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Proteins and Mechanisms Regulating Gap-Junction Assembly, Internalization, and Degradation

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Gap junctions (GJs) are the only known cellular structures that allow a direct cell-to-cell transfer of signaling molecules by forming densely packed arrays or “plaques” of hydrophilic channels that bridge the apposing membranes of neighboring cells. The crucial role of GJ-mediated intercellular communication (GJIC) for all aspects of multicellular life, including coordination of development, tissue function, and cell homeostasis, has been well documented. Assembly and degradation of these membrane channels is a complex process that includes biosynthesis of the connexin (Cx) subunit proteins (innexins in invertebrates) on endoplasmic reticulum (ER) membranes, oligomerization of compatible subunits into hexameric hemichannels (connexons), delivery of the connexons to the plasma membrane (PM), head-on docking of compatible connexons in the extracellular space at distinct locations, arrangement of channels into dynamic spatially and temporally organized GJ channel plaques, as well as internalization of GJs into the cytoplasm followed by their degradation. Clearly, precise modulation of GJIC, biosynthesis, and degradation are crucial for accurate function, and much research currently addresses how these fundamental processes are regulated. Here, we review posttranslational protein modifications (e.g., phosphorylation and ubiquitination) and the binding of protein partners (e.g., the scaffolding protein ZO-1) known to regulate GJ biosynthesis, internalization, and degradation. We also look closely at the atomic resolution structure of a GJ channel, since the structure harbors vital cues relevant to GJ biosynthesis and turnover.

Direct cell-to-cell communication is a pivotal cellular function of multicellular organisms. It is established by gap-junction (GJ) channels that bridge apposing plasma membranes (PMs) of neighboring cells. Typically, tens to thousands of GJ channels cluster into densely packed two-dimensional arrays, termed GJ plaques, that can reach several micrometers in diameter (FIGURE 1). In vertebrates, GJ channels are assembled from a ubiquitously expressed class of four-pass trans-membrane proteins, termed connexins (Cxs), with connexin 43 (Cx43) being the most abundantly expressed Cx type (168). In invertebrates, GJs are assembled from an unrelated class of four-pass transmembrane proteins, termed innexins (150). Three innexin relatives, termed *pannexin* 1–3, are also found in mammals (79). Six Cx polypeptides oligomerize into a ring to form a hexameric transmembrane structure with a central

hydrophilic pore, called a hemichannel or connexon. Once trafficked to the PM, two connexons, one provided by each of two neighboring cells, dock head-on in the extracellular space to form the complete double-membrane-spanning GJ channel that is completely sealed off to the extracellular space. Recruitment of additional GJ channels along the outer edge enlarges the GJ plaques, whereas simultaneous removal of older channels from plaque centers balances GJ channel turnover (see Ref. 166 and references cited below). Recent research, supported by classical ultrastructural observations, has shown that GJs are removed from the PM, internalized as double-membrane-spanning channel structures, and degraded by lysosomal pathways. It seems obvious that precise regulation of GJ biosynthesis and degradation is vitally important for physiological modulation of

GJ-mediated intercellular communication (GJIC), and much research over the past years has addressed the factors and mechanisms that regulate the function of this important cellular structure.

On electron microscopic images of classically stained (osmiumtetroxyde/uranylacetate/tannic acid) ultrathin sectioned specimens, GJs appear as typical, pentalaminar structures, well visible in the PM of cells (FIGURE 1D). However, accessory proteins potentially attached to their cytoplasmic surfaces are not well visible. This feature is very different, for example, from the appearance of adherens junctions (AdJ), another PM-located cell-cell adhesion structure that exhibits a dense layer of plaque proteins and an attached, pronounced actin-based cytoskeletal network on both cytoplasmic surfaces (FIGURE 1E), implying that GJs appear not to interact substantially with cytoplasmic proteins. Indeed, 10 years ago, only about a handful of proteins, including ZO-1, v- and c-Src, caveolin-1, α - and β -catenin, and tubulin were known to interact with GJs (reviewed, e.g., in Refs. 54, 67). More recently, however, this picture has changed dramatically with well over two dozen proteins now known to interact with GJs (extensively reviewed recently, e.g., in Refs. 68, 120) and in a number of other comprehensive recent reviews that discuss posttranslational modifications and GJ degradation (see citations below). However, structural components, such as microtubules and actin filaments that either interact directly with Cx43 as described for microtubules (56) or indirectly via the actin-binding protein drebrin (21), might be less abundant on GJs compared with the plaque proteins and linked actin filaments of AdJs shown here. Other Cx-binding partners are known to be

dynamic, only transiently recruited proteins that regulate GJ functions during all stages of the GJ life cycle. In addition, novel ultra-structural stains that better show less ordered and less dense assembled proteins, such as the ones developed to better visualize extracellular matrix (128), may in the future better reveal GJs including their binding partners.

In the following sections, we specifically review posttranslational modifications (e.g., phosphorylation and ubiquitination) and discuss in greater detail the binding of a scaffolding protein, zona occludens-1 (ZO-1), that are now well known to regulate the biosynthesis, trafficking, assembly, and especially internalization and degradation of GJs. Beforehand, however, we look closely at the three-dimensional (3D) atomic resolution structure of a Cx26 GJ channel and at the NMR solution structure of the isolated Cx43 COOH-terminal tail, because these 3D structures hold many functional cues that are highly relevant to the biosynthesis and turnover of GJs.

The 3D Structure of a GJ Channel Provides Functional Cues Relevant to GJ Biosynthesis and Turnover

Membrane proteins, due to their high content of hydrophobic amino acid residues, are notoriously difficult to crystalize. Thus GJ researchers feel fortunate that the crystal structure of a complete GJ channel has been resolved at atomic resolution revealing the location of most amino acid residues, giving cues to many functional features relevant to GJ assembly and turnover (124). In 1999, the pseudo-atomic structure of a GJ channel assembled from recombinant Cx43 with a COOH-terminal

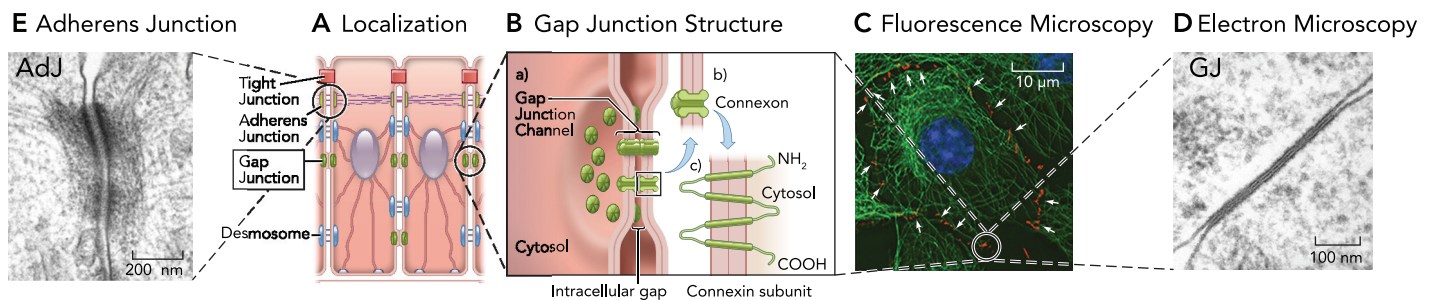


FIGURE 1. Location and structure of GJs

A: gap junctions (GJs) are assemblies of double-membrane-spanning hydrophilic channels termed “plaques” that bridge the apposing PMs of neighboring cells to provide direct cell-to-cell (or intercellular) communication (GJIC) as shown here for epithelial cells. B: GJ channels form by the head-on docking of two hemichannels or “connexons,” each assembled and trafficked to the PM by one of the two contacting cells. Connexons are assembled from six four-pass transmembrane proteins termed “connexins” (Cxs). C: GJ plaques can be seen by immunofluorescence light microscopy when stained with fluorescence-tagged antibodies, such as the ones shown here in T51B liver cells assembled from endogenously expressed Cx43 protein. D: GJ plaques, as the one assembled from exogenously expressed Cx43-GFP in HeLa cells shown here, appear as typical, pentalaminar structures in classically stained ultrathin sections when examined by electron microscopy, revealing the PMs that are closely aligned throughout the plaque, and the 2- to 3-nm-wide, name-giving “gap” separating the membranes. Regulatory proteins, if attached to the cytoplasmic plaque surfaces, are not well resolved. E: in contrast, another cell-cell junction type, adherens junctions (AdJs), are characterized by well visible plaque proteins and attached actin-based cytoskeletal filaments when stained by similar techniques as shown here also in HeLa cells.

truncation ($\Delta 263$) was resolved to 7.5 Å (membrane plane) by Unger et al. (199); and in 2007, a GJ channel assembled from Cx26 was resolved to 7 Å by Oshima et al. (140). Then, in 2009, a crystal structure of a Cx26 GJ channel at 3.5-Å atomic resolution was published by Maeda et al. (124), who describe the quaternary structure of the channel as a “tsuzumi” shape, a traditional Japanese drum that has a height without loops of 155 Å and an outer maximum diameter of 92 Å (FIGURE 2). The diameter of the hydrophilic channel is widest at the cytoplasmic surface (40 Å), narrows at the membrane/extracellular space interface forming the channel vestibule (14 Å), and widens again in the extracellular space (25 Å) (FIGURE 2, B AND C). Since Cxs share identical transmembrane topologies and all GJ channels are assembled from six Cx subunits, it is likely that the overall structure of the solved Cx26 GJ channel is representative for the structure of all GJ channels, a notion that is supported by the superimposition of the transmembrane cylinder models of Cx26 and Cx43 (140).

All four transmembrane (TM) regions of Cx proteins are α -helices that are organized such that TM1 and TM2 line the interior of the pore, and TM3 and TM4 form the exterior of the channel wall nearer to the surrounding lipid of the membrane (137, 199, 218) (FIGURE 2, A AND B). TM3, which initially was thought to line the pore, is highly aromatic and falls into the grooves of neighboring TM domains. Maeda et al. (124) speculate that the extracellular half of TM2 and TM4 and the extracellular loops are likely to

form the protein/protein interfaces that allow Cx subunits to oligomerize into hexameric connexons (homo-/heteromeric interactions), since these regions contain most of the interaction sites of Cx subunits. However, using TOXCAT assays to study the interaction of isolated transmembrane α -helices, Jara et al. (76) very recently identified a critical role of the first transmembrane domain of Cx26, and specifically of residues V³⁷VAA⁴⁰, in regulating Cx-dimerization, hexamer formation, and channel function. The extracellular loops (E1, E2) of two connected hemichannels (homo-/heterotypic interactions) form concentric, antiparallel β -barrels, which make a tight seal in the extracellular space between the opposing PMs (48, 147, 199) (FIGURE 2, B AND C). Between different Cxs, these E-loops are the most similar in sequence, yet they influence the specificity of Cx docking (38, 210).

Oshima et al. (140) isolated hCx26M34A hemichannels, which upon reconstitution were observed to dock together to form complete GJ channels. Their resulting structure showed a density in the center of the channel pore, and they speculated that the density represents the NH₂ terminus. Several electrophysiological reports (94, 138, 158, 203) have suggested that the NH₂ terminus resides inside the pore and functions in voltage gating, supporting the observations made by Oshima et al. These assumptions have been verified by the atomic resolution structure that also revealed the location and structure of the

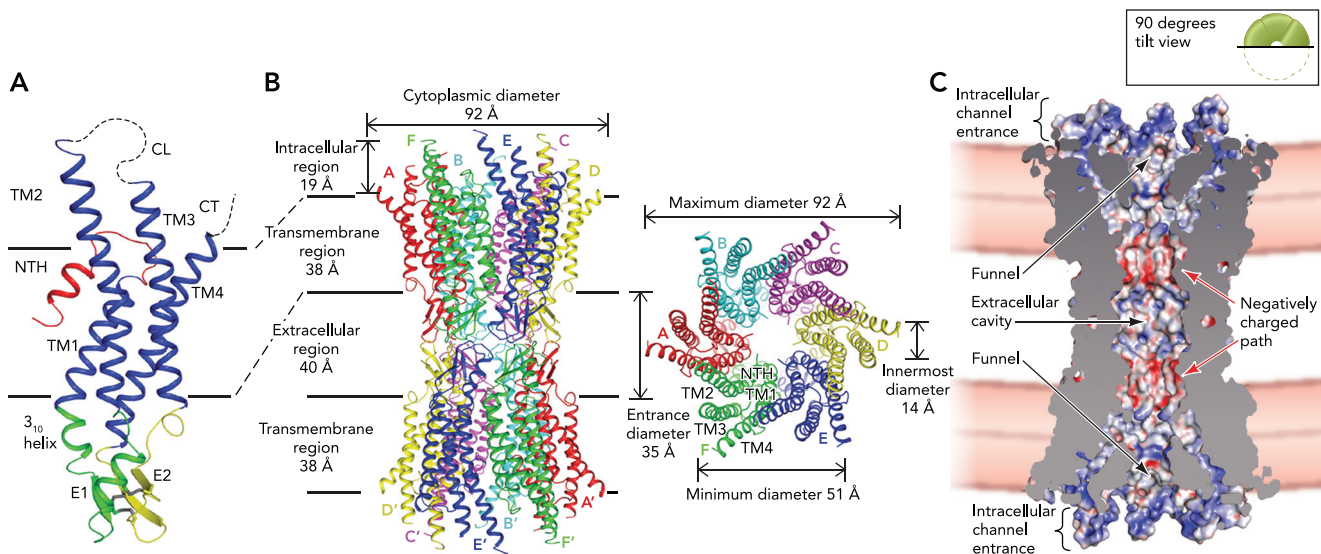


FIGURE 2. X-ray structure of a Cx26 GJ channel solved by Maeda et al.

