocon channel into the cytoplasm to be degraded by the proteasome [19].

Connexin proteasomal degradation was first demonstrated with Cx43, in Chinese hamster ovary (CHO) E36 and rat heart-derived BWEM cells [20]. Upon treatment of these cells with the proteasomal inhibitor, ALLN, Cx43 levels were increased, and correspondingly, turnover was reduced. Studies with ALLN and another proteasomal inhibitor, lactacystin, in neonatal rat ventricular myocytes, demonstrated that blocking proteasomal degradation reduced the loss of Cx43 that was induced by heat treatment [21], which confirmed further that Cx43 undergoes proteasomal degradation. ERAD-mediated degradation of Cx43 was also demonstrated in response to protein misfolding. ER stress to induce Cx43 misfolding (i.e. DTT treatment to break the disulfide bonds present in the Cx43 extracellular loops) in CHO and mouse S180 cells, promoted the dislocation of Cx43 from the ER into the cytosol and subsequently resulted in proteasomal degradation, as indicated by increased Cx43 protein levels after treatment with the proteasomal inhibitors ALLN, epoxomicin, or ZL_3VS [15,17]. In addition, siRNA-induced down-regulation of the ER resident chaperone ERp29 (a thioredoxin family protein that promotes protein folding and trafficking) decreased the total amount of exogenously expressed Cx43 in HeLa cells and increased the rate of Cx43 turnover as evidenced by a diminished half-life of 2.2 h compared to 4 h in control cells [22]. Lactacystin treatment restored the levels of Cx43 in the siRNA-treated ERp29 cells, confirming the role of proteasomal degradation in the turnover of misfolded Cx43 [22].

Our laboratory identified a novel interaction between Cx43 and CIP75 [23], a protein that belongs to the ubiquitin-like (UbL) and ubiquitin-associated (UBA) domain family. This protein family, via the UbL domain, is able to interact with subunits in the proteasome 19S regulatory particle, specifically the Rpn1/S2 and Rpn10/S5a proteins [24]. UbL–UBA proteins have been demonstrated to facilitate the proteasomal degradation of other substrates. For example, Rad23 promotes the degradation of the p53 tumor suppressor [25,26] and the CDK inhibitor Sic1 [27]. The ubiquitin-interacting UBA domain is the primary region supporting the interaction with the protein substrates. We have found that CIP75 is similarly able to interact with the Rpn1 and Rpn10 proteasomal proteins via its UbL domain, and with Cx43 via the UBA domain [23]. Both domains are necessary for facilitating Cx43 proteasomal degradation [23]. Recently, we have discovered that CIP75 mediates the degradation of misfolded Cx43 located in the ER, as the induction of protein misfolding by DTT treatment of HeLa cells expressing ER-localized Cx43 increased the CIP75–Cx43 interaction [28]. The interaction of Cx43 with the proteasomal subunit Rpn1 also increased with treatment, but this was dependent on CIP75 as the shRNA knockdown of CIP75 prevented the DTT-induced increase in Cx43–Rpn1 interaction [28], which indicated that CIP75 is essential for bridging Cx43 with the proteasome under conditions of ER stress.

Proteins bound for proteasomal degradation are typically marked with covalently attached polyubiquitin chains. These are thought to be lysine48 (K48)-linked tetraubiquitin chains. Ubiquitination is a multi-step process catalyzed by a cascade of ubiquitin-conjugating enzymes [29]. The evolutionarily-conserved 76-amino acid ubiquitin protein is first activated by the E1 ubiquitin-activating enzyme. Activated ubiquitin is then transferred to one of multiple E2 ubiquitin-conjugating enzymes that associate with an E3 ubiquitin ligase. This multimeric complex results in the covalent bonding of the ubiquitin protein with either the substrate target protein or, during the generation of a polyubiquitin chain, with another ubiquitin moiety at lysine residues. The ubiquitination of Cx43 has previously been demonstrated [20,30,31]; however, it is unclear whether ubiquitination is a necessary prerequisite for Cx43 proteasomal degradation. Although ubiquitinated Cx43 at the plasma membrane has been detected [30,32–34], the pool of Cx43 bound for ERAD has not been specifically assessed for ubiquitination. In our studies, we utilized Cx43 point mutants where lysine residues were mutated to arginine to prevent potential Cx43 ubiquitination [35]. We observed that CIP75 still interacted with the ubiquitin-deficient Cx43 mutants, revealing a rare situation where the ubiquitination of Cx43 is not an absolute requirement for ERAD. It is important to note that our studies did not eliminate the possibility that the pool of proteasomally-degraded Cx43 is at least partially ubiquitinated [35]. Based on our observations, we have proposed that CIP75 is required to mediate the proteasomal degradation of non-ubiquitinated ER-localized Cx43 by facilitating its interaction with the proteasome (Fig. 1).

3. Connexin internalization and intracellular trafficking

3.1. Endocytosis

After connexins are translated and properly folded in the ER, connexons are assembled from the connexin subunits either in the ER-Golgi intermediate compartment (ERGIC) or in the Golgi apparatus. These hexameric connexons can be formed from either the same connexin protein (homomeric) or different connexins (heteromeric). Connexons or hemichannels are then trafficked to the plasma membrane where they can dock with appositional connexons, via the disulfide bonds located in the extracellular loops. Connexons can dock with connexons formed from the same connexins (homotypic) or from different connexins (heterotypic). The docked connexons between adjacent cells form a complete gap junction channel. Large areas of docked connexons are termed plaques. It is generally thought that connexons are delivered to the periphery of the plaques and migrate laterally to the center of the plaque. Older gap junctions are removed from the center of the

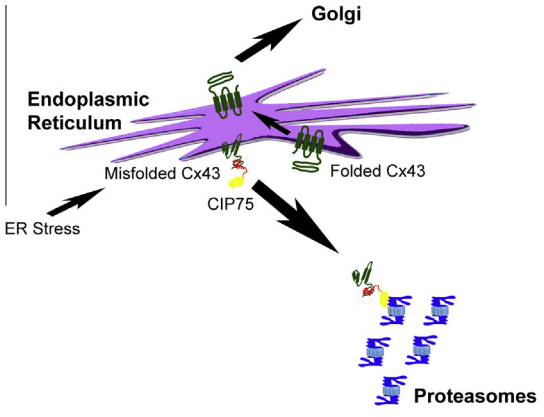

Fig. 1. Model for the CIP75-mediated proteasomal degradation of Cx43. Cx43 is co-translationally inserted into the ER where it is properly folded, then transported to the Golgi for oligomerization and transport to the plasma membrane. When Cx43 misfolding is induced, by ER stress for example, CIP75 interacts with the cytoplasmic C-terminal domain of Cx43 and facilitates the interaction of Cx43 with and subsequent degradation by the proteasome.

plaque by the internalization of double membraned structures called connexosomes or annular gap junctions (AGJs) that are subsequently degraded [36–38].

The level of GJIC is known to be regulated by multiple mechanisms- two of the major mechanisms are phosphorylation and endocytosis. Numerous studies have revealed the role of phosphorylation of various tyrosine and serine residues in the Cx43 C-terminal cytoplasmic domain in mediating channel opening and closing (reviewed in [39,40]). The endocytosis of gap junctions serves to physically remove channels from the plasma membrane, in effect reducing the amount of GJIC. Cx43 has been observed to colocalize with markers of endosomal compartments, such as EEA1, Rab5, and Rab7 [33,41–45]. Gap junction internalization has been explored with immense interest and great strides have been made in elucidating the mechanisms that regulate gap junction endocytosis.

3.2. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) has been found to play an integral role in gap junction internalization. Time-lapse studies of Cx43-GFP exogenously expressed in HeLa cells found internalized AGJs, which appeared to form from large areas of gap junction plaques. The larger AGJs were also observed to fragment into smaller AGJs [46]. Clathrin colocalized with Cx43-GFP at gap junction plaques and in the internalized AGJs. Furthermore, the formation of AGJs was dependent on clathrin, as the reduction of clathrin levels by siRNA significantly reduced the amount of internalized AGJs, which was similar to the effect resulting from the inhibition of CME by the treatment of the cells with hypertonic medium [46]. The clathrin adaptor proteins, AP-2 and Disabled-2 (Dab2), as well as the GTPase dynamin2 (Dyn2), which mediates the budding of vesicles from the membrane, were also found to colocalize with Cx43 gap junctions and AGJs [46]. Similar to clathrin, siRNA knockdown of these three proteins also significantly reduced the amounts of internalized Cx43 AGJs [47]. Dyn2 GTPase activity was specifically found to be important because the expression of the dominant-negative Dyn2K44A mutant and use of the dynasore, an inhibitor of dynamin, also reduced the internalization of Cx43 in AGJs [47,48].

