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Review

Gap junctional channels are parts of multiprotein complexes[☆]

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ABSTRACT

Gap junctional channels are a class of membrane channels composed of transmembrane channel-forming integral membrane proteins termed connexins, innexins or pannexins that mediate direct cell-to-cell or cell-to extracellular medium communication in almost all animal tissues. The activity of these channels is tightly regulated, particularly by intramolecular modifications as phosphorylations of proteins and via the formation of multiprotein complexes where pore-forming subunits bind to auxiliary channel subunits and associate with scaffolding proteins that play essential roles in channel localization and activity. Scaffolding proteins link signaling enzymes, substrates, and potential effectors (such as channels) into multiprotein signaling complexes that may be anchored to the cytoskeleton. Protein–protein interactions play essential roles in channel localization and activity and, besides their cell-to-cell channel-forming functions, gap junctional proteins now appear involved in different cellular functions (*e.g.* transcriptional and cytoskeletal regulations). The present review summarizes the recent progress regarding the proteins capable of interacting with junctional proteins and highlights the function of these protein–protein interactions in cell physiology and aberrant function in diseases. This article is part of a Special Issue entitled: The Communicating junctions, composition, structure and functions.

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Abbreviations of cell lines: 42GPA9, mouse Sertoli cell line; βTC-3, mouse pancreatic β cell line derived from insulinomas; A431, human squamous carcinoma cells; A7r5, rat aortic smooth muscle cell line; BICR/M1R_k, a permanently growing cell line derived from a spontaneous rat mammary tumor; BWEM, rat cardiomyocyte-derived cells; C2C12, murine myoblasts; C6, rat glioma cell line; CHST8 cells, immortalized mouse hepatocytes; COS7, monkey African green kidney cells; E36, Chinese hamster ovary cells; FT210, a mutant cell line derived from FM3A a murine mammary carcinoma cell line; HEK 293, human embryonic kidney 293 cells; IAR20, rat epithelial cell line; J774, murine macrophage cell line; Jeg3, human choriocarcinoma cell line; LNCaP, human prostate cancer epithelial cells; MDCK, epithelial Madin–Darby Canine Kidney cells; Neuro2A, mouse neuroblastoma cells; N/N1003A, rabbit lens epithelial cells; NIH 3T3, mouse fibroblasts; NRK, rat kidney cells; N2a, murine neuroblastoma cells; P3/22, mouse skin papilloma cell line and its derivatives P3E1 (in which E-cadherin gene is transfected); PC-12 cell, a cell line substitute neuron originally cloned from rat pheochromocytoma cells; ROS 17/2.8, rat osteosarcoma cells; S180, mouse sarcoma cells; SW-13, human adrenal cortical tumor cells; T51B, rat liver epithelial cells; TtT/GF, murine pituitary folliculo-stellate-like cell line; U251, human glioblastoma cells; WB-F344, rat liver epithelial cells

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1. Introduction

Intercellular communication is of paramount importance in allowing individual cells to successfully act in a co-ordinated manner in multicellular organisms and tissues. Gap junctional intercellular communication (GJIC) relies on intercellular protein channels which span the lipid bilayers of contiguous cells, allowing them to directly exchange ions and small molecules. All junctional channels have a similar overall structure but, unlike many other membrane channels, different gene families encode the membrane proteins that form them in different animal phyla. The intercellular junctional channel is made by end-to-end docking of two hemichannels, each of which is an oligomer of transmembrane protein subunits, termed innexins (Inxs) in invertebrates, and connexins (Cxs) and pannexins (Panxs) in chordates (see [1]), forming an aqueous pore in the lipid bilayer. If pannexins were found able to form cell-to-cell channels in paired oocytes [2], an uncertainty remains on this capability in native tissues. Intercellular dye or electrical couplings are indeed generally absent in a variety of pannexin-expressing host cells (see [3]) but a Ca^{2+} wave propagation between osteoblasts was recently ascribed to Panx3 gap junctions [4].

Cxs, Inxs and Panxs have a common topology, with four alpha-helical transmembrane domains, two extracellular loops, a cytoplasmic loop, and N- and C-termini located on the cytoplasmic membrane face. Historically, the unpaired hemichannels scattered in nonjunctional membranes were considered as remaining permanently closed to avoid cell death, but several data reported in the past decade revealed that at least some of them mediated paracrine signaling by providing a flux pathway for ions such as Ca^{2+}, for ATP, glutamate and plausibly for other compounds, in response to physiological and pathological stimuli.

The properties of single membrane channels and intercellular channels are, as those of other membrane channels, determined by their 3-dimensional structure and related interactions with regulatory proteins. Structures that include channel-subunits and regulatory proteins depend on the microdomains that incorporate protein constituents. Multiprotein complexes associate pore-forming structures with scaffolding proteins that link signaling enzymes, substrates, and potential effectors (including channels) into a signaling complex anchored to the cytoskeleton. Besides an obvious role in targeting the channel to a particular location on the cell membrane, there are several advantages to having a membrane channel in a multiprotein complex. Indeed, the substrate selectivity of some enzymes, essential to determining specificity in several signal transduction pathways, is largely determined by their subcellular location (*e.g.* for protein kinases and phosphatases). There is also a large increase in the efficiency of the reaction kinetics when an enzyme is localized with its substrate and effector in a microenvironment with restricted diffusion. Moreover, the anchoring of enzyme complexes to some channels may be necessary for the extremely rapid transmission of signals required to regulate the activity of these channels. However, if the proteins in signaling cascades are considered spatiotemporarily organized in such a way to achieve efficiency and specificity, the physical association of components to form signaling complexes or their close proximity to facilitate random collisions still remains poorly understood and largely focused on Cx43, owing in large part to its ubiquitous distribution in mammalian tissues.