A: Cx26 monomer. Transmembrane (TM) α -helical domains are shown in blue, NH₂-terminal helix in red, extracellular loops (E1 and E2) in green and yellow, respectively, and unstructured, flexible intracellular loop (I-loop) and COOH-terminal tail as dashed lines. Note the kink in the NH₂-terminal tail that positions the NH₂ terminus inside the channel, forming the funnel-shaped channel wall visible in C. B: overall structure of the Cx26 channel shown is as a side view and a top view. Note the six Cx26 monomers (shown in different colors) labeled A–E, and TM1, TM2, and NH₂ terminus (NTH) facing the hydrophilic pore, whereas TM3 and TM4 face the hydrophobic membrane environment (labeled in the green monomer in the top view). C: cross section of the Cx26 GJ channel demonstrating the electrostatic surface potential. The NH₂-terminal helices of each Cx26 monomer make up the funnel-shaped wall of the channel entrance. Figure is adapted from Ref. 124 and used here with permission.

NH₂-terminal domains (124). The short NH₂-terminal domains of the six Cx subunits form the funnel-shaped channel wall (FIGURE 2C), which is stabilized and held in place by a circular network of hydrogen bonds formed between the D2 residue on one Cx subunit and the backbone amide of T5 on another Cx subunit, as well as between W3 and M32 in TM1 within the same Cx subunit; yet the crystallographic data indicate that the NH₂ termini are the most mobile domains in the Cx26 channel structure. Indeed, the secondary structure of the NH₂ terminus can be broken down into two regions: an NH₂-terminal α -helix or hydrophobic domain followed by an open turn (FIGURE 2A, shown in red). Both Purnick et al. (157) and more recently Kalmatsky et al. (81, 82), using NH₂-terminal peptides of Cx32, have demonstrated the importance of the glycine residue at *position 12* in maintaining this open turn and allowing the first 10 residues of the NH₂-terminal domain to enter the pore. Mutations of G12 cause the normally flexible open turn to become stiff and more structured, likely causing the NH₂-terminal domain to be misplaced and to extend outward into the cytoplasm. The flexibility of the open turn may explain why NH₂-terminal fluorescent protein (FP)-tagged Cxs unexpectedly can oligomerize, traffic to the PM, and dock and assemble into GJ plaques, such that the flexibility allows the bulky FP-tag to locate outside of the channel in the cytoplasm, similar to COOH-terminal FP-tagged Cxs. However, similar to disease-causing D12 residue mutations that have misplaced NH₂-terminal domains (81), NH₂-terminal FP-tagged GJ channels are nonfunctional based on their inability to transfer the GJ-permeable dye lucifer yellow (99). Thus, although NH₂-terminal GFP-tagged Cxs may be useful to address certain questions (11, 23, 27, 60), their inability to form functional channels may be as destructive as the inability of COOH-terminal GFP-tagged Cxs to bind to the scaffolding protein ZO-1 (73) (see *Cx43/ZO-1 Binding* for more detail), and this needs to be taken into close consideration if meaningful results are to be obtained.

The intracellular loop and the COOH-terminal domain are the least conserved domains among Cxs and contribute to the difficulties of crystallizing and structurally resolving GJ channels. Maeda et al. (124) succeeded solving the structure of a GJ channel assembled from Cx26, which has the second shortest COOH-terminal domain of all Cxs (19 residues); and Unger et al. (199) used a Cx43 with 119 COOH-terminal residues truncated. These domains also resisted crystallographic resolution, indicating that they are unstructured and highly flexible (FIGURE 2A, shown as dashed lines).

Sorgen et al. (184, 185) used NMR to elucidate the secondary structure of a peptide representing

almost the entire COOH-terminal tail of Cx43 (*residues S255–I382*) (see FIGURE 4). They found it to have a random coil structure with two short α -helices. The authors speculate that the random coil may be important in numerous interactions with many different proteins, whereas the α -helices may allow for the formation of higher-order structures. They further demonstrated that the interaction of proteins well known to bind to and phosphorylate the COOH terminus of Cx43, such as ZO-1 and c-Src (see *Cx43 Phosphorylation and Cx43/ZO-1 Binding* for more details), can cause dramatic structural changes to the COOH-terminal domain outside of the immediate region at which they bind and/or modify (180). These structural changes are of particular interest, since they likely allow or prevent regulatory partner proteins to bind to the Cx COOH-terminal tail to initiate forward trafficking, GJ assembly, and potentially internalization and degradation. Recently, Sorgen and colleagues reported initial structural analyses of a longer peptide encompassing the entire Cx43 COOH-terminal domain plus the fourth transmembrane spanning domain (TM4-Cx43CT; D196–I382) (64). Since the NH₂ terminus of the previous peptide is anchored to TM4 and thus is not “free,” the authors feel that the new peptide is a better model than the previous soluble Cx43CT and might more closely represent the native folded Cx43 COOH terminus. Initial analyses indicate that the α -helicity of the new peptide is increased (64). Thus, although the molecular structure of GJ channels is beginning to unfold, still only limited information is available about these different structural conformations of the flexible COOH-terminal tail that appear responsible for most regulatory Cx binding protein interactions. Some of these interactions and resulting posttranslational modifications of the Cx43 polypeptide relevant to biosynthesis and GJ turnover will be discussed further in the following sections.

Cx43 Phosphorylation

Kinase-mediated posttranslational phosphorylation of serine (S), threonine (T), and tyrosine (Y) residues is a major regulatory event under physiological as well as pathological conditions. Phosphorylation may lead to alterations in hydrophobicity, charge, and structural organization of target proteins, either promoting or inhibiting normal function (79). Not surprisingly, phosphorylation also plays a significant role in GJ function, mediating almost all stages of the Cx43 life cycle, altering its oligomerization behavior, forward trafficking from the ER to Golgi and PM, as well as GJ assembly, gating, internalization, degradation, and regulation of the

cell cycle (comprehensively reviewed in Refs. 88, 97, 102, 129, 141, 177, 179, 180, 208) (FIGURE 3).

Cx43 phosphorylation has been extensively studied, although less is known about the phosphorylation of other Cxs. Cx26, Cx32, Cx36, Cx45, Cx46, and Cx50 have also been shown to be phosphorylated (reviewed in Ref. 102). However, since the focus of this review is largely on Cx43, phosphorylation of other Cxs is not further discussed. Cx43 contains a total of 66 serine, tyrosine, and threonine residues (32 found in its COOH-terminal tail alone) (see FIGURES 4 AND 5C). Firm evidence for at least 15 different phosphorylation events in its COOH terminus and regulation by a large number of different kinases has been obtained (Table 1). Cx43 does not contain serine residues within its intracellular loop domain, and threonine residues

present in this region do not conform to any known consensus phosphorylation motifs. Therefore, Cx43 is not modified directly by phosphorylation within its intracellular loop domain (79, 179). In general for Cx43, CK1, PKA, and Akt phosphorylation increase and PKC, MAPKs, Src, and CDC2 reduce GJIC (79, 141, 144, 177–179) (FIGURE 3; Table 1). Although, surprisingly, none of these COOH-terminal phosphorylation sites are required for Cx43 assembly, trafficking, and the formation of dye-transferring GJ plaques [deletions at amino acid 239 and higher traffic to the cell membrane and form GJs (209)], precise regulation of GJ function in situ is likely dependent on phosphorylation/dephosphorylation at these sites, with multiple events probably occurring simultaneously. For example, homozygous Cx43K258Stop mice die shortly after birth due

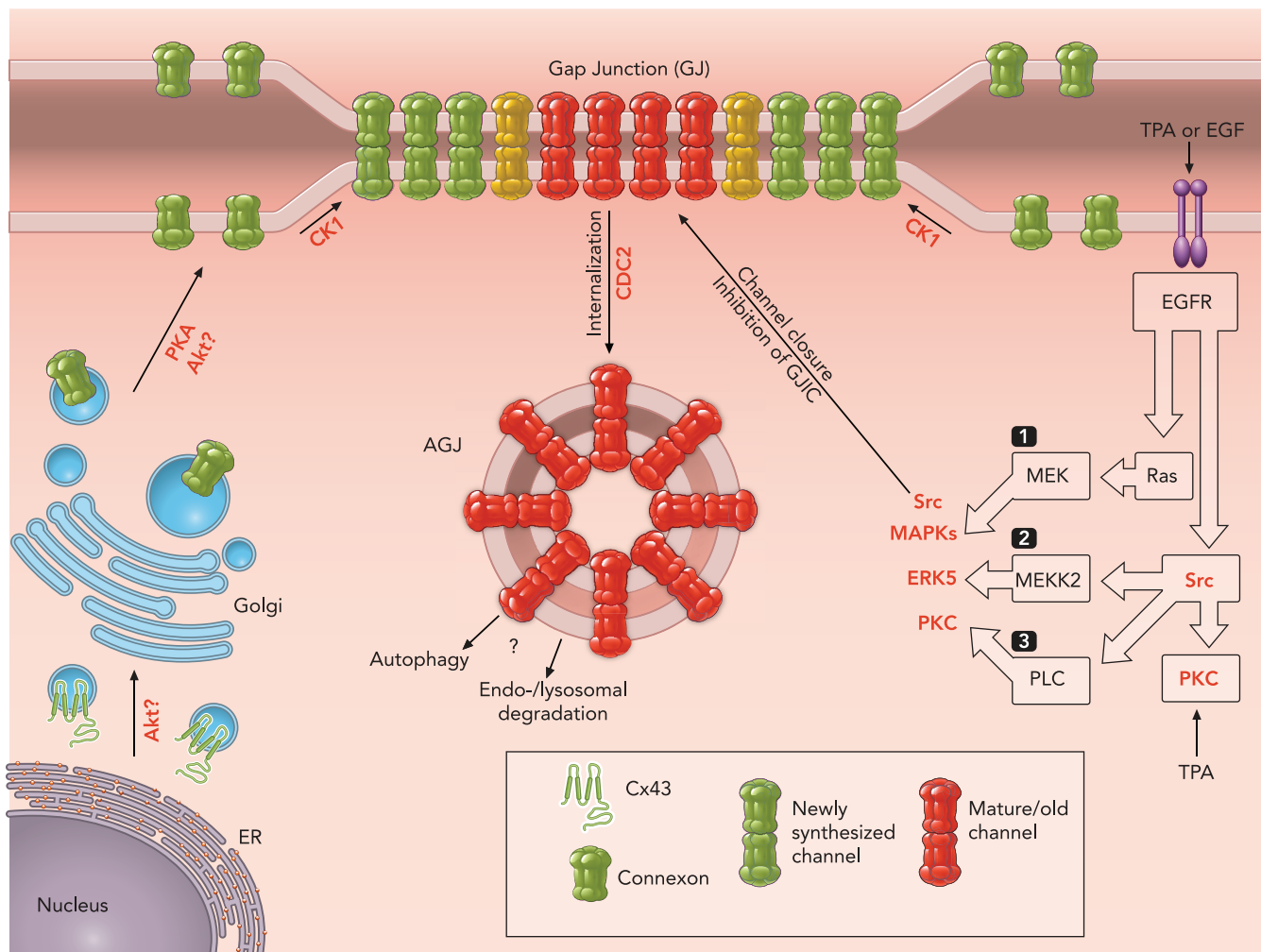


FIGURE 3. Kinases involved in the regulation of the Cx43 life cycle

Kinases discussed in this review are labeled red. Forward trafficking of Cx43 monomers from the ER to the Golgi apparatus may be regulated through Akt phosphorylation (and interaction with 14-3-3 protein) (left). Trafficking of connexons toward the PM is regulated through phosphorylation by PKA, whereas assembly of PM-localized connexons into newly synthesized channels in GJ plaques is regulated via CK1 phosphorylation. Src, PKC, and MAPK activation can be achieved through the activation of the EGF receptor (EGFR) in an EGF- or TPA-dependent manner (right). MAPKs (i.e., ERKs, JNKs, or p38) are activated through Ras, Raf, and MEK (pathway 1). Src can be activated directly by the EGFR, which in turn can activate ERK5 through MEK2 (pathway 2). Src can also activate PKC through diacylglycerol (DAG) that is generated by phospholipase C (PLC)-mediated phosphatidylinositol 4,5-bisphosphate (PIP₂) cleavage (pathway 3). PKC can also be activated directly by TPA. Activated kinases (Src, MAPKs, ERK5, and PKC) regulate downregulation of GJIC, channel closure, and possibly internalization, formation of annular GJs (AJGs), and degradation through either autophagosomal and/or endo-/lysosomal pathways (middle). CDC2 phosphorylates Cx43 at the onset of mitosis, also leading to GJ internalization and degradation.

to defective epidermal barrier function and perturbation in keratinocyte differentiation, supporting the fact that the COOH-terminal domain of Cx43 is necessary for proper physiological functions (121). In addition, mice with a single Cx43K258Stop allele survived, but the loss of COOH-terminal domain altered the spatial organization of Cx43 in intercalated discs, affected Cx43 channel function, increased the average diameter of GJ plaques, and prolonged Cx43 half-life (122).