Clathrin does not interact directly with the cargo found in the clathrin-coated vesicles formed during CME. Instead, cargo is recognized by adaptor proteins which recruit CME machinery to facilitate the endocytosis. As previously described, AP-2 and Dab2 colocalized with Cx43 and several experiments helped to identify AP-2 as a Cx43 binding protein. First, a C-terminal tyrosine-based sorting motif (YXXΦ), Y^{286}KLV, in Cx43 was identified as an important signal for Cx43 degradation. The Cx43Y286A and Cx43V289D mutants were expressed at elevated total levels compared to the wild-type control or other point mutations in the nearby region (G285A and P283L) [49]. Second, yeast two-hybrid analysis revealed the direct interaction between the C-terminal domain of Cx43 with the μ2 subunit of the AP-2 adaptor protein complex, which was abolished in the Y286A, V289D, and Y286A/V289D mutants [50]. Two additional YXXΦ sequences were identified in the Cx43 C-terminus, Y^{230}VFF and Y^{265}AYF, although the first was thought to be too close to the last transmembrane domain to be a functional AP-2 binding region [51]. Mutation and deletion analysis of the Y^{265}AYF and Y^{286}KLV sites (referred to as S2 and S3, respectively) revealed that mutating either site reduced the interaction of Cx43 with Dab2 and clathrin [51]. Mutating both sites virtually completely abolished the interaction with Dab2 and its colocalization [51]. These AP-2 binding mutants also exhibited increased half-lives from 1 h for wild-type to 2 h for the single S2 or S3 mutants and 4 h for the double S2 + S3 mutant [51]. HeLa cells expressing the single site mutants also had twice as many gap junction channels, while the double mutants had three times as many as the wild-type Cx43 expressing cells [51]. In addition, when the mutant-expressing cells were paired with the wild-type Cx43-expressing cells, AGJs were more often internalized in the wild-type Cx43 cells, with the double site mutant again exhibiting a stronger effect [51]. Thus, these results indicated that the interaction of AP-2 with the sorting signal YXXΦ motif of Cx43 at the Y^{265}AYF and Y^{286}KLV sites mediates Cx43 internalization via CME.

Eps15 (epidermal growth factor (EGF) receptor substrate 15), a protein that is involved in mediating EGF endocytosis and is found at clathrin-coated pits, was also demonstrated to be involved in the internalization of Cx43 [30,32]. Interestingly, while Cx43 ubiquitination was not found to be an absolute requirement for the proteasomal degradation of Cx43, it may have a role in Cx43 internalization. Eps15 and Cx43 interaction appeared to be dependent on the ubiquitin interacting motif (UIM) of Eps15, as suggested by the inability of an Eps15 mutant with a deletion of the UIM to bind to Cx43. Furthermore, Cx43 colocalized with Eps15 at the plasma membrane of rat NRK cells. In addition to the Eps15 UIM requirement for the interaction with Cx43, the ubiquitination of Cx43 is also required. A previous study had demonstrated that the E3 ubiquitin ligase, Nedd4, colocalized with Cx43 at the plasma membrane and interacted with Cx43 [52]. Nedd4 was also found to be required for Cx43 internalization, as the siRNA knockdown of Nedd4 resulted in the accumulation of Cx43 in the membrane and in gap junction plaques [52]. Nedd4 was subsequently demonstrated to be responsible for Cx43 ubiquitination [30]. Knockdown of Nedd4 reduced the amount of ubiquitinated Cx43, and also reduced the interaction of Cx43 and Eps15 [30].

The Cx43-interacting protein of 85 kDa (CIP85) was identified by our laboratory to be a novel Cx43 binding partner, interacting with the C-terminal domain of Cx43. Initial studies demonstrated that CIP85 colocalized with Cx43 at the plasma membrane [53]. CIP85 was subsequently demonstrated to be involved in Cx43 internalization from the plasma membrane [54]. Overexpression of CIP85 in HeLa cells increased the amount of internalized Cx43, observed by both immunofluorescence microscopy as well as biochemically, by the reduction in the amount of TritonX-100-insoluble Cx43 [54]. The TritonX-100-insoluble protein is considered to represent gap junction plaques, while TritonX-100-soluble Cx43 represents protein that is intracellularly localized [39,55,56]. A direct interaction of CIP85 with Cx43 may be required for the increased internalization, as this effect was not observed when a non-interacting CIP85 truncation mutant was expressed instead of wild-type [54]. Interestingly, CIP85 was also observed to interact and colocalize with clathrin at the plasma membrane by coimmunoprecipitation and immunofluorescence experiments [54]. The nature of the CIP85 interaction with clathrin, however, is unclear. For example, the coimmunoprecipitation did not indicate whether CIP85 was directly binding to clathrin or if this occurs through an adaptor protein. Also, the precise functional role of CIP85 in Cx43 internalization is currently unknown. We speculate that CIP85 might be involved in Cx43 CME, perhaps acting in the recruitment of the clathrin machinery to Cx43 gap junction plaques. Further studies of the interaction of CIP85 with the clathrin machinery, as well as CIP85 loss-of-function studies on Cx43 CME internalization studies will be required to address some of these issues.

3.3. Post-endocytic trafficking of Cx43

After internalization, Cx43 is trafficked intracellularly to different destinations. Studies have demonstrated that internalized Cx43 can be degraded by the lysosome [15,21,44,49,56–63], but can also be recycled to the membrane [33,41,48,61,64]. Ubiquitination is thought to play an important role in the sorting of internalized Cx43.

During the study of Cx43 internalization, the ubiquitination of plasma membrane localized Cx43 has been observed. Cellular exposure to the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate) or to EGF causes the internalization of Cx43 from the plasma membrane, observed as decreased levels of membrane localized Cx43. Cx43 ubiquitination was also detected after treatment [31,34]. Both mono- and polyubiquitinated forms of Cx43 (detected with ubiquitin-specific antibodies) were observed biochemically, particularly in response to TPA treatment [34]. Multi-monoubiquitinated Cx43 was also observed under normal untreated conditions [30]. The ubiquitination of Cx43 in gap junction plaques was inferred in immunoelectron microscopy experiments, where 50% of plaques were ubiquitinated when Cx43 trafficking to the plasma membrane was blocked by brefeldin A (BFA) treatment, demonstrating the presence of ubiquitin at the plaques [65]. In addition, two E3 ubiquitin ligases, Nedd4 and Smurf2 (smad ubiquitination regulatory factor-2), were identified to affect cell surface Cx43 [52,66] and GJIC levels [66]. A third E3 ligase, TRIM21, was subsequently identified to interact with Cx43, colocalizing at the cell surface where Cx43 phosphorylation appeared to induce TRIM21-mediated Cx43 ubiquitination and degradation [67].

Initial studies suggested that the proteasome was actively involved in the turnover of Cx43 gap junctions. Many of these studies came to this conclusion after utilizing general proteasomal inhibitors which resulted in the accumulation of Cx43 at the plasma membrane with increased amounts of gap junction plaques [15,20,31,34,58,60,61,68–71]. The identification of ubiquitinated Cx43 further supported this idea because ubiquitination is known to be a modification that marks a substrate for proteasomal degradation. However, a more recent study suggested otherwise. Utilizing the previously described lysine to arginine Cx43 mutant that presumably is unable to be ubiquitinated, the mutant was still able to be trafficked through the secretory pathway to the plasma membrane, form intact gap junctions, and participate in GJIC (although at a lower level) [72]. Proteasomal inhibition again revealed increased levels of membrane Cx43, although it was discovered that the loss of Akt/protein kinase B proteasomal degradation was primarily responsible for the stabilization of Cx43 [72]. Akt phosphorylation of Cx43 is responsible for the reduction of gap junctional Cx43 [72]. Thus, it is more likely that other mechanisms, such as lysosomal degradation, are responsible for the turnover of Cx43 in gap junctions.