As mentioned above, phosphorylation likely allows or prevents interactions of Cx43 with many of its binding partners, such as scaffolding proteins and chaperones (discussed below). Precise regulation of binding is crucial, since many of the Cx-binding proteins that regulate GJ function are not interacting with Cxs at all times but are recruited to and interact with Cx43-based GJs only at certain stages of their life cycle. Below, we discuss selected characteristics of Cx43 phosphorylation that are related to trafficking, GJ assembly and stability, and, particularly, GJ internalization and degradation.

Cx43 Trafficking, Assembly into GJs, and Stability Are Regulated by Akt, PKA, and CK1

Phosphorylation of Cxs, especially of Cx43, was shown to take place in the early stages of Cx trafficking. Work by Park et al. (143, 144) suggests that S369 and S373 are Akt kinase (PKB) phosphorylation sites and that Cx43 phosphorylation by Akt regulates binding of 14-3-3 proteins (FIGURES 3 AND 4;

Table 1). The 14-3-3 family members are small, acidic, dimeric proteins that are known to interact with over 200 different partners in a phosphorylation-dependent manner, and dimerization of 14-3-3 was shown to be necessary for their function (reviewed in Ref. 176). Binding of 14-3-3 proteins to their phosphorylated partners may lead to changes in partner protein conformation, scaffolding with other proteins, or masking of ER retention sequences to promote forward trafficking toward Golgi and the PM. Cx43 is the only Cx that has a 14-3-3 binding motif, R-S-X-pS/pT-X-P, where the phosphorylated serine residue (pS) is S373 in human Cx43. Cx43 was also shown to colocalize with Akt1 and 14-3-3 at the edges of GJ plaques (144). These results suggest that S373-mediated Akt phosphorylation and subsequent binding of 14-3-3 to Cx43 may play a role in facilitating Cx43 trafficking from the ER to the Golgi apparatus and on to the PM, leading to the incorporation of connexons into GJ plaques (144). However, it is unclear what it is about Cx43 trafficking that favors the interaction with 14-3-3, whereas other Cxs traffic without 14-3-3. One possibility is that 14-3-3 protein interaction may be responsible for Cx43 oligomerization that only occurs en route to the PM in the trans-Golgi network (TGN) (Ref. 134; reviewed in Ref. 93), whereas most other Cxs already oligomerize in the ER. Interestingly, Chen et al. (27) showed that phosphorylation of serine 373 by Akt decreased the

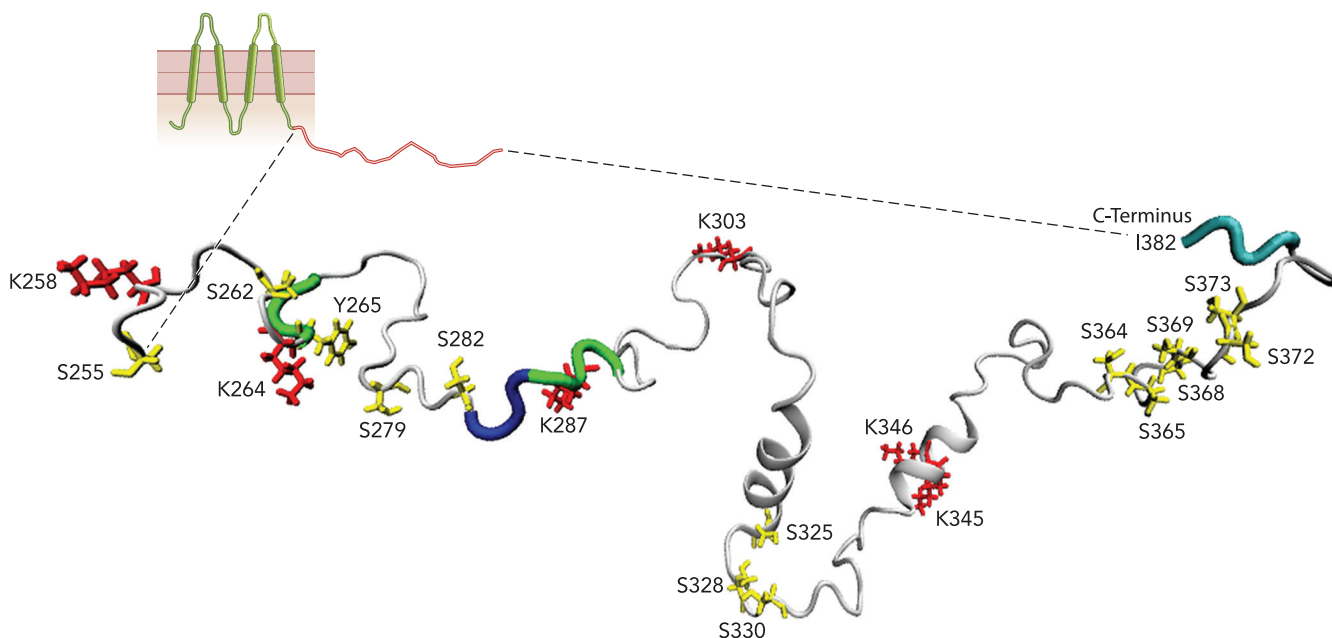


FIGURE 4. Lowest energy 3D solution NMR structure of the Cx43 COOH-terminal tail revealing the location of post-translational modifications and protein-binding sites
 The structure of almost the entire COOH-terminal tail (residues S255–I382) as reported by Sorgen et al. (184) is depicted. Sites of Cx43 modification discussed in this review (highlighted in color) are shown in relative spatial context to each other. Sites of phosphorylation are shown as yellow side chains, with serine (S) and tyrosine (Y) residues numbered. Lysine (K) residues (possible ubiquitination sites) are labeled in red. The Nedd4 ubiquitin-E3-ligase binding site (S283PPGY²⁸⁶) is labeled in blue. Tyrosine-based sorting signals (S265YAYF²⁶⁸ and S286YKLV²⁸⁹) are highlighted in green. The minimal COOH-terminal ZO-1 recognition motif (S379DLG1³⁸²) is shown in cyan. (The figure was generated from the Protein Data Bank accession file 1R5S deposited by Sorgen et al. (184), using VMD software, version 1.8.7, for Macintosh.)

Table 1. Cx43 phosphorylation sites and kinase recognition motifs

Known In Situ Cx43 Phosphorylation Sites			
Residue	Kinase	Regulatory effect	References
Y247	vSrc/cSrc	Channel closure/ ↓ GJIC	178
S255	MAPKs/CDC2	Channel closure/ ↓ GJIC	101, 174
S262	MAPKs/CDC2	Channel closure/ ↓ GJIC	83
Y265	vSrc/cSrc	Channel closure/ ↓ GJIC	178
S279	MAPKs	Channel closure/ ↓ GJIC	114, 148, 154
S282	MAPKs	Channel closure/ ↓ GJIC	114, 148, 154
S325	CK1	GJ plaque assembly	31
S328	CK1	GJ plaque assembly	31
S330	CK1	GJ plaque assembly	31
S364	PKA	Trafficking to PM	170, 192
S365	PKA	Trafficking to PM	181
S368	PKC	Channel closure/ ↓ GJIC	103, 181
S369*	Akt/PKA		144
S372*	PKC		162a
S373	Akt/PKA	Forward trafficking (to Golgi and PM), 14-3-3/ZO-1 binding	143, 144

Established Phosphorylation Motifs for Serine/Threonine Kinases Discussed in this Review		
Kinase	Motif [†]	References
	<small>[−5][−4][−3][−2][−1][0][+1][+2][+3]</small>	
Akt	R-X-R-X-X-S	176
PKA	R-X-X-S	176
CK1	pS-X-X-S	183
PKC	R-X-X-S-X-R-X	176
MAPKs	P-X-S-P	183
CDC2	S-P-X-R/K or S-P-K	183 83

Sequence Alignment of Cx43 Around Serine 262[‡]

Rat	²⁵⁵ SPSKDCGSPKYAYF ²⁶⁷
Human [§]	²⁵⁵ SPSKDCGSQKYAYF ²⁶⁷

*S369 and S372 were shown to be phosphorylated in vitro. A regulatory effect of these phosphorylation events in situ was not demonstrated. [†]The phospho-acceptor serines (at [0] position) are shown in bold. X, any amino acid; pS, phosphorylated serine. [‡]S262 is labeled in bold, and the residue 263 is underlined. [§]MAPKs, as well as CDC2 require a proline residue (P) at the [+1] position (186). Therefore, it is unlikely that these kinases will phosphorylate serine 262 in human Cx43 with a glutamine residue (Q) at the [+1] position.

interaction between the Cx43 COOH terminus and the scaffolding protein ZO-1 (see below). Thus phosphorylation by Akt may regulate whether Cx43 interacts with 14-3-3 during trafficking from the ER or with ZO-1 once it reaches the PM. Although Cx43 phosphorylation on S373 by Akt was shown both in vitro and in vivo, S369 only appears to be a substrate of Akt in vitro, and it is not a predicted 14-3-3 binding site (144). In addition, Dunn et al. (36) recently showed that Akt activity and phosphorylation of Cx43 contribute to the stability of GJ plaques in the PM.

Protein kinase A (PKA) and casein kinase 1 (CK1) are the other two kinases that have been extensively shown to increase the amount of Cx43 in GJ plaques at the PM (179). PKA is activated when cAMP levels are increased and can phosphorylate Cx43 on S364, S365, S369, and S373 (192) (FIGURES 3 AND 4; Table 1). Phosphorylation by PKA in vivo on S364 and S365 increases GJIC, stabilizes GJ

plaques, and allows for assembly of new GJs (145, 170). Importantly, phosphorylation of S365 by PKA was demonstrated to be a “gate-keeper” event that stabilized GJ plaques through prevention of phosphorylation on S368 by PKC and subsequent downregulation of GJIC (181). It is interesting to note that PKA and Akt share common phosphorylation sites on Cx43 (S369, S373), and it is currently unclear whether PKA and Akt phosphorylation events may be cell-cycle or cell-type specific.

CK1 interacts with Cx43 and phosphorylates it on S325, S328, and S330 in vitro and in vivo (31) (FIGURE 4; Table 1). Use of a CK1-specific inhibitor demonstrated that Cx43 localization in the PM was increased but GJ formation was reduced. This suggests that CK1 phosphorylation plays an important role in the assembly of PM-localized connexons into GJ plaques (31) (FIGURE 3). In addition to efficient GJ assembly, phosphorylation at S325, S328, and S330 is

required for efficient dye transfer, playing a substantial role in channel permselectivity (100).

In resting/untreated cells, Cx43 exhibits multiple electrophoretic forms in SDS-PAGE analyses due to specific conformational changes induced by phosphorylation events: a faster migrating P “zero” (P0) form and at least two slower migrating forms (P1 and P2) that correspond to differently phosphorylated Cx43 polypeptides dependent on the involved kinase types (reviewed in Ref. 177). Initial phosphorylation of Cx43 was shown to occur as early as 15 min after protein synthesis (98, 179). A P1 form has been linked to PKA-mediated phosphorylation of S364 and S365 (180), whereas a P2 form has been linked to CK1-mediated phosphorylation of S325, S328, and S330 (100). GJ plaques that contain the P2 isoform are Triton X-100 insoluble (133), suggesting that Cx43 present in GJ plaques are phosphorylated on S325, S328, and/or S330. This supports the concept that Cx43 phosphorylation by CK1 is important for the assembly and/or maintenance of GJ plaques (100). Taken together, it is likely that phosphorylation of Cx43 by Akt, PKA, and CK1 allows for a more efficient and regulated control of trafficking and GJ assembly.

Channel Closure, Inhibition of GJIC, and GJ Internalization Are Regulated by CDC2, MAPKs, PKC, and Src

Once Cx43 is incorporated into GJ plaques, several phosphorylation events can trigger channel closure, inhibition of GJIC, and likely internalization and degradation of GJs (FIGURE 3). CDC2, a cyclin-dependent serine kinase that is active during mitosis, was shown to phosphorylate Cx43 on S255 (83, 101) and S262 (83) (FIGURE 4). This phosphorylation event has been shown to lead to decreased GJIC and rapid GJ internalization, leading to mitosis (83, 101).

S/T-P-X-K/R and S-P-K have been identified as CDC2 kinase-consensus phosphorylation motifs

where the proline (P) residue at the +1 position (underlined) is essential for recognition by this kinase (Table 1) (136, 183). Several reviews, as well as primary research articles have identified serine 262 as a phosphorylation site of CDC2 (83, 97, 141, 179). Even though serine 262 is conserved between rodents, humans, and primates, proline 263 is not: in rat Cx43, residue 263 is a proline, whereas in humans this residue is a glutamine (Table 1; FIGURE 5C). Therefore, it is unlikely that S262 will be phosphorylated by CDC2 in humans because the obligatory proline at the +1 position is not present. In vivo work on CDC2 phosphorylation of Cx43 was conducted in rat fibroblasts, and in vitro work was carried out using rat Cx43 cDNA, where Lampe et al. (101) only identified S255 as a CDC2 site, whereas Kanemitsu et al. (83) identified phosphorylation of both S255 and S262 (Table 1). Thus it is probable that, in humans, phosphorylation at S255 by CDC2 is sufficient to trigger GJ internalization at the onset of mitosis.

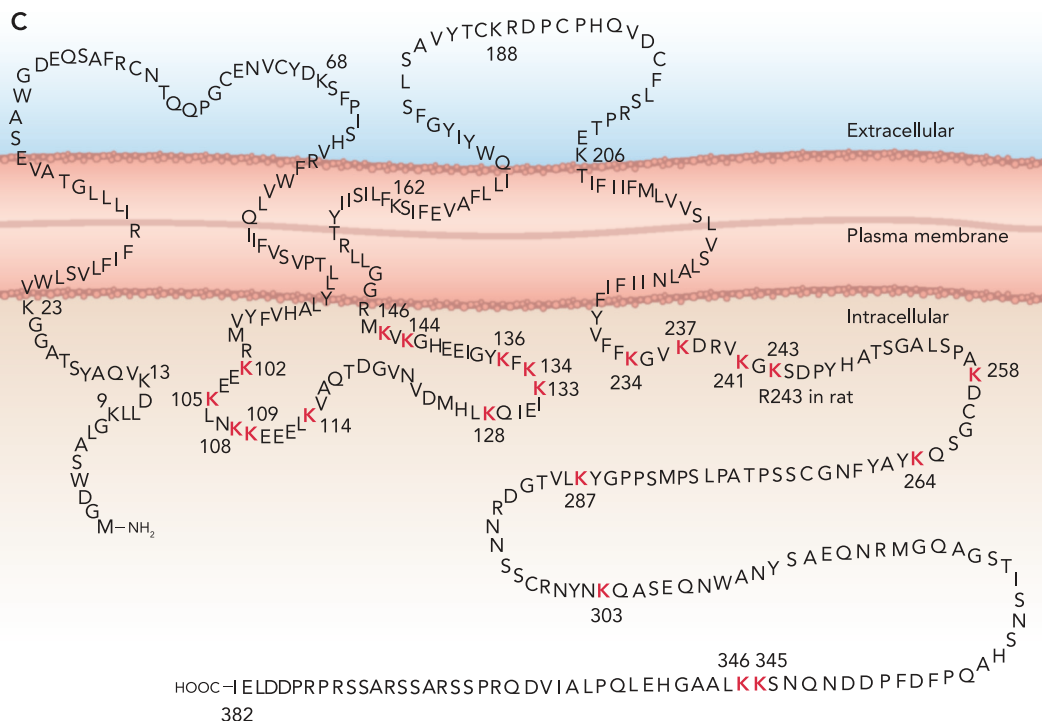
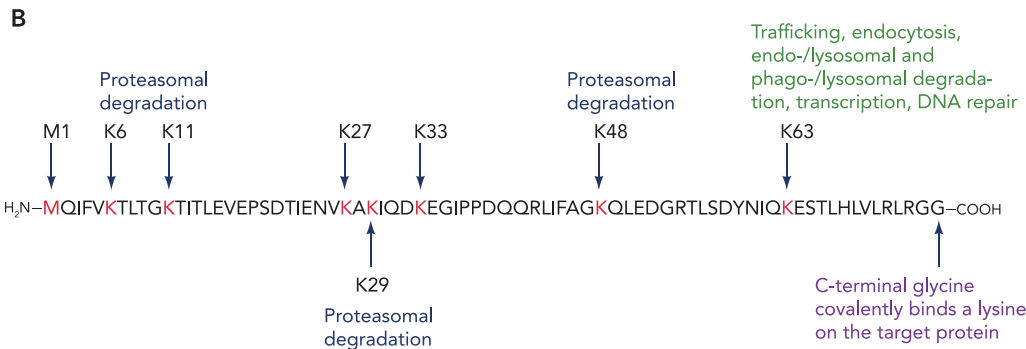
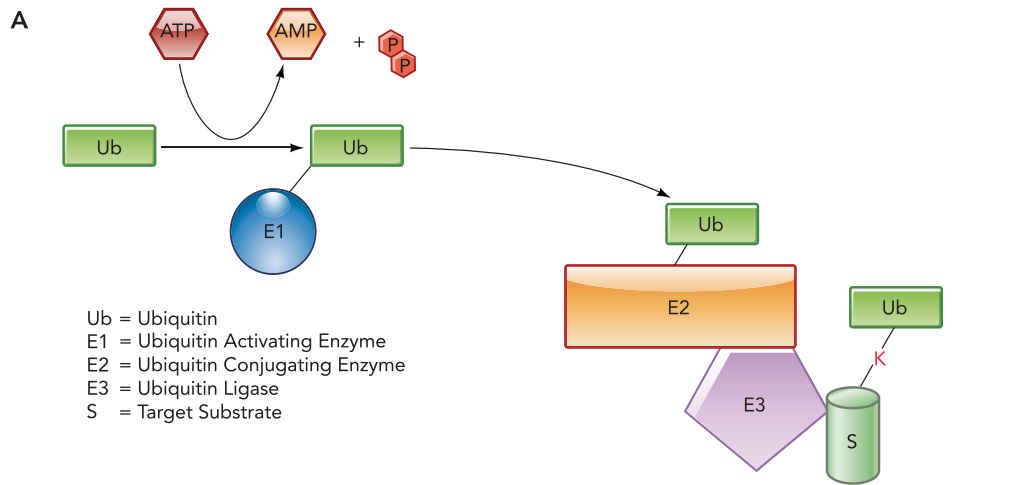
Due to cross talk between MAPKs, PKC, and Src signaling cascades, these three kinases are best discussed together. It has been shown that all three signaling pathways can contribute to GJ channel closure and downregulation of GJIC when Src is active (97, 141, 174, 178, 208). FIGURE 3 depicts the cross talk between PKC, Src, and MAPK in response to 12-*O*-tetradecanoylphorbol 13 acetate (TPA), an analog of the second messenger diacylglycerol (DAG) that is known to elevate levels of Cx43 phosphorylation and ubiquitination, or epidermal growth factor (EGF) treatment and activation of the EGF receptor (FIGURE 3). EGF-mediated activation of its receptor triggers activation of c-Src, which in turn mediates direct phosphorylation of Cx43 on Y247 and Y265 (Table 1; FIGURE 4). Both c-Src and v-Src can phosphorylate Cx43 directly on these residues and inhibit GJIC via a reduction of open-channel probability (155). The SH3 domain of v-Src has been shown to bind to the proline-rich region of Cx43 (P²⁷⁴TAPLSPMSPP²⁸⁴; discussed

FIGURE 5. Ubiquitin and ubiquitination of Cx43

A: ubiquitin targeting cascade. E1 (ubiquitin activating enzyme) binds to ubiquitin (Ub) in an ATP-dependent process. E1 transfers ubiquitin to E2 (ubiquitin conjugating enzyme), which moves the ubiquitin moiety to the lysine (K) of the target protein. E3 (ubiquitin ligase) is bound to the target protein and aids in the transfer of ubiquitin from E2 to the target substrate and in its covalent attachment. B: lysine residues of the ubiquitin amino acid sequence involved in the formation of polyubiquitin chains. Ubiquitin has 76 amino acids, eight of which are involved in forming polyubiquitin chains. Of the seven lysines (K), K6, K11, K29, and K48 linkages lead to proteasomal degradation, whereas K63 linkages lead to trafficking, endocytosis, endo-/lysosomal and phago-/lysosomal degradation, transcription, and DNA repair. The functions of K27 and K33 linkages have yet to be elucidated. In addition to the seven lysines, methionine 1 (M1) also can link ubiquitin moieties together to form linear chains. The COOH-terminal glycine (G) residue is responsible for the covalent linkage of the ubiquitin moiety to the lysine of the target protein. C: potential ubiquitination and SUMOylation sites in the human Cx43 polypeptide sequence. Twenty-four lysine residues (K) are potentially available in the cytoplasmically located domains (NH₂ terminus, intracellular loop, and COOH-terminal tail) of Cx43 for ubiquitination or SUMOylation (highlighted in red in the intracellular loop and COOH-terminal tail). Based on the structural organization of GJ channels discussed above, with the Cx NH₂ termini being located inside the channel (see FIGURE 2), lysine-residues in the NH₂ terminus are not likely to be ubiquitinated in assembled connexons or GJ channels and to serve as signals for GJ internalization and/or degradation. The lysine present at position 243 is not conserved and is an arginine (R) in rat Cx43. Lysine 9 and 144 are known sites of SUMOylation (90); and lysine 9 and 303 have been identified as potential ubiquitination sites (205). C was adapted from Ref. 190 and used with permission.

further in *GJ Internalization*), leading to phosphorylation of Y265. Subsequently, the SH2 domain of v-Src docks with the phosphorylated Y265, leading

to phosphorylation of Y247, causing channel closure (141, 208). Sorgen et al. (184) showed, using NMR studies, that binding of the SH3 domain of



Src to Cx43 could interfere with and partially disrupt the binding of the PDZ2 domain of ZO-1 to the extreme COOH-terminal end of Cx43 (see below). It was proposed that the disruption of this interaction was likely due to long-range conformational changes that affected residues A367–S372 that were caused by the upstream Src binding (to residues P274–P283) (184). Toyofuku et al. (194) showed that Src regulates the interaction between Cx43 and ZO-1 through Src-mediated tyrosine phosphorylation and the binding of its SH2 (and not of its SH3) domain to Cx43. This regulatory mechanism has not been found in Cx40, since binding of Src to Cx40 had no effect on the Cx40/ZO-1 interaction (16).

MAP kinases have been shown to phosphorylate Cx43 on S255, S279, S282 (206, 207), and S262 (174) (Table 1; FIGURE 4). As in the case of v-Src, phosphorylation by MAP kinases was found to cause channel closure through a reduction in open-channel probability (32, 208) (FIGURE 3). ERKs (ERK1, ERK2, and ERK5), p38, and JNK are all members of the MAP kinase family, and all have been reported to phosphorylate Cx43 (22, 148, 154). Unfortunately, most primary literature on MAP-kinase-mediated Cx phosphorylation ambiguously uses the term “MAPK” without defining precisely which MAPK was actually studied (206, 207). This caveat significantly hampers the interpretation of published observations, since ERKs are usually active under nonstressed conditions, whereas p38 and JNK are usually more active during oxidative stress (28). Most published work on MAP kinase-mediated phosphorylation of Cx43 demonstrated that treatment of cells with EGF or TPA, and subsequent activation of the EGF-receptor, led to a rapid disruption of GJIC, concomitant with an increase in Cx43 serine phosphorylation. Absence of Cx43-tyrosine phosphorylation demonstrated that the EGF receptor did not phosphorylate Cx43 directly but instead activated downstream MAP kinase cascades through Ras GTPases (106, 208) (FIGURE 3). Strong Cx43 phosphorylation by ERK2 (and weaker phosphorylation by p38 or JNK) was observed on S279 and S282 (114) (FIGURE 4). It was also found that ERK1/2 and p38 may regulate Cx43 function in cardiomyocytes upon activation of endothelin I and angiotensin II G-protein-coupled receptors (GPCRs) (154). Petrich et al. (148) demonstrated activation of JNK-mediated downregulation of Cx43 in cardiomyocytes, whereas work by Cameron et al. (22) revealed phosphorylation of Cx43 on S255 by Erk5 in EGF-treated cells (FIGURES 3 AND 4). As discussed previously for S262-phosphorylation by CDC2, phosphorylation of human Cx43 by MAP kinases on S262 also appears unlikely, since MAP kinases prefer to phosphorylate serine residues with a proline (and not a glutamine

[Q] as present in the human Cx43 sequence) located at the +1 position (S/T-P or P-X-S/T-P) (183) (Table 1; FIGURE 5C).