In addition to the Akt study, Cx43 has also been reported to be monoubiquitinated [34]. This is significant because while the K48 ubiquitin chain is generally believed to be a marker for proteasomal degradation, monoubiquitination and also K63 polyubiquitination are more likely to be involved in other cellular processes, such as protein internalization and trafficking [73,74]. Importantly, Cx43 interacts with components of the ESCRT (endosomal sorting complex required for transport) machinery, specifically with UIM-containing proteins, Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and Tsg101 (tumor susceptibility gene 101) [33,75]. The ESCRT machinery is responsible for the endosomal sorting of ubiquitinated membrane proteins (reviewed in [76]) and consists of four ESCRT complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, which are composed of different subunits. Hrs comprises the ESCRT-0 complex along with STAM (signal transducing adaptor molecule) and in human cells, ESCRT-0 associates with Eps15b. ESCRT-0 acts to retain ubiquitinated membrane proteins in the endosomal membrane. Tsg101 comprises the ESCRT-I complex with three other proteins and the Tsg101 UEV (ubiquitin E2 variant) domain binds to ESCRT-0, mediating the recruitment of ESCRT-I to the endosome. ESCRT-II is recruited to ESCRT-I, and which then recruits the ESCRT-III complex. The ESCRT machinery serves to sort ubiquitinated membrane proteins from the endosome into intraluminal vesicles which prevents recycling, retrograde transport to the secretory pathway, or endosomal retention.

Hrs and Tsg101 were found to mediate the intracellular trafficking of Cx43 from endosomes to lysosomes [33]. When combined with both the TPA-stimulated internalization of Cx43 and a cyclohexamide block in protein synthesis, knockdown of Hrs and Tsg101 by siRNA increased the amount of Cx43 at the plasma membrane as well as the level of GJIC. In addition, reduced Hrs and Tsg101 levels produced an accumulation of ubiquitinated Cx43. It was proposed that Hrs and Tsg101 were responsible for directing Cx43 towards degradation instead of being recycled back to the plasma membrane. Cx43 recycling was previously demonstrated to occur during mitosis [41] and situations of cellular stress [61]. These studies indicated that Cx43 may be subject to the highly regulated ESCRT machinery which would direct ubiquitinated Cx43 towards degradation and allow non-ubiquitinated Cx43 to be recycled back to the plasma membrane. Thus, Cx43 ubiquitination has a key role in determining the amount of Cx43 in gap junction plaques, and therefore, the level of GJIC.

In addition to the ubiquitin modification, acetylation and SUMOylation have also been demonstrated to affect Cx43 localization and GJIC [77,78]. Cx43 acetylation in mouse hearts caused the relocalization of Cx43 from the intercalated discs to lateral membranes, an effect found in diseased hearts [77]. GJIC was also negatively affected by Cx43 acetylation [77]. Modification with the ubiquitin-like small protein SUMO (small ubiquitin-related modifier) had the opposite effect of increasing GJIC, while also increasing both the amount of Cx43 found at the plasma membrane at gap junctions and intracellular Cx43 [78]. Acetylation and SUMOylation modifications also occur at lysine residues, thus, it is possible that cross-talk between the pathways regulating ubiquitin, acetyl group, and SUMO attachment regulates Cx43 localization, trafficking, and GJIC. Cx43 post-translational modifications are clearly important in modulating Cx43 activities.

3.4. Degradation of internalized connexins

Once internalized, Cx43 gap junctions are likely to be destined for degradation to reduce the level of Cx43 and thus, indirectly regulating the amount of GJIC. A multitude of studies have demonstrated the involvement of lysosomal degradation in the turnover of Cx43. Early electron microscopy studies identified structures that resembled lysosomes fused with internalized AGJs and Cx43 [79–81]. Subsequent studies primarily used a variety of lysosomal inhibitors which produced an accumulation of Cx43 in lysosomes as well as an overall increase in the total amount of Cx43, and blocked the degradation of cell surface Cx43 [15,21,44,49,56–63].

More recently, a number of reports detailed the role of autophagy in Cx43 turnover. Autophagy, specifically macroautophagy, involves the formation of a characteristic intracellular double membrane autophagosome that fuses with the lysosome for the degradation of its cargo that can include large structures and organelles [82]. The Atg autophagy-related proteins are primarily responsible for executing autophagy. The Atg1/13/17 complex assists in the induction of autophagosome formation where vesicle nucleation is mediated by Atg6 (mammalian Beclin-1)/14 and Vps34/15, followed by vesicle elongation mediated by the Atg8 or Atg12 complexes. During autophagosome maturation, Atg8 (mammalian LC3 or microtubule-associated protein 1 light chain 3) is conjugated to phosphatidylethanolamine (Atg8-PE/LC3-II) which stably associates with the autophagosomal vesicle [82]. The Atg proteins serve as useful markers of autophagosomes and can also be used to inhibit or disrupt autophagy.

The possible involvement of autophagy in regulating connexin or gap junctional levels was first revealed with the observation of

structures resembling autophagosomes via electron microscopy [81,83,84]. Electron microscopy also revealed the presence of Cx43 in autophagosomal structures in the failing canine heart myocardium [85]. In addition, Cx43-GFP, exogenously-expressed in HeLa cells, colocalized with LC3 [85]. Induction of autophagy by starvation also revealed the localization of Cx43 within autophagosomes, observed as vesicles containing LC3 in the vesicular membrane as well as a reduction in Cx43 levels [86]. Inhibition of autophagy, either with a lysosomal inhibitor or by siRNA knockdown of Atg5, prevented both this colocalization and the loss of Cx43 [86]. Furthermore, Cx43 also colocalized with the ubiquitin-binding protein p62, known to have a role in autophagy [86]. These studies revealed a role for autophagy in Cx43 degradation, although the pool of Cx43 targeted for autophagic degradation had not been determined.

Two of the most recent autophagy studies demonstrated that the pool of Cx43 in plasma membrane gap junction plaques is targeted for degradation by autophagy [87,88]. In one study, using Cx43-GFP expressing HeLa cells, the formation of AGJs in live cells was studied [88]. These Cx43-GFP containing AGJs were found in various stages of autophagosomal formation in ultrastructural analyses. Cx43 specifically in AGJs also colocalized with autophagosomal markers, such as LC3 and p62. Inhibition of autophagy by siRNA knockdown of multiple proteins required for the autophagic process (Beclin-1/Atg6, LC3B, LAMP-2, p62) or the pharmacological inhibition of autophagy (with 3-methyladenine (3MA), wortmannin, or Bafilomycin A1) resulted in a threefold increase of total levels of exogenously-expressed Cx43-GFP/YFP over the siRNA control treated cells and a significant increase in cytoplasmic AGJs. Beclin-1 and p62 knockdown was also observed to result in the reduction of colocalization of AGJs and autophagosomes [88].