Activation of PKC has been shown to phosphorylate Cx43 on S368 and S372 (Table 1; FIGURE 4). PKC activity can be stimulated in more than one way: PKC can be phosphorylated and activated directly by Src, as well as through Src-mediated phospholipase C (PLC) activation and generation of DAG (141) (FIGURE 3). PKC-mediated phosphorylation of Cx43 has been reported to lead to decreased GJ assembly, downregulation of GJIC, and a reduced half-life of Cx43 (103). Closure of GJ channels after PKC-mediated Cx43 phosphorylation was demonstrated to result in a reduction in the unitary conductance rather than open-channel probability (103). A more recent study by Sirnes et al. (174) showed that, in TPA-treated cells, PKC was activated, triggering activation of the MAPK cascade. This study provided evidence that downregulation of GJIC was due not only to phosphorylation by PKC (on S368) but also through phosphorylation of Cx43 by MAPKs (on S255 and S262) (174) (FIGURE 3). Cx43 in TPA-treated cells also exhibits varying electrophoretic mobilities, with phosphorylated serine 368 (pS368) identified as the P0 form, pS255/pS279/pS282 as a P1 form, and pS262 as a P2 form (Ref. 180; reviewed in detail in Ref. 177).

In summary, it is important to emphasize that many of the Cx43 phosphorylation events have been shown to cause large conformational changes of the Cx43 COOH-terminal tail. For example, Solan et al. (181) found that a phosphomimetic mutation (S365D) at a PKA site had a dramatic effect on the structure of the Cx43 COOH terminus affecting far-upstream residues located in the polyproline-rich region that harbors AP-2 and Nedd4 binding sites (P²⁷⁴TAPLSPMSPP²⁸⁴; mentioned above and discussed in *GJ Internalization and Cx43 Ubiquitination*), and prevented phosphorylation of S368 by PKC. Also, as discussed above, Sorgen et al. (184) found that binding of Src to residues 274–283 apparently has a dramatic effect on ZO-1 binding that involves the last residues of Cx43 (S372–I382) (discussed in *Cx43/ZO-1 Binding*). Furthermore, using structure-specific antibodies, Sosinsky et al. (186) were able to demonstrate that the conformation (and likely the phosphorylation state) of S364 and S365 residues (PKA sites) is important for intracellular localization of Cx43, whereas the conformation of P375–D379 (near the Akt site S373) dictates the targeting of connexons into GJ plaques. All these results conform to the hypothesis that phosphorylation-induced conformational changes in the COOH-terminal tail lead to regulated protein/protein interactions during the Cx43 life cycle and may contribute to the modulation of GJ internalization and degradation. The

following sections will specifically discuss additional posttranslational Cx modifications (ubiquitination) and Cx-binding proteins (AP-2, Dab2, dynamin, ZO-1) that lead to downregulation of GJIC and (clathrin-mediated) GJ endocytosis.

GJ Internalization

GJs have a half-life of only 1–5 h (9, 13, 42), unexpectedly short for a structural membrane protein, suggesting that GJ channels and plaques are turned over constantly. This assumption has been verified by various life-cell techniques that showed that newly synthesized channels are accrued to the outer edges of existing GJ plaques, whereas older channels are simultaneously removed from plaque centers (40, 52, 107). Interestingly, when connexons of two opposing cells have docked to form complete double-membrane-spanning GJ channels and plaques, they were found to be inseparable under physiological conditions (53, 63). To remove their PM GJs, cells were observed to internalize portions of or entire plaques (40, 80, 152). GJ internalization generates cytoplasmic double-membrane vesicles, termed annular GJs (AGJ) or connexosomes. GJ internalization is supported by numerous earlier ultrastructural analyses that detected double-membrane AGJ vesicles in the cytoplasm of cells *in situ*, especially occurring in differentiating tissues and sometimes in association with lysosomes (59, 69, 80, 105, 108, 126, 149, 169). Importantly, recently, these dynamic GJ assembly and internalization observations have also been revealed to occur at a vertebrate electrical synapse *in vivo* as well as in an established GJ assembly system with experimentally re-aggregated cells in culture, using modern freeze-fracture replica immunolabeling (FRIL) methods (46, 78). Potential alternative mechanisms of GJ removal, such as GJ splitting or GJ dispersal (104), have not been observed by others or by us in life-cell recordings and, therefore, are not considered efficient mechanisms of GJ removal.

Clathrin-Mediated GJ Internalization

Removal of GJ plaques from the PM is a complex cellular process. It has been described to involve clathrin and other relevant endocytic proteins (40, 45, 65, 152). By immunofluorescence staining, Piehl et al. (152) demonstrated that Cx43-GFP-based GJs colocalize with key components of the clathrin-mediated endocytosis (CME) pathway, including clathrin, the clathrin adaptors AP-2 and Dab2, dynamin2, myosin VI, and actin. In addition, when Gumpert et al. (65) knocked down the expression of clathrin, AP-2, Dab2, and dynamin2, a significant reduction in the number of internalized AGJ vesicles and an overall increase of GJ plaque

size was observed. Together, these results provide strong evidence to support the role of CME in GJ internalization. More recently, we demonstrated that Cx43-GFP co-immunoprecipitates with clathrin heavy chain, AP-2, Dab2, and dynamin2. By mutating the potential AP-2 binding site motifs (YXX Φ ; see below) in Cx43 and transiently expressing these constructs in HeLa cells, we found that these Cx43 mutants exhibit longer protein half-lives and insufficiently internalize GJ plaques (Fong J, Falk M, unpublished data). Recently, a report describing a Cx43 COOH-terminal domain comprised of residues cysteine 271 to asparagine 302 playing a significant role in GJ internalization has been published (209). This report is consistent with our own findings described above.

CME has been studied extensively, and it is the best-characterized endocytosis pathway. Clathrin, besides its classical role in the endocytosis of vesicles from the PM, also has been implicated in the internalization of large membrane structures, such as pathogens, latex beads, and GJs (reviewed in Ref. 146). In CME, clathrin adaptors are required to bridge and link cargo (e.g., membrane proteins) with clathrin coats. Adaptor protein complex 2 (AP-2), a classical clathrin adaptor that functions on the PM, is a hetero-tetrameric protein complex composed of α -, β 2-, μ 2-, and σ 2-subunits. The NH₂-terminus of the α -subunit binds to the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) to anchor AP-2 on the PM (30, 51), whereas its COOH terminus can interact with other endocytic accessory proteins such as Hip1/Hip1R, AP180/CALM, Epsin, NUMB, Dab2, and Eps15 (156, 175, 196, 197). The β 2-subunit interacts with the terminal domain (TD) of clathrin heavy chain and clathrin-adaptors such as ARH and β -Arrestin (156, 175, 196, 197). The μ 2-subunit directly interacts with the cargo via tyrosine-based sorting signals (YXX Φ and NPXY, with X being any amino acid residue and Φ being a residue with a bulky hydrophobic side chain such as L, W, V, F, M, or I) and dileucine-based sorting signals ([D/E]XXXL[L/I] that also can bind to the β -subunit) to recruit clathrin (15). The μ 2-subunit is also found to bind to PIP₂. The σ 2-subunit does not seem to participate in clathrin-coat assembly but appears to serve as a structural subunit for the AP-2 protein complex (15).

Three potential AP-2 binding sites (YXX Φ) are present in the COOH-terminal tail of Cx43 (Y²³⁰VFF, Y²⁶⁵AYF, and Y²⁸⁶KLV; the human sequence is represented) and in the COOH-terminal domain of many other Cxs (FIGURE 4; Table 2). Y²³⁰VFF is located immediately adjacent to the PM, and thus it is unlikely for AP-2 to interact with Cx43 via this signal (YXX Φ signals in general are located 10–40 amino acid residues away from transmembrane

regions) (15). No dileucine-based sorting signal is found in cytoplasmically located domains of Cx43 (however, dileucine-based sorting signals are present in the Cx32 COOH-terminal tail and in the COOH-terminal domain of a few other Cxs) (Table 2). Disabled 2 (Dab2), an alternative clathrin adaptor that has been shown to efficiently bind to Cx43-based GJs (65, 152), is also known to interact with cargo via the NPXY consensus sequence (15). No NPXY signals are found in Cx43 or in any other Cx (NPXY signals are generally only found in single-spanning membrane proteins) (15). Still, cryptic PXY sequence motifs are present in the Cx43 COOH terminus and in the COOH terminus of eight other Cxs (152). However, substituting N, P, or Y by alanine in the NPXY signaling motif was found to abolish function (15), making it less likely that Dab2 interacts directly with the Cx protein sequence. On the other hand, Dab2 can also interact with the α -subunit appendage of AP-2 via its D²⁹³PFRDDPF sequence (130) and its F⁴⁸⁰LDLF (FXDXF) motif (17). Dab2 is also known to interact with the retrograde actin motor myosin VI (2, 34), and recruiting myosin VI to newly forming AGJ vesicles for efficient transport deeper into

the cytoplasm for subsequent degradation might be Dab2's primary role in GJ internalization (152). Thus Dab2 may be recruited to internalizing GJs either via its interaction with AP-2, PIP₂, or clathrin.

The GTPase activity of dynamin2 (Dyn2) has also been found to be involved in GJ endocytosis by completing AGJ vesicle budding. Cx43-GFP GJ plaque endocytosis was significantly inhibited when cells were either transfected with a dominant-negative form of Dyn2 (Dyn2K44A) or treated with the dynamin GTPase inhibitor, dynasore, or the non-hydrolysable GTP analog GTP- γ S (57, 65). In addition to the conventional functions of pinching the coated vesicle off the PM, more recently, Dyn2 has also been proposed to play a role in controlling Cx43 GJ plaque recycling and degradation by interacting with Cx43 (57). Dyn2 was found to traffic from the cytosol to the PM and interact with Cx43 at an early stage of membrane invagination. Based on these observations, it is reasonable to hypothesize a role for Dyn2 in mediating GJ plaque curvature.

Clathrin-Independent GJ Internalization

Beside the role of AP-2, Dab2, dynamin2, and myosin VI in CME of GJs, Eps15 (another multifunctional endocytic adaptor protein) was reported to interact with ubiquitinated Cx43 and to mediate GJ plaque internalization (23, 60). The proline-rich PY motif (XP²⁸³PXY) in the Cx43 COOH-terminal domain was reported to interact with the ubiquitin ligase, Nedd4, via its WW domains (115) (FIGURE 4). Girao et al. (60) found that once ubiquitin is conjugated to Cx43, ubiquitinated Cx43 is recognized by Esp15 through its ubiquitin-interacting motif, proposed to result in its internalization. These findings suggest that cells may use more than one pathway for GJ internalization. Different upstream cell signaling events and resulting modifications of Cxs in GJ plaques may determine which pathway cells ultimately pursue to efficiently internalize GJs (see *Cx43 ubiquitination* for more details). Interestingly, the PY motif in Cx43 is overlapping with the YXX Φ -tyrosine-based sorting signal (P²⁸³PGYKLV²⁸⁹), and Thomas et al. (193) found that substituting P²⁸³ with leucine (L) in the PY motif resulted in a modest increase in the Cx43 steady-state pool by 1.7-fold. In contrast, substituting V²⁸⁹ of the tyrosine-based sorting signal with aspartic acid (D) resulted in a more significant, 3.5-fold increase. No additive effect was found in either Cx43 double mutant P²⁸³L/V²⁸⁹D or Y²⁸⁶A (Y286 is crucial for both the PY motif and the tyrosine-based sorting signal), supporting a predominantly CME-based mechanism for GJ internalization.

Cx43 and other Cx isoforms (Cx32, Cx36, and Cx46) have also been reported to associate with Caveolin-1, suggesting the possibility of Cx proteins to localize

Table 2. Tyrosine- (YXX Φ) and dileucine-based ([D/E]XXXL[L/I]) sorting signals of the human Cx protein family in cytoplasmically located Cx domains (NH₂ terminus, intracellular loop, and COOH terminus)

Cx	YXX Φ	[D/E]XXXL[L/I]
Cx23	Y¹¹²TII; Y²⁰⁰FPF*	None
Cx25	None	None
Cx26	Y²¹²LLI	E²⁰⁹LCYLL
Cx30	Y²¹²LLL	E²⁰⁹LCYLL; E²⁴³MNELI
Cx30.2	Y ⁹⁷ HVI	E ¹¹¹ EETLI
Cx30.3	Y ¹⁶ STV; Y ¹¹⁷ DNL	None
Cx31	Y ¹⁶ STV; Y ¹¹⁷ DNL	None
Cx31.1	Y ⁹⁷ REV	None
Cx31.9	None	E²⁰⁸LGHLL; E²³³AOKLL
Cx32	Y ⁷ TLL; Y²¹¹LII	E²⁰⁸VYLI; E²⁴⁷INKLL
Cx36	Y ¹⁰⁹ STV	None
Cx37	Y²⁶⁶LPV; Y²⁸¹NGL	E²²⁷LVHLL
Cx40	None	None
Cx40.1	None	E ² GVDLL
Cx43	Y²³⁰VFF; Y²⁶⁵AYF; Y²⁸⁶KLV	None
Cx45	Y ¹⁴¹ PEM; Y ³⁰¹ TEL	None
Cx46	None	None
Cx47	Y³³⁰SLV	None
Cx50	Y⁹⁹VRM; Y²⁶⁶QLL; Y²⁷⁸FPL; Y³²³AQV	None
Cx59	Y²⁸⁴NLL; Y²⁹⁷PSL	None
Cx62	Y⁵¹³VCV	None

*Sorting signals in the COOH-terminal domain are highlighted in bold.

within lipid rafts (119, 165). However, in general, GJs are much larger than lipid rafts, and currently it is not clear whether alternative clathrin-independent GJ internalization pathways, such as caveolae-dependent endocytosis, exist. Apparently, cells have developed numerous independent endocytic pathways, making such a hypothesis feasible. In the following sections, we will discuss ubiquitination and the binding of Cx43-protein partners such as ZO-1 that may “flag” and “render” Cx43 polypeptides in plaques to bind endocytic proteins and thus may lead to the internalization and degradation of GJs.

Cx43 Ubiquitination

Ubiquitin is a small, long-lived 76-amino acid protein that is covalently linked to lysine residues of target proteins as a posttranslational modification signaling for trafficking, degradation, transcription, and even DNA damage repair (reviewed in Refs. 49, 92, 213). Linkage of ubiquitin to a target protein (and removal by deubiquitinases for attachment of the ubiquitin moiety to another target protein) involves multiple enzymes (FIGURE 5A). The activating enzyme E1 initially binds to ubiquitin in an ATP-dependent manner. Then E1 aids in transferring the ubiquitin moiety to a cysteine residue of the conjugating enzyme, E2, by the COOH terminus of ubiquitin. The ligating enzyme E3, which is bound to E2 and the target protein, finally facilitates the translocation of ubiquitin from E2 to a lysine residue of the target protein (FIGURE 5A). This linkage is mediated via the COOH-terminal glycine of ubiquitin and a specific lysine of the target protein. Ubiquitin can be linked to target proteins as a single ubiquitin, multiple monoubiquitins, or polyubiquitin chains. Ubiquitin itself has seven lysine residues, Lys 6, 11, 27, 29, 33, 48, and 63, with lysines 48 and 63 being the best characterized (92) (FIGURE 5B). The first ubiquitin residue, methionine 1, is also known to link linear chains of ubiquitin together. Each one of the above-mentioned residues is capable of binding other ubiquitin moieties to form polyubiquitin chains. These chains can be linear or branched, adding to the complexity of protein ubiquitination (49, 213). Lysine 6, 11, 29, and especially 48 linkages are known as signals targeting to the 26S proteasomal pathway of protein degradation (Refs. 10, 204, 214, 216; reviewed in Ref. 49), whereas lysine 63 linkages represent signals for protein trafficking and endocytosis (signaling to clathrin-mediated endocytosis) (7, 12, 66, 123), targeting to endo-/lysosomal (70, 71, 171) and phago-/lysosomal degradation (14, 29, 142), and DNA repair and transcription (reviewed in Refs. 27, 49, 92) (FIGURE 5B).

Cx43 has 23 lysine residues in its cytoplasmic domains (3 in the NH₂ terminal, 11 in the intracellular loop, and 9 in the COOH terminal), and all of these can potentially be linked to ubiquitin moieties (FIGURE 5C). However, based on structural characteristics discussed above (*The 3D Structure of a GJ Channel Provides Functional Cues Relevant to GJ Biosynthesis and Turnover*), ubiquitination of NH₂-terminal lysines in assembled connexons and GJ channels appears unlikely. Laing and Beyer, and others (60, 95, 110–112) have shown that Cx43 polypeptides can be posttranslationally modified by ubiquitin, but the details of the linkages of ubiquitin and what these different linkages mean for Cx43 function have yet to be elucidated.

Another protein similar in structure to ubiquitin, SUMO (small ubiquitin-like modifier), can also be added to lysine residues of target proteins. Recently, Cx43 has also been shown to interact with SUMO 1, 2, and 3 via lysine 144 (located in the intracellular loop) and lysine 237 (located in the COOH-terminal domain), which led to increased GJIC (90) (FIGURE 4 AND 5C). Since ubiquitin and SUMO both require covalent bonds to lysine residues in the target protein, there is increasing evidence that SUMO and ubiquitin can co-regulate substrate proteins as well as cross regulate each other's SUMO/ubiquitin-targeting cascade (reviewed in Ref. 212).

Ubiquitination Signaling Proteasomal Degradation of Cx43 Polypeptides

The proteasome is a cytoplasmic, 26S protein complex that degrades individual, unfolded polypeptides in an ATP-dependent manner. Target proteins with lysine 48-linked polyubiquitin chains attached are recruited to the proteasome, unfolded, and finally degraded into small peptide fragments by “spooling” the protein through the 20S core of the proteasome (reviewed in Ref. 61). Cx43 polypeptides have been found to be ubiquitinated in a proteasome-dependent manner and to be degraded by the proteasome (95, 96). It should be mentioned here that, based on the structure and digestive mechanism of the proteasome, internalized GJs due to their complex architecture do not qualify as targets for proteasomal degradation. Nevertheless, Musil and coworkers found that cross talk exists between proteasomal degradation and PM GJ plaques, since inhibition of proteasomal degradation resulted in an increased amount of GJ plaques in the PM (135, 201, 202). More recently, Cx43 has been shown through mass spectrometry of trypsin-digested cell lysates to be ubiquitinated on lysine 9 and lysine 303 (205) (FIGURE 5C). However, ubiquitination of Cx43 for proteasomal degradation may not be required, since Cx43 interaction with the ubiquitin-like protein CIP75

has been shown recently to also target non-ubiquitinated Cx43 to proteasomal degradation (Refs. 36, 190; reviewed in Refs. 188, 189). CIP75, or connexin interacting protein of 75 kDa [which is 100% identical to the mouse UBIN protein (188, 189), a member of the ubiquilin family], has a UBL and a UBA domain (ubiquitin-like and ubiquitin-associated, respectively), and CIP75 has been shown to interact with ER-associated Cx43. Taken together, these findings suggest that different Cx43 ubiquitin-modifications likely serve as signals for both proteasome-mediated Cx43 polypeptide degradation (e.g., lysine 48-linked) as well as GJ internalization and subsequent degradation (e.g., lysine 63-linked).

Ubiquitination Signaling Internalization of Cx43-Based GJs

As described in *GJ Internalization*, internalization of Cx43 GJs has been shown to be dependent on tyrosine 286 of the tyrosine-based sorting signal YXX_p (193), allowing GJs to be targeted to CME, an endocytic process that, as mentioned above, does not require but can be linked to ubiquitination (7, 12, 66, 123). However, Cx43 assembled in GJ plaques at the PM also has been shown to be ubiquitinated (23, 50, 60, 109–111, 162), and Girao et al. (60) gained evidence that Cx-ubiquitination can also result in the internalization of GJs in an Eps15-dependent manner. Recently, Catarino et al. (23) obtained further evidence suggesting ubiquitination as an additional GJ internalization signal based on the observation that a mutation of tyrosine 286 significantly increases the half-life of Cx43 at GJ plaques. Interestingly, as discussed in *GJ Internalization* above, tyrosine 286 of Cx43 overlaps the PY recognition motif of the E3 ubiquitin ligase Nedd4–1 (discussed below) (FIGURE 4). Taken together, these findings imply that the internalization of Cx43 may not solely be based on the tyrosine-based sorting signal but also on the ubiquitination status of Cx43 at GJ plaques. A similar observation has been made for the EGF receptor that can switch between CME and a CME-independent internalization process. Unlike CME, the CME-independent pathway is ubiquitin-mediated and lipid raft-dependent, and the EGF receptor concentration present in the experiments determines which pathway is ultimately chosen (1, 172).

Ubiquitination of Cx43 signaling GJ internalization, as well as the factors that bind to and traffic internalized GJ plaques to degradation are not yet understood well; however, accessory proteins interacting with ubiquitinated Cx43 have been found in recent years that provide clues as to how this process might be mediated. As mentioned above, Cx43 contains a PY motif (XPPXY, ²⁸²S-P-P-G-Y²⁸⁶)

in its COOH-terminal domain. The E3 ligase, Nedd4–1 has three WW domains (WW1, 2, and 3) and binds to the PY motif of Cx43 via its WW2 domain (115). E3 ligases have been classified into three major families: HECT, RING, and U-box (Ref. 151; also see Ref. 50). Nedd4 and Smurf2 (see below) are members of the HECT family. WW domains are protein-protein interaction modules of 38–40 residues in length containing two highly conserved tryptophan residues and a conserved proline residue (26, 191), and according to their ligand preference have been grouped into four classes (87). Within the PY motif of Cx43, proline 283 has been shown to be responsible for this Nedd4-1 interaction (60). Additionally, Nedd4-mediated ubiquitination of Cx43 is necessary for subsequent Eps15 interaction (23). Eps15 is an endocytic protein that harbors a ubiquitin-binding domain. This UIM (ubiquitin interacting motif) domain of Eps15 is responsible for binding to Cx43 (60). On the other hand, in cells that were treated with TPA, the E3-ligase Smurf2 (Smad ubiquitination regulatory factor 2) was recently found to bind to and interact with phosphorylated and ubiquitinated Cx43 (50). The knockdown of Smurf2 with and without the addition of TPA has been shown to increase Cx43 protein levels at GJ plaques and GJIC (50). However, the exact role of Smurf2 in regulating the internalization and degradation of Cx43 GJ plaques is not yet known.

How ubiquitinated Cx43 GJs are trafficked once internalized is also being elucidated. Tsg101 is a member of the ESCRT (endosomal sorting complex required for sorting) machinery that also binds to intracellular Cx43. As a member of the ESCRT-1 complex (one of the four ESCRT complexes; reviewed in Ref. 159), Tsg101 is responsible for sorting ubiquitinated cargo for lysosomal degradation. It directly binds to Cx43 and has a role in Cx43 internalization and degradation, although the ubiquitination and phosphorylation status of Cx43 is unknown (3). Tsg101 and Hrs (an ESCRT-0 complex protein) have both been found to colocalize with Cx43 intracellular vesicles during TPA-treatment and the subsequent depletion of both proteins leads to an increase of the Cx43-phosphorylated P2 isoform (109). Taken together, these observations might indicate that Nedd4-ligase and Eps15 ubiquitinate, internalize, and target Cx43-based GJs to autophagic degradation (11), whereas HRS, TSG101, and Smurf2-ligase target internalized Cx43-based GJs to endo-/lysosomal degradation (see below). It is tempting to speculate that targeting to these two different cellular degradation pathways could be dependent on the levels of Cx43 phosphorylation and/or ubiquitination, since it is known that TPA, an analog of DAG, results in elevated Cx43

phosphorylation and ubiquitination (see Ref. 41 for further discussion).