The second study confirmed Cx43 autophagy and described the role of ubiquitination in Cx43 autophagy [87]. Using mouse liver tissue, immunogold staining revealed the presence of Cx43 in autophagic compartments (lysosomes, autophagosomes, autophagolysosomes). Under starvation conditions to stimulate autophagy, an enrichment of Cx43 was found in the autophagic compartments, as well as an increase in intracellular vesicles positive for Cx43, and LC3 or p62. Starvation-induced autophagy also decreased the amount of Cx43 at the plasma membrane, decreasing the half-life of gap junctional Cx43 from 6 h to 3 h. The effects of starvation were reduced or prevented by inhibitors of autophagy, with either pharmacologic treatment with 3MA or lysosomal inhibitors, chloroquine, ammonium chloride, leupeptin or genetically with Atg7 knockdown or using Atg5 null mouse embryonic fibroblasts or MEFs. Significantly, 3MA or chloroquine treatment of starved cells increased the amount of TritonX-100 insoluble (i.e. gap junctional) Cx43, indicating that blocking autophagy specifically prevented the loss of Cx43 at the plasma membrane. Furthermore, a combination of 3MA treatment with either Atg7 knockdown or using the Atg5 null MEFs had no additive effect, demonstrating that it was indeed autophagy that was being affected. Internalization and lysosomal degradation of Cx43 (induced by lindane or γ-hexachlorocyclohexane treatment [57,89]) was also reduced when autophagy was inhibited (both pharmacologically and genetically). To further assess the relationship between autophagy and Cx43 internalization, the study utilized the previously described Cx43 Y286A endocytosis mutant [32,49]. Both induction (by starvation or pharmacologically with rapamycin treatment) and inhibition of autophagy (via Atg7 knockdown) had no effect on the Y286A mutant, which remained at the plasma membrane. This suggested a dependence of Cx43 gap junctional autophagic degradation on endocytosis. The role of Cx43 ubiquitination was also assessed. Starvation conditions led to increased Cx43 ubiquitination. Nedd4 had been demonstrated to ubiquitinate plasma membrane localized Cx43. Depletion of Nedd4 by siRNA knockdown also blocked the starvation induced Cx43 degradation, indicating that Cx43 ubiquitination mediated by Nedd4 was required for the autophagic degradation. Autophagy inhibition did not affect Cx43 ubiquitination, however, suggesting that the incorporation of Cx43 gap junctions into autophagosomes occurred after ubiquitination. To confirm that Cx43 ubiquitination was required for autophagy, the study utilized a Cx43-ubiquitin (Cx43-Ub) fusion protein. Nedd4 knockdown had no effect on this protein since it was already ubiquitinated, while 3MA treatment or Atg7 knockdown to block autophagy prevented Cx43-Ub internalization and localization in autophagy compartments, confirming the positive relationship between Cx43 ubiquitination and autophagy. Similar to the other studies, Cx43 colocalized and coimmunoprecipitated with p62, with an increased interaction observed upon starvation. Nedd4 depletion reduced the Cx43-p62 interaction, while the Cx43-Ub protein had significantly more interaction with p62. Since p62 contains the UBA ubiquitin-binding domain, the interaction between Cx43 and p62, and thus, Cx43 association with autophagosomes, could rely on the binding of a Cx43 ubiquitin modification with the p62 UBA domain. The role of Eps15, already demonstrated to be involved in the internalization of ubiquitinated Cx43 [30,32], in Cx43 autophagy was also explored. The induction of autophagy promoted the interaction of Cx43 with Eps15, which facilitated the endocytosis of ubiquitinated Cx43, although it appeared that this event occurred prior to the recruitment of autophagic proteins. However, the inhibition of autophagy did not have a significant effect on the high level of Cx43-Eps15 interaction. Reduction of Eps15 by siRNA knockdown inhibited the starvation-induced Cx43 degradation. Further analysis of the Eps15-Cx43 interaction confirmed that Cx43 ubiquitination was required for Eps15 binding as Nedd4 knockdown prevented the Eps15-Cx43 interaction and Eps15 failed to interact with the Cx43 Y286A mutant. The PY motif containing the Y286 residue was previously demonstrated to be involved in Nedd4 interaction [52]. Interestingly, the study also demonstrated that Eps15 could interact with LC3, but not p62, suggesting that Eps15 is an intermediate adaptor molecule which bridges ubiquitinated Cx43 to the autophagic machinery. This would also provide a mechanism to link the internalization of ubiquitinated Cx43 with the autophagy degradation pathway. The effect of 3MA inhibition of autophagy on GJIC in NIH3T3 cells was also studied under both normal and starvation conditions, where, GJIC (measured by dye transfer) was increased by 3MA treatment, which was further enhanced by starvation [87].

Recent reports on the involvement of autophagy in Cx43 gap junction turnover provides new information on the possible mechanisms that regulate the levels of Cx43 at the plasma membrane and Cx43 channel activity. Previous reports detailing Cx43 lysosomal degradation, for the most part, assumed that the lysosome was the end point for endo/lysosomal degradation, which is the pathway that utilizes classical endocytic mechanisms (early, late endosomes) and ending with proteins degraded by the lysosome. However, these studies did not attempt to distinguish between endo/lysosomal degradation and autophagy (phago/lysosomal). Lysosomal inhibitors are known to block both pathways of degradation, which would not allow the distinction of the different pathways. Thus, it is possibly that the results of earlier studies included effects from the inhibition of autophagosomal degradation. Lysosomal degradation of Cx43 gap junctions, either via the endo/lysosome or autophagy, has been demonstrated to directly affect GJIC [33,87]. Therefore, it is vital to fully elucidate the processes regulating Cx43 lysosomal degradation. Based on these reports, we speculate on two distinct possibilities for the internalization, trafficking, and degradation of Cx43 gap junctions by either endosomes/lysosomes or autophagosomes.

In an endo/lysosomal model (Fig. 2), Cx43 traffics to the cell surface and becomes ubiquitinated in a process mediated by the

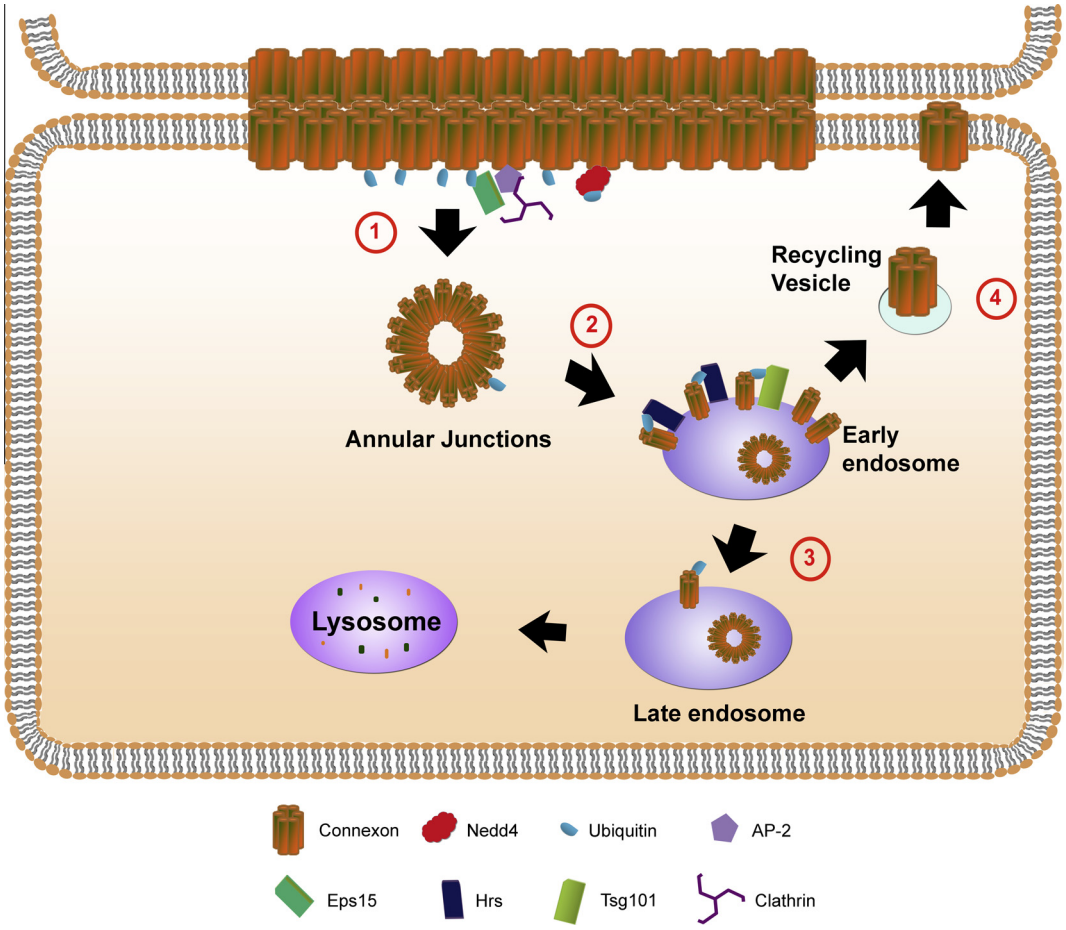

Fig. 2. Model for the endo/lysosomal degradation of gap junctional Cx43. Cx43 assembled into connexons in gap junction plaques are ubiquitinated by the Nedd4 E3 ligase, which then interact with Eps15. AP-2 binds to the cytoplasmic C-terminal domain of Cx43 and recruits the clathrin machinery. Cx43 plaques are internalized as double-membrane annular gap junctions (1). The ESCRT-0 subunit Hrs binds to ubiquitinated Cx43 and sorts it through the endocytic pathway (2) to be degraded by the lysosome (3). Non-ubiquitinated Cx43 is recycled back to the plasma membrane (4).