Ubiquitination Signaling Degradation of Cx43-Based GJs

As described above, proteasomal (ubiquitin-lysine 48-linked, CIP75-mediated) and endo-/lysosomal degradation (e.g., ubiquitin-lysine 63-linked, HRS/Tg101, Smurf2-mediated) of Cx43 polypeptides and GJ plaques has been demonstrated (Refs. 50, 95, 96, 109–111; reviewed in Refs. 41, 89, 113). More recently, molecular evidence for the autophagic degradation of internalized Cx43-based GJs has been discovered (11, 47, 69, 118). Macroautophagy (or simply autophagy) is a cellular degradation system that is designed specifically for the degradation of cytoplasmically localized protein aggregates, multi-protein complexes, and cytoplasmic organelles. These structures are engulfed by newly formed membrane vesicles that fuse with lysosomes for content degradation. Since internalized AGJ vesicles are cytoplasmic, highly oligomeric, multi-subunit protein complexes, they qualify as likely targets for autophagosomal degradation. Historically, autophagy has been known as a cellular degradation pathway that is essential for cell survival following nutrient depletion. However, substantial research over the past decade has indicated that autophagy represents a much more common and highly conserved degradation pathway that is active at all times of physiological and pathological stages of cellular life (Refs. 14, 72, 153, 160; reviewed in Ref. 41). Remarkably, although autophagic degradation of GJs had been described quite some time ago in several ultrastructural analyses of various cells and tissues *in situ* including heart, dermis, and liver (108, 126, 149, 169), until recently, not much attention was attributed to this fundamental GJ degradation pathway. Autophagic degradation of GJs plays a significant role in the regulation of GJ function. Inhibition of cellular autophagy increases GJIC, prevents internalization of GJs, slows down the degradation of Cxs, and causes cytoplasmic accumulation of internalized AGJ vesicles *in situ*, as well as in cultured cells expressing either endogenous or exogenous Cx proteins (11, 47, 118). Under normal, unstarved conditions, internalized Cx43-based GJs are degraded by autophagy and colocalize with multiple autophagy markers (such as LC3, p62, and Beclin1) in HeLa and primary endothelial cells (11, 47, 118). Upon inhibition of autophagy by pharmacological means, or by protein knockdowns, the number of internalized AGJ vesicles was shown to increase significantly (11, 47). Under starvation conditions, Cx43-based AGJ vesicles are degraded by autophagy, as indicated by colocalization of AGJs with LC3-positive autophagosomes. This is also

evidenced by an increase in Cx43 protein levels when lysosomal function was inhibited or when autophagy-essential proteins (such as ATG5) were knocked down (118). Autophagic degradation of Cx43 is also evident in diseased states, as ailing canine myocardium exhibits increased autophagic degradation of internalized, Cx43-based AGJ vesicles (69). Furthermore, internalized Cx43-based AGJ vesicles were found to colocalize with a protein, termed p62 (also called sequestosome1 or SQSTM1) (11, 47, 118). p62/SQSTM1 is a ubiquitin-binding protein that recognizes and interacts via its UBA-domain with polyubiquitinated proteins (29, 167, 211) and delivers poly-ubiquitinated (Lys63-linked) oligomeric protein complexes to the autophagic degradation pathway (14, 142). The findings that Cx43-based GJs can become ubiquitinated, the known affinity of p62/SQSTM1 for ubiquitinated protein complexes, its colocalization with PM-localized GJs in HeLa, COS7, and PAE cells (11, 47, 118) suggests that Nedd4 may ubiquitinate Cx43 at GJ plaques, leading to recruitment of Eps15 and GJ internalization, and that these initial endocytic events are followed by the interaction of ubiquitinated AGJ vesicles with p62 for sequestration and targeting for subsequent autophagic degradation (11, 41, 47).

From the observations described above, it is clear that ubiquitination of unassembled and assembled Cxs plays an important role in their functional control. Besides signaling for proteasomal degradation of Cx polypeptides, mounting evidence suggests that ubiquitination of Cxs assembled in GJ plaques serves as an important signal regulating GJ internalization and degradation by endo-/lysosomal and phago-/lysosomal pathways. Much future research is required to determine the potentially numerous types (multiple mono-ubiquitins, Lys48- and Lys63-linked poly-ubiquitins, etc.) and functions of Cx ubiquitin and ubiquitin-like signals in the maintenance and degradation of GJs (also see Refs. 68, 79, 89, 113, 189 for recent reviews that discuss Cx-ubiquitination).

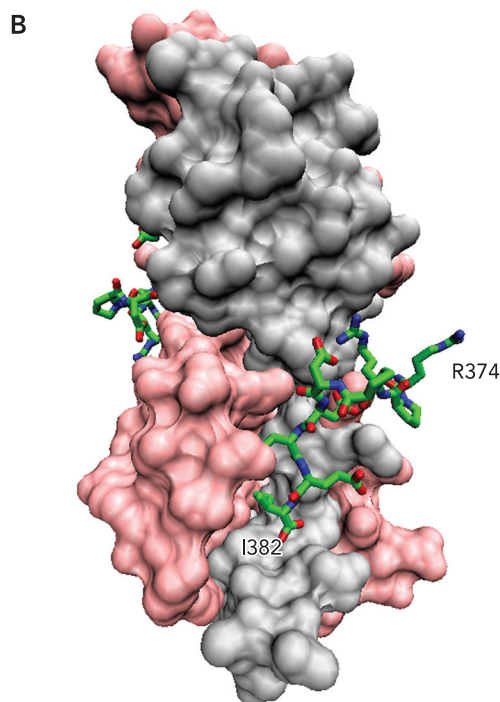
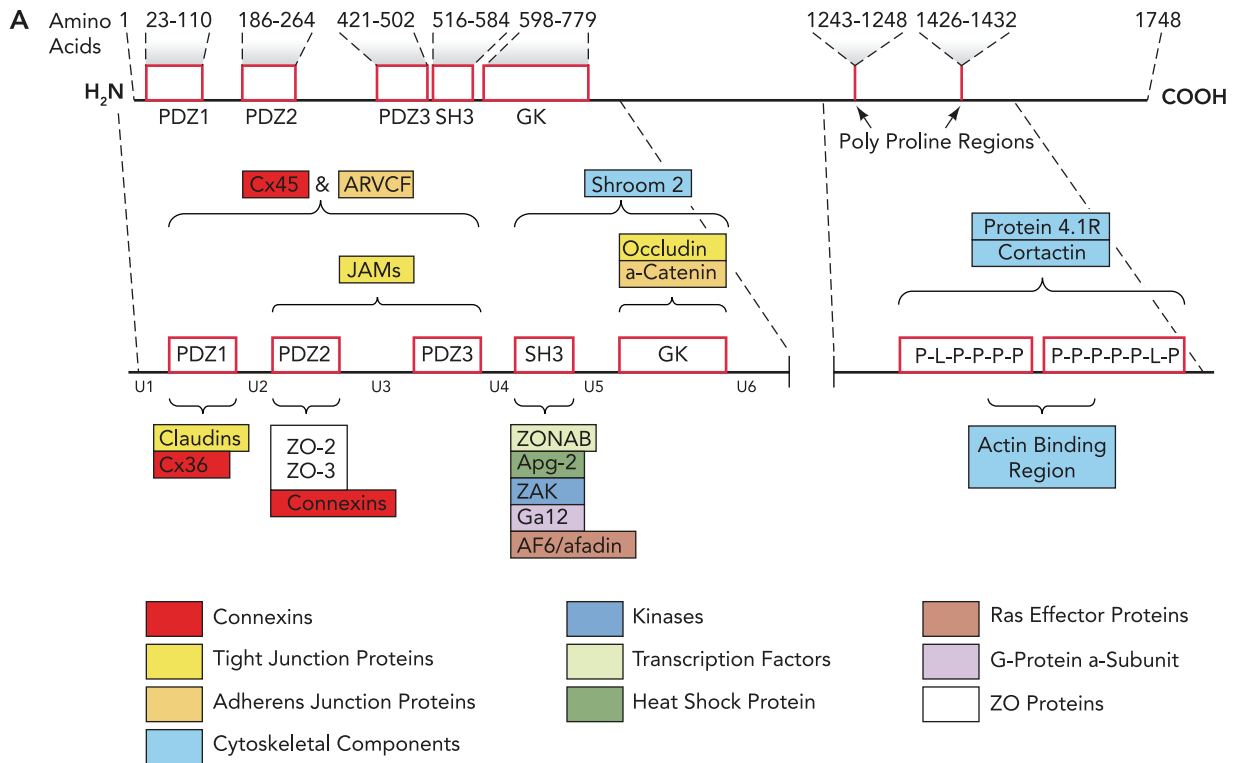
Cx43/ZO-1 Binding

Zona occludens-1 (ZO-1) is a very large, 220-kDa protein (almost the size of an entire Cx43-based connexon) that has emerged as a prominent player in GJ regulation because of its established interaction with Cx43 (55, 195). ZO-1, together with ZO-2 and ZO-3, are members of the membrane-associated guanylate kinase (MAGUK) family, scaffolding proteins that aid in the assembly of multi-protein complexes at PMs via their numerous protein interaction sites. ZO-1 contains three NH₂-terminal PDZ domains, followed by an SH3 domain, an inactive guanylate kinase (GK) domain, and two

polyproline regions (FIGURE 6A). PDZ-domains are 80- to 90-amino acid-long protein domains that generally bind the very COOH-terminal residues of target proteins (182). The interaction between Cx43 and ZO-1 was originally shown by Giepman and Moolenaar (55) and Toyofuku et al. (195) in 1998 to be through ZO-1's second PDZ domain, and, since then, numerous other Cxs have been shown to interact with ZO-1 via the PDZ2

domain (68) (Table 3), with the notable known exceptions being Cx36, which seems to only interact with ZO-1's PDZ1 domain (117, 120), and Cx45, which is reported to interact with all three ZO-1 PDZ domains (86).

As expected, ZO-1 interacts through the extreme COOH terminus of Cx43, with the last four residues being the most important (77) (FIGURES 4 AND 6B). Jin et al. (77) further demonstrated the importance



not only of the last four amino acids but of the very end, zero-position isoleucine (I382) and showed through yeast two hybrid screens that deletion of this amino acid would result in Cx43 being unable to bind to ZO-1. Chen et al. (27) later firmly demonstrated the need for an undisturbed Cx43 PDZ motif by solving the crystal structure of isolated PDZ2 domains in the presence of a peptide that represented the last nine amino acid residues of Cx43. These analyses revealed a hydrophobic cleft (or pocket) in ZO-1's PDZ2 domain that specifically accommodates a hydrophobic Cx43 COOH-terminal end (27) (FIGURE 6B). These analyses further revealed that any structural alterations of the Cx43 COOH terminus (such as amino acid deletions within the binding site or additions as is the case in COOH-terminal fluorescence protein or otherwise COOH-terminal-tagged Cxs) would disturb the highly specific Cx43/ZO-1 interaction (FIGURE 6B). The Cx43/PDZ2 interaction requires that two ZO-1 proteins undergo a structural dimerization known as "domain swap" as they bind two separate Cx43 COOH-terminal peptides (FIGURE 6B), resulting in a structural assembly that is believed to make the PDZ2/Cx43 interaction Cx43 specific (27). Notably, these results indicate that at least two ZO-1 proteins will simultaneously interact with a connexon of a GJ channel, a 440-kDa complex that dwarfs the size of the underlying bound connexon, a remarkable structural arrangement that needs to be considered when investigating GJ/binding partner interactions. Interestingly, peptide binding experiments showed that substituting S373 with glutamine (E) caused a sevenfold decrease in PDZ2 binding affinity, whereas glutamine at *position 372* resulted in a more moderate, 2.5-fold decrease, offering a potential control mechanism of ZO-1

binding through Cx43 phosphorylation (likely by Akt or PKA) (27).

Most Cxs that have been shown to bind to ZO-1 contain a variation of a PDZ binding motif referred to as *class 1* and *class 2* PDZ binding motifs (see below), with Cx30, Cx32, and Cx36 as potential exceptions (Table 3). Cx32's (and potentially Cx30's and Cx36's) proposed interaction with ZO-1 has created somewhat of a quandary in the GJ field. Cx32's last four amino acids, -C-S-A-C-COOH, do not conform with known *class 1* (-X-S/T-X-V/I/L-COOH) or *class 2* (-X-V/I/L-X-V/I/L-COOH) PDZ-binding consensus sequences (68, 182), since it conspicuously lacks a valine (V), isoleucine (I), or leucine (L) residue at its zero position, yet it is widely believed to interact with ZO-1 (Table 3). Cx32 was first shown to interact with ZO-1 in immunofluorescence colocalization and co-immunoprecipitation (Co-IP) experiments by Kojima et al. (91) in rat hepatocytes. Li et al. (116) also showed immunofluorescence colocalization of Cx32 with ZO-1 in mouse hippocampus and thalamus cells. However, yeast two hybrid screens of the Cx32 COOH terminus (*residues 220–283*) and the PDZ2 domain of ZO-1 (*residues 163–310*) did not confirm an interaction between these two domains (77). One possibility is that the Co-IP conducted by Kojima et al. (89) may not have shown a direct interaction between Cx32 and ZO-1. It may merely have demonstrated that Cx32 interacts with a larger protein complex, of which ZO-1 is a member. This notion may be supported by the observation of Wu et al. (215) who reported 1) evidence for the formation of a catenin/ZO-1/Cx43 complex in rat cardiomyocytes and 2) that binding of catenins to ZO-1 is required for Cx43 transport to the PM during the assembly of GJs. Alternatively, Cx32 may

FIGURE 6. ZO-1 and its interaction with Cx43

A: the known protein-binding domains of ZO-1 drawn to scale highlighting specific binding partners. The 220-kDa scaffolding protein ZO-1 consists of 1,748 amino acid residues. It includes three NH₂-terminal PDZ domains, followed by an SH3 domain, an inactive guanylate kinase (GK) domain, and two further COOH-terminal polyproline regions (shown as red boxes). Regions labeled "U1" through "U6" located between these domains denote regions that are unique to the ZO-1 protein sequence (8). Connexins (labeled red) known to bind to ZO-1 preferably bind to the PDZ2 domain, with the notable exceptions of Cx36, reported to only bind to the PDZ1 domain (117), and Cx45, reported to bind to all three PDZ domains (86) (also see Table 3). ZO-1's tight junction partners (shown in yellow) include claudins, which bind to PDZ1 (75, 217), JAMS, which attach to PDZ2 and PDZ3 (37), and occludins, which bind to the GK domain (43, 164). Adherens junction proteins (shown in orange), ARVCF, and α -catenin bind to the PDZ domains (85) and to the GK domain (131), respectively. The cytoskeletal component (light blue) Shroom2 binds to the SH3 and GK domains (39). Other cytoskeletal components, namely cortactin (84), protein 4.1R (125), and actin (44), bind to the polyproline regions. The SH3 domain is the interaction site for the transcription factor ZONAB (6) (light green), the heat shock protein Apg-2 (198) (dark green), the ZO-1-associated kinase-1 (ZAK) (5) (dark blue), the G-protein α -subunit G α 12 (127) (gray), and the Ras effector AF6/afadin (139) (brown). ZO-1 can also bind to other ZO proteins (ZO-2, ZO-3) via its PDZ2 domain (200) (white). (References 8 and 62 provide additional useful information on ZO-1 binding partners.) B: crystal structure of the ZO-1 PDZ2/Cx43 peptide-complex solved by Chen et al. (27). Each PDZ2 domain monomer is shown as a space-filling model (pink and gray), whereas the two nine-residue-long COOH-terminal Cx43 peptides ($-^{374}\text{RPRPDDLEI}^{382}\text{-COOH}$) (one seen in the front, whereas the other one can be partially seen in the back) are shown as stick models (green). The "domain-swapped" dimer of the two PDZ2 domains forms two specific binding clefts (or pockets) for the two Cx43 peptides, indicating that deletions within this region, as well as any additional amino acids/protein tags added to the COOH terminus of Cx43 will interfere with its ZO-1 binding. The figure was generated from the Protein Data Bank accession file 3CY7 using VMD software, version 1.8.7 for Macintosh. B was adapted from Ref. 27 and used with permission.