E3 ligase Nedd4. Ubiquitinated Cx43 is recognized by the UIM of Eps15. Perhaps in a complex with Eps15, Cx43 is also recognized by AP-2 through its C-terminal YXXΦ motifs, which then recruits the clathrin machinery (i.e. clathrin, Dab2, Dyn2) to the membrane. Cx43 gap junctions are then endocytosed as AGJs. Once internalized, the ubiquitinated Cx43 is recognized by the UIM of Hrs in the ESCRT-0 complex. Through the ESCRT machinery, ubiquitinated Cx43 is sorted through the endosomal pathway, moving from early endosomes to late endosomes to lysosomes where Cx43 is then degraded. Non-ubiquitinated Cx43 is not retained in the endosomal membrane by the ESCRT machinery, and is therefore available to be recycled back to the plasma membrane.

In a model for autophagy (Fig. 3), Eps15 is recruited to ubiquitinated Cx43 gap junctions that are assembling into clathrin-coated pits when autophagy is induced (for example, under starvation conditions). Then, either during or immediately after vesicle formation, Eps15 begins to recruit the autophagic machinery to the AGJs, perhaps recruiting LC3 which then recruits other autophagy-related proteins such as p62. Autophagosomes form around AGJs, fuse with the lysosome to form the autophagolysosome where its contents (i.e. the AGJs) are degraded.

While progress has been made in elucidating the mechanisms and pathways that contribute to the downregulation of GJIC via Cx43 gap junction levels, there are many questions that remain unanswered and gaps to be closed in current models. One major question is how do the CME, endo/lysosomal, and autophagosomal pathways interact to facilitate the internalization, trafficking, degradation, and recycling of Cx43? And, specifically, which signals determine the destinations that Cx43 is trafficked to after internalization and what is the role(s) that Cx43 binding proteins, such as CIP85, play in the internalization and degradation of Cx43? Another question focuses on the interaction of Cx43 with AP-2, which has been implicated in Cx43 CME, and with Eps15, which is involved in Cx43 internalization. It would be enlightening to study both of these proteins and the mechanism(s) that determines how these pathways might intersect to mediate Cx43 internalization. Ubiquitination and ubiquitin-binding proteins (e.g. Eps15) are clearly involved in the internalization process, but, what determines whether Cx43 gap junctions are ubiquitinated or not? Furthermore, what is the fate of non-ubiquitinated Cx43? Cx43 ubiquitination does not appear to be required for GJIC since non-ubiquitinated Cx43 can still traffic to the plasma membrane and participate in GJIC [72], although the loss of Nedd4-mediated Cx43 ubiquitination diminishes the internalization of Cx43 from the plasma membrane [30,52]. It is clear that Cx43-interacting proteins have a major role in regulating Cx43 trafficking and turnover. It will be of great interest to determine if these proteins can affect the interaction of Cx43 with other binding partners, and possibly, alter the fate of Cx43.

4. Human diseases

Given that gap junctions interconnect cells in solid tissues, connexins and GJIC provide the mechanism for the exchange of

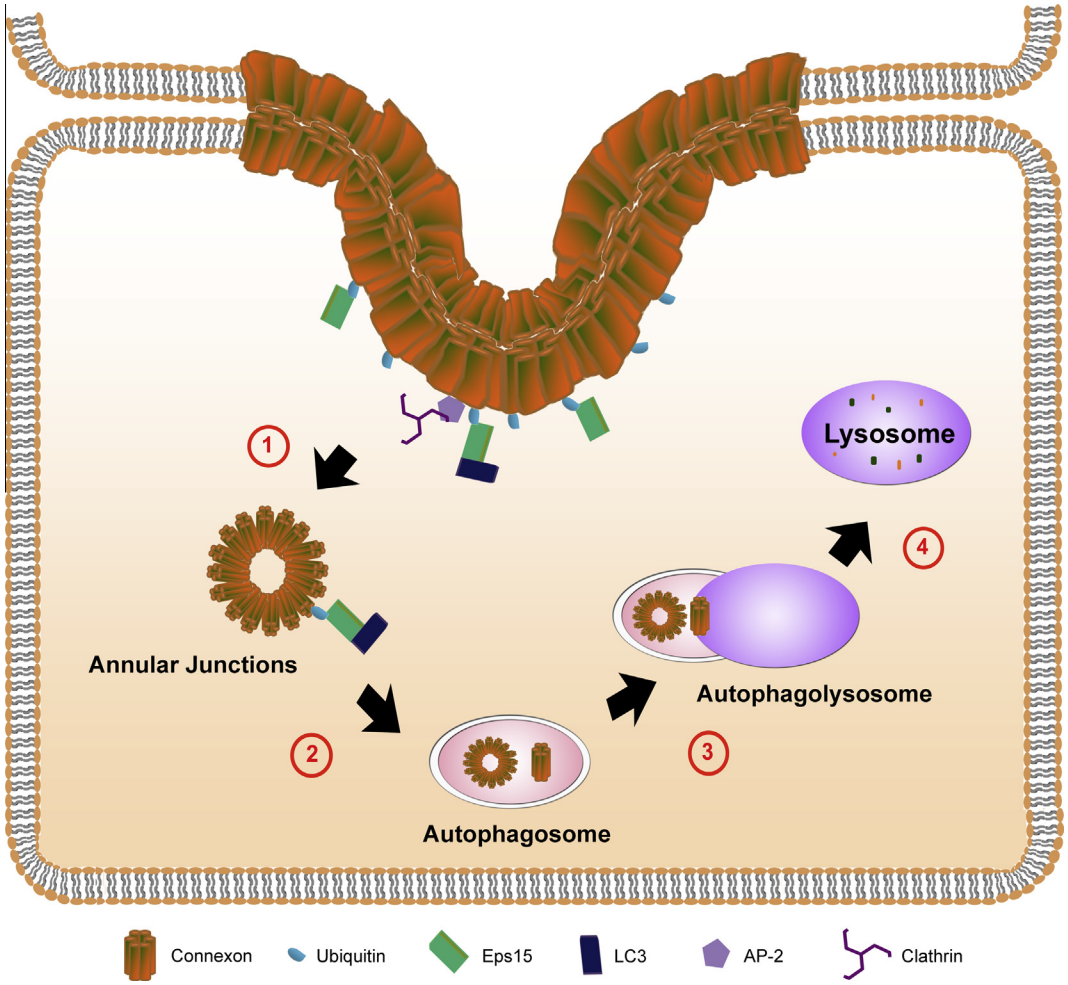

Fig. 3. Model for the autophagosomal degradation of gap junctional Cx43. Ubiquitinated Cx43 assembled into connexons in gap junction plaques are recognized by Eps15 during or after the internalization of the plaque into annular gap junctions via clathrin-mediated endocytosis (1). Eps15 interacts with LC3 which recruits other autophagic proteins to induce the formation of an autophagosome around the annular gap junction (2). The autophagosome fuses with the lysosome (3) resulting in the degradation of the autophagolysosome contents (4).

molecules in most embryonic and adult organs and tissues. The composition of connexins in various tissues and cell types can differ widely. With 21 different human connexins, there is still much to understand about how they interact with one another and how they are regulated in terms of cellular levels, channel activity, subcellular localization, and degradation. These processes are important because mutations in different connexin genes have been identified in human diseases arising in different tissues where there may be the differential expression of more than one connexin. For example, in the cardiovascular system, Cx40, Cx43, and Cx45 are expressed, and these gap junctions facilitate the propagation of the action potentials responsible for the rhythmic contraction of the heart [2]. These connexins are expressed differentially, however, with Cx43 in the ventricular myocardium, and all three in atrial cardiomyocytes. Gap junctions are normally localized to the intercalated disks in heart tissue [2]. A reduction in Cx43 expression and relocalization of Cx43 to lateral membranes has been found in arrhythmias and under conditions of abnormal impulse conduction [7–9]. A reduction in the expression of Cx43 has also been observed in myocardial infarctions [7–9]. The importance of Cx43 in heart disease has been demonstrated with the use of certain experimental therapies, for example the use of rotigaptide, which enhances GJIC, and gap26, a Cx43 structural mimetic peptide (first extracellular loop), which protects against ischemic injury [90]. Cx40 also appears to be critical in heart function, as Cx40 mutations have been identified in patients with atrial fibrillation [91,92].

While the effects of altered GJIC are evident in the heart, connexin alterations are also found in other tissues and diseases that are not clearly GJIC-dependent. Connexins have been implicated in human cancers. The downregulation of connexins at the plasma membrane has been observed in human and mouse breast cancer and rat glioma cells [59,80,93], while Cx43 overexpression reduced proliferation and tumorigenesis [80]. Given that undocked hemichannels can affect cell growth and migration [94,95], the role of connexins in cancer may be due to hemichannel or gap junction activities, or possibly, a combination of both types of channels. Cx43 mutations that affect its trafficking and localization have also been found in the human developmental disease, oculodentodigital dysplasia [10,96]. Cx32 mutations (e.g. E208K that exhibits trafficking defects and elevated proteasomal degradation) have been identified in the human neurological disorder Charcot–Marie–Tooth disease (CMTx) [97]. Lastly, Cx50 mutations linked to hereditary congenital cataracts are defective in trafficking and proteasomal degradation [98,99].

The trafficking and degradation of connexins is clearly important in a number of human diseases. Thus, proper regulation of these processes has important consequences for the pathways

and processes that are mediated by connexins, hemichannels, and gap junctions.

5. Conclusion

While much progress has been made toward elucidating the mechanisms and pathways that regulate connexin trafficking, localization, and degradation (i.e. the connexin life cycle), more questions remain about these critical events. Experimental systems are evolving as evidenced by some of the recent studies detailed here. However, future studies are necessary to determine how proteasomal degradation affects connexin functions, and whether lysosomal degradation consists of multiple divergent pathways. It will also be necessary to determine whether these mechanisms affect other connexins and whether these are general connexin regulatory pathways or specific to only certain connexins, particularly as more connexin mutations are identified in different human diseases. For example, although autophagy has also been implicated in Cx50 [86], Cx26, and Cx32 [87] degradation, at this time, knowledge of these connexins is not as extensive or as detailed as known for Cx43. Knowledge of the factors and signals that contribute to the proper trafficking of connexins and the regulation of connexin levels will aid in the understanding of connexin-related disease pathologies, and perhaps in the future, contribute to the treatment of human disease arising from connexin abnormalities.

Acknowledgements

This work was supported in part by Grants 11POST5460028 from the American Heart Association (to V.S.), 11ADVC-49235 from the Hawaii Community Foundation (to V.S. and A.F.L.), CA052098 (to A.F.L.) from the National Cancer Institute, National Institutes of Health, 2G12RR003061-26 from the National Center for Research Resources, 8G12MD7601-27 from the National Institute on Minority Health and Health Disparities, and support from the Pacific Biosciences Research Center.

References

[1] Goodenough, D.A., Goliger, J.A. and Paul, D.L. (1996) Connexins, connexons, and intercellular communication. Annu. Rev. Biochem. 65, 475–502.
[2] Nielsen, M.S., Nygaard Axelsen, L., Sorgen, P.L., Verma, V., Delmar, M. and Holstein-Rathlou, N.H. (2012) Gap junctions. Compr. Physiol. 2, 1981–2035.
[3] Vinken, M., Vanhaecke, T., Papeleu, P., Snykers, S., Henkens, T. and Rogiers, V. (2006) Connexins and their channels in cell growth and cell death. Cell. Signal. 18, 592–600.
[4] Wei, C.J., Xu, X. and Lo, C.W. (2004) Connexins and cell signaling in development and disease. Annu. Rev. Cell Dev. Biol. 20, 811–838.
[5] White, T.W. and Paul, D.L. (1999) Genetic diseases and gene knockouts reveal diverse connexin functions. Annu. Rev. Physiol. 61, 283–310.
[6] Naus, C.C. and Laird, D.W. (2010) Implications and challenges of connexin connections to cancer. Nat. Rev. Cancer 10, 435–441.
[7] Martin, P.E. and Evans, W.H. (2004) Incorporation of connexins into plasma membranes and gap junctions. Cardiovasc. Res. 62, 378–387.
[8] Severs, N.J., Bruce, A.F., Dupont, E. and Rothery, S. (2008) Remodelling of gap junctions and connexin expression in diseased myocardium. Cardiovasc. Res. 80, 9–19.
[9] van Veen, A.A., van Rijen, H.V. and Opthof, T. (2001) Cardiac gap junction channels: modulation of expression and channel properties. Cardiovasc. Res. 51, 217–229.
[10] Laird, D.W. (2008) Closing the gap on autosomal dominant connexin-26 and connexin-43 mutants linked to human disease. J. Biol. Chem. 283, 2997–3001.
[11] Beardslee, M.A., Laing, J.G., Beyer, E.C. and Saffitz, J.E. (1998) Rapid turnover of connexin43 in the adult rat heart. Circ. Res. 83, 629–635.
[12] Darrow, B.J., Laing, J.G., Lampe, P.D., Saffitz, J.E. and Beyer, E.C. (1995) Expression of multiple connexins in cultured neonatal rat ventricular myocytes. Circ. Res. 76, 381–387.
[13] Fallon, R.F. and Goodenough, D.A. (1981) Five-hour half-life of mouse liver gap-junction protein. J. Cell Biol. 90, 521–526.
[14] Laird, D.W., Puranam, K.L. and Revel, J.P. (1991) Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. Biochem. J. 273 (Pt 1), 67–72.
[15] Musil, L.S., Le, A.C., VanSlyke, J.K. and Roberts, L.M. (2000) Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. J. Biol. Chem. 275, 25207–25215.
[16] Stout, C., Goodenough, D.A. and Paul, D.L. (2004) Connexins: functions without junctions. Curr. Opin. Cell Biol. 16, 507–512.
[17] VanSlyke, J.K. and Musil, L.S. (2002) Dislocation and degradation from the ER are regulated by cytosolic stress. J. Cell Biol. 157, 381–394.
[18] Voges, D., Zwickl, P. and Baumeister, W. (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. 68, 1015–1068.
[19] Smith, M.H., Ploegh, H.L. and Weissman, J.S. (2011) Road to ruin: targeting proteins for degradation in the endoplasmic reticulum. Science 334, 1086–1090.
[20] Laing, J.G. and Beyer, E.C. (1995) The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. J. Biol. Chem. 270, 26399–26403.
[21] Laing, J.G., Tadros, P.N., Green, K., Saffitz, J.E. and Beyer, E.C. (1998) Proteolysis of connexin43-containing gap junctions in normal and heat-stressed cardiac myocytes. Cardiovasc. Res. 38, 711–718.
[22] Das, S. et al. (2009) ERp29 restricts connexin43 oligomerization in the endoplasmic reticulum. Mol. Biol. Cell 20, 2593–2604.
[23] Li, X., Su, V., Kurata, W.E., Jin, C. and Lau, A.F. (2008) A novel connexin43-interacting protein, CIP75, which belongs to the UbL–UBA protein family, regulates the turnover of connexin43. J. Biol. Chem. 283, 5748–5759.
[24] Su, V. and Lau, A.F. (2009) Ubiquitin-like and ubiquitin-associated domain proteins: significance in proteasomal degradation. Cell. Mol. Life Sci. 66, 2819–2833.
[25] Brignone, C., Bradley, K.E., Kisselev, A.F. and Grossman, S.R. (2004) A post-ubiquitination role for MDM2 and hHR23A in the p53 degradation pathway. Oncogene 23, 4121–4129.
[26] Glockzin, S., Ogi, F.X., Hengstermann, A., Scheffner, M. and Blattner, C. (2003) Involvement of the DNA repair protein hHR23 in p53 degradation. Mol. Cell. Biol. 23, 8960–8969.
[27] Verma, R., Oania, R., Graumann, J. and Deshaies, R.J. (2004) Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin–proteasome system. Cell 118, 99–110.
[28] Su, V., Hoang, C., Geerts, D. and Lau, A.F. (2014) The connexin43-interacting protein of 75 kDa (CIP75) mediates the endoplasmic reticulum dislocation of connexin43. Biochem. J. 458, 57–67.
[29] Fang, S. and Weissman, A.M. (2004) A field guide to ubiquitylation. Cell. Mol. Life Sci. 61, 1546–1561.
[30] Girao, H., Catarino, S. and Pereira, P. (2009) Eps15 interacts with ubiquitinated Cx43 and mediates its internalization. Exp. Cell Res. 315, 3587–3597.
[31] Leithe, E. and Rivedal, E. (2004) Epidermal growth factor regulates ubiquitination, internalization and proteasome-dependent degradation of connexin43. J. Cell Sci. 117, 1211–1220.
[32] Catarino, S., Ramalho, J.S., Marques, C., Pereira, P. and Girao, H. (2011) Ubiquitin-mediated internalization of connexin43 is independent of the canonical endocytic tyrosine-sorting signal. Biochem. J. 437, 255–267.
[33] Leithe, E., Kjenseth, A., Sirnes, S., Stenmark, H., Brech, A. and Rivedal, E. (2009) Ubiquitylation of the gap junction protein connexin-43 signals its trafficking from early endosomes to lysosomes in a process mediated by Hrs and Tsg101. J. Cell Sci. 122, 3883–3893.
[34] Leithe, E. and Rivedal, E. (2004) Ubiquitination and down-regulation of gap junction protein connexin-43 in response to 12-O-tetradecanoylphorbol 13-acetate treatment. J. Biol. Chem. 279, 50089–50096.
[35] Su, V., Nakagawa, R., Koval, M. and Lau, A.F. (2010) Ubiquitin-independent proteasomal degradation of endoplasmic reticulum-localized connexin43 mediated by CIP75. J. Biol. Chem. 285, 40979–40990.
[36] Gaietta, G. et al. (2002) Multicolor and electron microscopic imaging of connexin trafficking. Science 296, 503–507.
[37] Lauf, U., Giepmans, B.N., Lopez, P., Braconnot, S., Chen, S.C. and Falk, M.M. (2002) Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. Proc. Natl. Acad. Sci. U.S.A. 99, 10446–10451.
[38] Falk, M.M., Baker, S.M., Gumpert, A.M., Segretain, D. and Buckheit 3rd., R.W. (2009) Gap junction turnover is achieved by the internalization of small endocytic double-membrane vesicles. Mol. Biol. Cell 20, 3342–3352.
[39] Solan, J.L. and Lampe, P.D. (2009) Connexin43 phosphorylation: structural changes and biological effects. Biochem. J. 419, 261–272.
[40] Thevenin, A.F., Kowal, T.J., Fong, J.T., Kells, R.M., Fisher, C.G. and Falk, M.M. (2013) Proteins and mechanisms regulating gap-junction assembly, internalization, and degradation. Physiology (Bethesda) 28, 93–116.
[41] Boassa, D., Solan, J.L., Papas, A., Thornton, P., Lampe, P.D. and Sosinsky, G.E. (2010) Trafficking and recycling of the connexin43 gap junction protein during mitosis. Traffic 11, 1471–1486.
[42] Gilleron, J., Fiorini, C., Carette, D., Avondet, C., Falk, M.M., Segretain, D. and Pointis, G. (2008) Molecular reorganization of Cx43, Zo-1 and Src complexes during the endocytosis of gap junction plaques in response to a non-genomic carcinogen. J. Cell Sci. 121, 4069–4078.
[43] Govindarajan, R., Chakraborty, S., Johnson, K.E., Falk, M.M., Wheelock, M.J., Johnson, K.R. and Mehta, P.P. (2010) Assembly of connexin43 into gap junctions is regulated differentially by E-cadherin and N-cadherin in rat liver epithelial cells. Mol. Biol. Cell 21, 4089–4107.
[44] Leithe, E., Brech, A. and Rivedal, E. (2006) Endocytic processing of connexin43 gap junctions: a morphological study. Biochem. J. 393, 59–67.