Table 3. Predicted and experimentally found human (mouse) connexin/ZO-1 interactions

Connexin	COOH-Terminal aa Sequence*	Predicted to Bind ZO-1/PDZ2†	Experimental Findings	References
Cx23	LFKR	No		
Cx25	<u>VLSV</u> (2)	Yes		
Cx26	KKPV	No	No	91
Cx30	GFPS	No	Unclear	146a, Yes; 158a, No
Cx30.3	GGYP	No		
Cx31	<u>LTPI</u> (1)	Yes		
Cx31.1	<u>KTIL</u> (1)	Yes		
Cx31.3	PRDA	No		
Cx31.9	<u>DLAI</u> (2)	Yes	Yes	135b
Cx32	<u>CSAC</u>	No	Unclear	91, 116, Yes; 77, No
Cx36	<u>SAYV</u>	No‡	Yes	117
Cx37	<u>KQYV</u>	No		
Cx40	<u>DLSV</u> (2)	Yes	Yes	16
Cx40.1	<u>SEWV</u>	No		
Cx43	<u>DLEI</u> (2)	Yes	Yes	55, 77a, 184, 195
Cx45	<u>SVWI</u> (2)	Yes§	Yes	86, 95a
Cx46	<u>DLAI</u> (2)	Yes	Yes	135a
Cx47	<u>TVWI</u> (2)	Yes	Yes	116
Cx50	<u>DLTV</u> (2)	Yes	Yes	24
Cx59	<u>DLQI</u> (2)	Yes		
Cx62	KFNS	No		
mCx33	<u>SVCI</u> (2)	Yes	Yes	22a

*Amino acid residues that conform with residues of known class 1 and class 2 PDZ-binding consensus sequence motifs are shown in bold and underlined. Class 1- or class 2-type motifs are given in brackets. †Based on the presence of amino acid residues on positions 0 and -2 conforming with the two known PDZ-binding consensus motifs (class 1: -X-**S/T**-X-**V/I/L**-COOH; class 2: -X-**V/I/L**-X-**V/I/L**-COOH). ‡Cx36 only binds to the PDZ1 domain and not to the PDZ2 domain of ZO-1. §Cx45 can bind to all three PDZ domains.

interact directly with ZO-1 but via a site that is different from the assumed PDZ2 domain. Indeed, Duffy et al. (35) showed with yeast two hybrid screens that Cx32 could, noncanonically yet directly, interact with the SH3 domain of Dlg1, another MAGUK family member, leaving open the possibility of alternative yet direct interactions between Cx32 and ZO-1. The COOH terminus of Cx36 (-SAYV-COOH) also does not conform to either class 1 or 2 binding sites normally associated with Cx/ZO-1 interaction. However, many claudins associate with ZO-1 via a COOH-terminal “YV” motif that is also present in Cx36 (217). Since claudins and Cx36 both associate with ZO-1 through the PDZ1 domain, the YV motif may enable the Cx36/ZO-1 PDZ1-domain interaction (FIGURE 6A). Regardless, more research must be conducted to settle the Cx32/ZO-1 (and Cx30 and Cx36/ZO-1) interaction issue(s).

Despite extensive research, the role of ZO-1 in GJ function (ranging from being involved in GJ assembly to degradation) is far from clear, although clues, albeit contradictory, have been emerging. One simple explanation to clarify this confusion is that ZO-1 may play multiple roles in the GJ life-cycle, which would not be surprising for a scaffolding protein that functions in the assembly of multi-protein complexes. When tagged with a COOH-terminal GFP, Cx43 plaques grow to unusually large sizes in

HeLa cells compared with untagged plaques (74). The reason for this did not become clear until 2005 when Hunter et al. (73) showed that a mimetic/synthesized fragment of the Cx43 COOH terminus would compete for ZO-1 with the wild-type COOH termini of Cx43 when expressed in cultured cells. The resulting plaques of wild-type Cxs competing for ZO-1 were the same size as Cx43 tagged with GFP. It was believed that larger GJ plaques produced by Cxs unable to bind to ZO-1 occurred because the turnover rates of these plaques were longer than those of plaques assembled from wild-type Cxs. However, pulse/chase experiments not only showed that this was not the case (73) but that Cx43 incapable of binding ZO-1 actually had a shorter turnover time compared with wild-type Cx43 capable of binding ZO-1 (2 h vs. 3–5 h, respectively) (73). Therefore, although ZO-1 was shown to be important for Cx43 size regulation, the results suggested that the mechanism by which it worked was not via internalization of GJ plaques. It should be noted here that the large plaque size generated by expressed Cx43-GFP actually was a welcome side-effect, because much of the structural and spatio-temporal information of GJs known today could not have been acquired with GJ plaques assembled from untagged Cxs that usually exhibit a size that is not much larger than

the resolution limit of a standard fluorescence light microscope.

Fluorescence microscopy, for example, has shown that ZO-1 localizes primarily to the edge of GJ plaques and not in their centers (4, 58, 73) with on average a partial 26.6% overlap (219). Recently, it was discovered that a “halo” of connexons attached to ZO-1 appears to exist in the periphery of Cx43-based GJ plaques (161). This pool of connexons surrounding the plaques, termed the “perinexus,” is believed to be the source from which GJ plaques recruit new connexons to dock into complete GJ channels (161). It is also possible that this pool of connexons represents the source of connexons that initially fuels the assembly of early GJ plaques, termed “formation plaques” (78). Additionally, as shown by Bruce et al. (19), there also appears to be an increase in interaction between Cx43 and ZO-1 in heart tissue from congestive heart failure patients, whose GJs have been shown to form smaller plaques. These observations also hint at a role of ZO-1 in stimulating plaque formation rather than in limiting plaque size.

However, complicating this issue, Cx reliance upon ZO-1 for plaque formation has been shown to vary between isoforms and experimental cell types. Cx43, rendered “ZO-1 binding-incompetent” with either its extreme most isoleucine or most of its COOH terminus deleted, has been shown to still be capable of forming plaques in HeLa cells, albeit slightly less efficiently (24). At least in the case of Cx43, as long as the microtubule-interaction region in the COOH terminus remains intact for trafficking to the PM, Cx43 plaques can still assemble without ZO-1 (56, 163, 209). However, the same form of deletions in Cx50 results in the inability of the cells to form Cx50-based plaques. Interestingly, it was shown that if only the last seven amino acids of a truncated Cx50 were added back on, this isoform could again form plaques (24). Also, with COOH terminus truncated, and presumably ZO-1 binding-incompetent, Cx43 and Cx32 mutants were unable to form plaques in A431D cells (25). However, these same mutants were able to form plaques in LNCaP cells, and Cx43 was able to form plaques in HeLa cells, which underscores how the selection of cell type can alter results with regard to plaque formation. Finally, Cx43 has also been reported to bind to the PDZ2 domain of the related scaffolding protein, ZO-2 (173), suggesting that different ZO-proteins may compete for the Cx43-COOH terminus to regulate Cx43 GJ function.

Despite these sometimes confusing results, it is clear that ZO-1 binds to the Cx43 COOH terminus in a highly regulated fashion. It is tempting to speculate that ZO-1, as expected of a scaffolding protein, may coordinate the assembly of a multi-protein complex

on Cx43-based GJ plaques to recruit proteins, such as kinases, ubiquitin-ligases, clathrin-based endocytic components, etc. to aid in GJ internalization. Further complicating attempts to understand the role of ZO-1 in GJ function is the fact that ZO-1 can interact with many other PM-localized structures, such as tight and adherens junctions, which often spatially overlap with GJs (FIGURE 6A), and that many of the myriad functions of ZO-1 may be exhibited and may be recognizable only when in association with distinct cellular structures or cellular conditions or when examined in appropriate cell types.

Low Open-Probability of GJ Channels

Bukauskas and colleagues investigating electrical coupling of cells expressing a Cx43-GFP fusion protein construct found that only a small portion (<10%) of GJ channels of a GJ plaque appear to be open at any given time (20). In addition, active channels exhibit a very high open probability (18, 187), suggesting that electrical transmission likely results from a small number of active channels rather than from a larger number of channels with a low open probability. More recently, Pereda and colleagues found that even a smaller fraction of GJ channels (only ~0.1–1%) present in electrical synapses of teleosts (Cx35, afferent terminals on the goldfish Mauthner cell) and mammals [Cx36, rat brain mesencephalic trigeminal nucleus (MesV) neurons] appears to be conductive and contributes to electrical transmission at any given time (33, 46). Although currently it is unknown and may be puzzling why only a small portion of the channels of a GJ plaque appear to be active (mechanical stability relevant for the maintenance of functional GJs has been discussed; see Ref. 33), it is tempting to speculate that channel activity is also regulated by above-described regulatory mechanisms, e.g., Cx-phosphorylation and ZO-1 binding. Since 1) newly synthesized GJ channels (likely to be active and open) appear to be accrued along the outer periphery of GJ plaques (40, 52, 107), while older channels (likely to be inactive and closed) are endocytosed simultaneously from their centers (40, 52, 107), 2) Cx phosphorylation is increased in GJ plaques (132), and 3) ZO-1 colocalizes with the outer rim of Cx43-based GJ plaques (73, 161), one can speculate that ZO-1 binds to connexons not only to regulate channel accretion (161) but also to keep newly accrued channels open. Specific Cx-phosphorylation events may then displace ZO-1, followed by permanent channel closure and removal of channels from plaque centers and the PM. It would be interesting to know whether (certain) Cx-phosphorylations conform with

such a hypothetical distribution within plaques and whether Cx phosphorylation patterns match with ZO-1 binding that is known to exhibit such a hierarchical binding pattern.

Concluding Remarks

GJ channels are unique cellular structures that play important functions in many aspects of multicellular life, including direct cell-to-cell communication (their hallmark function), intra-/extracellular signaling (based on hemichannel/connexon function), cell guiding, and cell-cell adhesion (based on head-on connexon docking). It is clear that Cx biosynthesis, GJ assembly, function, and degradation need to be controlled precisely to ensure accurate physiological performance of this versatile cellular component. The sections above discuss regulatory mechanisms that likely control these critical steps of the GJ life cycle. They include post-translational modifications, such as phosphorylation of COOH-terminal Cx43 serine and tyrosine residues, ubiquitination of lysine residues, as well as interactions with regulatory binding proteins, such as ZO-1. Open questions that future research will need to address not only include the identification of such signals and the physiological conditions under which they are applied but importantly address which Cx polypeptides and channels in a GJ plaque will actually be modified to trigger the desired events. For example, in general, not all Cxs of a GJ plaque and not all GJ plaques of a cell will be ubiquitinated or will be able to interact with endocytic proteins such as AP-2 at all times, but Cx modification and accessory protein binding will need to occur spacially and be temporally controlled. Substantial future research is necessary to further elucidate the numerous, functionally relevant Cx-modifications, Cx protein/protein interactions, and molecular signals that accurately regulate these events. Unfortunately, as described above, addition of fluorescent protein tags, such as GFP, will eliminate the binding of certain regulatory proteins, such as ZO-1, when placed on the Cx43 COOH terminus and render assembled channels nonfunctional when placed on its NH₂ terminus. Without this commonly used tool, it will be more challenging to investigate GJ function, especially in living cells, forcing the development of new tools and techniques to explore these regulatory Cx modifications in greater detail. ■

This review provides only a sampling of the literature that is available on this topic, and we apologize to authors whose original works have not been cited.

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