[45] Segretain, D. et al. (2003) Sequestration of connexin43 in the early endosomes: an early event of Leydig cell tumor progression. Mol. Carcinog. 38, 179–187.
[46] Piehl, M., Lehmann, C., Gumpert, A., Denizot, J.P., Segretain, D. and Falk, M.M. (2007) Internalization of large double-membrane intercellular vesicles by a clathrin-dependent endocytic process. Mol. Biol. Cell 18, 337–347.
[47] Gumpert, A.M., Varco, J.S., Baker, S.M., Piehl, M. and Falk, M.M. (2008) Double-membrane gap junction internalization requires the clathrin-mediated endocytic machinery. FEBS Lett. 582, 2887–2892.
[48] Gilleron, J., Carette, D., Fiorini, C., Dompierre, J., Macia, E., Denizot, J.P., Segretain, D. and Pointis, G. (2011) The large GTPase dynamin2: a new player in connexin 43 gap junction endocytosis, recycling and degradation. Int. J. Biochem. Cell Biol. 43, 1208–1217.
[49] Thomas, M.A., Zosso, N., Scerri, I., Demaurex, N., Chanson, M. and Staub, O. (2003) A tyrosine-based sorting signal is involved in connexin43 stability and gap junction turnover. J. Cell Sci. 116, 2213–2222.
[50] Johnson, K.E., Mitra, S., Katoch, P., Kelsey, L.S., Johnson, K.R. and Mehta, P.P. (2013) Phosphorylation on Ser-279 and Ser-282 of connexin43 regulates endocytosis and gap junction assembly in pancreatic cancer cells. Mol. Biol. Cell 24, 715–733.
[51] Fong, J.T., Kells, R.M. and Falk, M.M. (2013) Two tyrosine-based sorting signals in the Cx43 C-terminus cooperate to mediate gap junction endocytosis. Mol. Biol. Cell 24, 2834–2848.
[52] Leykauf, K., Salek, M., Bomke, J., Frech, M., Lehmann, W.D., Durst, M. and Alonso, A. (2006) Ubiquitin protein ligase Nedd4 binds to connexin43 by a phosphorylation-modulated process. J. Cell Sci. 119, 3634–3642.
[53] Lan, Z., Kurata, W.E., Martyn, K.D., Jin, C. and Lau, A.F. (2005) Novel rab GAP-like protein, CIP85, interacts with connexin43 and induces its degradation. Biochemistry 44, 2385–2396.
[54] Cochrane, K., Su, V. and Lau, A.F. (2013) The connexin43-interacting protein, CIP85, mediates the internalization of connexin43 from the plasma membrane. Cell Commun. Adhes. 20, 53–66.
[55] Musil, L.S. and Goodenough, D.A. (1991) Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. J. Cell Biol. 115, 1357–1374.
[56] Sirnes, S., Kjenseth, A., Leithe, E. and Rivedal, E. (2009) Interplay between PKC and the MAP kinase pathway in connexin43 phosphorylation and inhibition of gap junction intercellular communication. Biochem. Biophys. Res. Commun. 382, 41–45.
[57] Guan, X. and Ruch, R.J. (1996) Gap junction endocytosis and lysosomal degradation of connexin43-P2 in WB-F344 rat liver epithelial cells treated with DDT and lindane. Carcinogenesis 17, 1791–1798.
[58] Laing, J.G., Tadros, P.N., Westphale, E.M. and Beyer, E.C. (1997) Degradation of connexin43 gap junctions involves both the proteasome and the lysosome. Exp. Cell Res. 236, 482–492.
[59] Qin, H., Shao, Q., Igdoura, S.A., Alaoui-Jamali, M.A. and Laird, D.W. (2003) Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and -competent breast tumor cells. J. Biol. Chem. 278, 30005–30014.
[60] Simeckova, P., Vondracek, J., Andrysik, Z., Zatloukalova, J., Krcmar, P., Kozubik, A. and Machala, M. (2009) The 2,2′,4,4′,5,5′-hexachlorobiphenyl-enhanced degradation of connexin 43 involves both proteasomal and lysosomal activities. Toxicol. Sci. 107, 9–18.
[61] VanSlyke, J.K. and Musil, L.S. (2005) Cytosolic stress reduces degradation of connexin43 internalized from the cell surface and enhances gap junction formation and function. Mol. Biol. Cell 16, 5247–5257.
[62] Berthoud, V.M., Minogue, P.J., Laing, J.G. and Beyer, E.C. (2004) Pathways for degradation of connexins and gap junctions. Cardiovasc. Res. 62, 256–267.
[63] Sirnes, S., Leithe, E. and Rivedal, E. (2008) The detergent resistance of connexin43 is lost upon TPA or EGF treatment and is an early step in gap junction endocytosis. Biochem. Biophys. Res. Commun. 373, 597–601.
[64] Xie, H., Laird, D.W., Chang, T.H. and Hu, V.W. (1997) A mitosis-specific phosphorylation of the gap junction protein connexin43 in human vascular cells: biochemical characterization and localization. J. Cell Biol. 137, 203–210.
[65] Rutz, M.L. and Hulser, D.F. (2001) Supramolecular dynamics of gap junctions. Eur. J. Cell Biol. 80, 20–30.
[66] Fykerud, T.A. et al. (2012) Smad ubiquitination regulatory factor-2 controls gap junction intercellular communication by modulating endocytosis and degradation of connexin43. J. Cell Sci. 125, 3966–3976.
[67] Chen, V.C., Kristensen, A.R., Foster, L.J. and Naus, C.C. (2012) Association of connexin43 with E3 ubiquitin ligase TRIM21 reveals a mechanism for gap junction phosphodegron control. J. Proteome Res. 11, 6134–6146.
[68] Fernandes, R., Girao, H. and Pereira, P. (2004) High glucose down-regulates intercellular communication in retinal endothelial cells by enhancing degradation of connexin 43 by a proteasome-dependent mechanism. J. Biol. Chem. 279, 27219–27224.
[69] Girao, H. and Pereira, P. (2003) Phosphorylation of connexin 43 acts as a stimuli for proteasome-dependent degradation of the protein in lens epithelial cells. Mol. Vis. 9, 24–30.
[70] Girao, H. and Pereira, P. (2007) The proteasome regulates the interaction between Cx43 and ZO-1. J. Cell. Biochem. 102, 719–728.
[71] Kimura, K. and Nishida, T. (2010) Role of the ubiquitin–proteasome pathway in downregulation of the gap-junction protein connexin43 by TNF-{alpha} in human corneal fibroblasts. Invest. Ophthalmol. Vis. Sci. 51, 1943–1947.
[72] Dunn, C.A., Su, V., Lau, A.F. and Lampe, P.D. (2012) Activation of Akt, not connexin 43 protein ubiquitination, regulates gap junction stability. J. Biol. Chem. 287, 2600–2607.
[73] Clague, M.J. and Urbe, S. (2010) Ubiquitin: same molecule, different degradation pathways. Cell 143, 682–685.
[74] Hicke, L. and Dunn, R. (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19, 141–172.
[75] Auth, T. et al. (2009) The TSG101 protein binds to connexins and is involved in connexin degradation. Exp. Cell Res. 315, 1053–1062.
[76] Raiborg, C. and Stenmark, H. (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature 458, 445–452.
[77] Colussi, C. et al. (2011) Nepsilon-lysine acetylation determines dissociation from GAP junctions and lateralization of connexin 43 in normal and dystrophic heart. Proc. Natl. Acad. Sci. U.S.A. 108, 2795–2800.
[78] Kjenseth, A. et al. (2012) The gap junction channel protein connexin43 is covalently modified and regulated by SUMOylation. J. Biol. Chem. 287, 15851–15861.
[79] Murray, S.A., Larsen, W.J., Trout, J. and Donta, S.T. (1981) Gap junction assembly and endocytosis correlated with patterns of growth in a cultured adrenocortical tumor cell (SW-13). Cancer Res. 41, 4063–4074.
[80] Naus, C.C., Hearn, S., Zhu, D., Nicholson, B.J. and Shivers, R.R. (1993) Ultrastructural analysis of gap junctions in C6 glioma cells transfected with connexin43 cDNA. Exp. Cell Res. 206, 72–84.
[81] Sasaki, T. and Garant, P.R. (1986) Fate of annular gap junctions in the papillary cells of the enamel organ in the rat incisor. Cell Tissue Res. 246, 523–530.
[82] Levine, B. and Kroemer, G. (2008) Autophagy in the pathogenesis of disease. Cell 132, 27–42.
[83] Mazet, F., Wittenberg, B.A. and Spray, D.C. (1985) Fate of intercellular junctions in isolated adult rat cardiac cells. Circ. Res. 56, 195–204.
[84] Severs, N.J., Shovel, K.S., Slade, A.M., Powell, T., Twist, V.W. and Green, C.R. (1989) Fate of gap junctions in isolated adult mammalian cardiomyocytes. Circ. Res. 65, 22–42.
[85] Hesketh, G.G. et al. (2010) Ultrastructure and regulation of lateralized connexin43 in the failing heart. Circ. Res. 106, 1153–1163.
[86] Lichtenstein, A., Minogue, P.J., Beyer, E.C. and Berthoud, V.M. (2011) Autophagy: a pathway that contributes to connexin degradation. J. Cell Sci. 124, 910–920.
[87] Bejarano, E., Girao, H., Yuste, A., Patel, B., Marques, C., Spray, D.C., Pereira, P. and Cuervo, A.M. (2012) Autophagy modulates dynamics of connexins at the plasma membrane in a ubiquitin-dependent manner. Mol. Biol. Cell 23, 2156–2169.
[88] Fong, J.T., Kells, R.M., Gumpert, A.M., Marzillier, J.Y., Davidson, M.W. and Falk, M.M. (2012) Internalized gap junctions are degraded by autophagy. Autophagy 8, 794–811.
[89] Defamie, N. et al. (2001) Disruption of gap junctional intercellular communication by lindane is associated with aberrant localization of connexin43 and zonula occludens-1 in 42GPA9 Sertoli cells. Carcinogenesis 22, 1537–1542.
[90] Fontes, M.S., van Veen, T.A., de Bakker, J.M. and van Rijen, H.V. (2012) Functional consequences of abnormal Cx43 expression in the heart. Biochim. Biophys. Acta 1818, 2020–2029.
[91] Chaldoupi, S.M., Loh, P., Hauer, R.N., de Bakker, J.M. and van Rijen, H.V. (2009) The role of connexin40 in atrial fibrillation. Cardiovasc. Res. 84, 15–23.
[92] Delmar, M. and Makita, N. (2012) Cardiac connexins, mutations and arrhythmias. Curr. Opin. Cardiol. 27, 236–241.
[93] Qin, H., Shao, Q., Curtis, H., Galipeau, J., Belliveau, D.J., Wang, T., Alaoui-Jamali, M.A. and Laird, D.W. (2002) Retroviral delivery of connexin genes to human breast tumor cells inhibits in vivo tumor growth by a mechanism that is independent of significant gap junctional intercellular communication. J. Biol. Chem. 277, 29132–29138.
[94] Kameritsch, P., Pogoda, K. and Pohl, U. (2012) Channel-independent influence of connexin 43 on cell migration. Biochim. Biophys. Acta 1818, 1993–2001.
[95] Vinken, M., Decrock, E., Leybaert, L., Bultynck, G., Himpens, B., Vanhaecke, T. and Rogiers, V. (2012) Non-channel functions of connexins in cell growth and cell death. Biochim. Biophys. Acta 1818, 2002–2008.
[96] Laird, D.W. (2010) The gap junction proteome and its relationship to disease. Trends Cell Biol. 20, 92–101.
[97] Kelly, S.M., VanSlyke, J.K. and Musil, L.S. (2007) Regulation of ubiquitin–proteasome system mediated degradation by cytosolic stress. Mol. Biol. Cell 18, 4279–4291.
[98] Minogue, P.J., Beyer, E.C. and Berthoud, V.M. (2013) A connexin50 mutant, CX50fs, that causes cataracts is unstable, but is rescued by a proteasomal inhibitor. J. Biol. Chem. 288, 20427–20434.
[99] Lichtenstein, A., Gaietta, G.M., Deerinck, T.J., Crum, J., Sosinsky, G.E., Beyer, E.C. and Berthoud, V.M. (2009) The cytoplasmic accumulations of the cataract-associated mutant, connexin50p88s, are long-lived and form in the endoplasmic reticulum. Exp. Eye Res. 88, 600–609.