Many signaling pathways and regulatory systems in eukaryotic cells are controlled by proteins with multiple interaction domains that mediate specific protein–protein and protein–phospholipid interactions. Pairs of interacting partners at protein–protein (or protein–peptide) interfaces interact via modular protein domains that are well demarcated and independently folded domains, typically comprising 40 to 200 amino acids. These domains are non-catalytic and bind specifically to short continuous peptide sequences in their binding partner(s) via one or more exposed, ligand-binding surfaces ("ligand recognition pockets"). Ligands interact with their complementary domains through short and generally continuous sequence motifs. In some cases, certain amino acids of the ligands must be post-translationally modified (phosphorylated, acetylated, methylated,

etc.) before recognition and binding can occur (see for example [5]). The protein–protein interactions may act in concert as "molecular switches", changing the properties of the channels (or their ability to be regulated by other factors) in response to modifications in the channel microenvironment.

2. Intramolecular interactions between gap junction proteins

The assembly of gap junction molecules into a connexon, an innexon or a pannexon, made of single (homomeric) or multiple (heteromeric) protein isotypes is based on specific signals located within the polypeptides. Cxs, Inx and Panx1 assemble into hexamers and Panx2 into octamers (the oligomeric number of a Panx3 membrane channel has not yet been determined; see [6]). These proteins have conserved cysteine residues in their extracellular loops (six in Cxs (except Cx23; [7]), four in Inxs, Panx1 and Cx23) that form intra-protein disulfide bonds (for review, see for example [8]). The head-to-head binding between two connexons or two innexons occurs via non-covalent interactions between the extracellular loops of apposing hemichannels. Pannexins, in contrast with both Inxs and Cxs, contain glycosylation sites in their extracellular loops [9]. Presumably, such glycosylation not only plays a role in trafficking of Panx1 to the membrane, but also poses a steric barrier to formation of pannexon linkage across the extracellular space (see above). Thus, the role of pannexin channels is most likely to involve exchange from extracellular space, rather than between cells, directly or through associations with several P2 purinergic receptors (see Section 3.5).

Mutagenesis studies suggest that the docking region contains beta structures, and may resemble to some degree the beta-barrel structure of porin channels. The hemichannels that compose one intercellular channel are rotationally staggered by ~30° relative to each other so that the alpha helices of each connexin monomer are axially aligned with the alpha helices of two adjacent monomers in the apposed hemichannel (for review, see [10]). The second extracellular (E2) domain [11] together with the middle cytoplasmic portion [12] determine the heterotypic compatibility between connexins. The docking process of connexons could be hindered by competition with oligopeptides homologous to specific regions of the extracellular loops of connexins (for recent review, see [13]).

Formation of heterotypic channels, *i.e.* docking of two hemichannels composed of different connexin isotypes was at first controversial but several associations have now been observed *in situ* (see Table 1). In A7r5 cells for example, He et al. [14] reported that Cx40 and Cx43 failed to form functional heterotypic channels but built heteromeric channels with unique functional properties (conductances, gating behaviors or selectivities) relative to the homomeric forms.

Connexin intramolecular interactions have been suggested to be responsible for the interruption of the cell-to-cell communication elicited by different stimuli (including pH, insulin, and the fast component of voltage-dependent gating for example), via interactions between the channel pore and a discrete C-terminal domain of the connexin serving as gating particle, a "ball and chain", model [15–22] analogous to the inactivation of Shaker-type K^+ channels [23].

Several lines of evidence indeed imply that the CT domain of Cxs and Inxs is a movable structure, able to behave like an independent domain. Indeed, truncation of this domain in Cx40 or Cx43 resulted in alterations in their single channel activity. However, normal functions were restored by co-expression of the CT domain, *even of another connexin*. Thus, C-tails of other connexin family members can functionally substitute, for example Cx40 truncated at amino acid 248 (Cx40tr248) function is – partially – restored by separate co-expression of the heterologous Cx43-CT fragment [21,24,25]. Both Cx40CT and Cx43CT were reported to be intrinsically disordered proteins, being able to interact with each other; the CT domains of Cx40 and Cx43 can indeed interact with the CL domains of Cx40 and Cx43 (and *vice versa*, respectively; [26]).

These hetero-domain interactions (occurring between the CT domain of one connexin isotype and the pore-forming region of another) suggest that, despite their differences in primary sequence, the CT domains of Cx40 and of Cx43 can reach a similar conformation, allowing their interaction with a common binding domain. Such intramolecular interaction, by analogy with other biological reactions involving two separate molecules (*e.g.* ligand and receptor), is viewed as an intramolecular noncovalent interaction of the CT domain (acting as a ligand) with a domain in or near the mouth of conductive path (acting as a receptor). The second half of the cytoplasmic loop of Cx43 (amino acids 119 to 144) able to bind to the CT domain of the same protein, has been proposed to act as the receptor for the gating particle [22,27,28]. Consequently, mimetic peptides were able to prevent the gating of channels. Peptides containing the sequence "RXP" as a consensus Cx43CT binding motif prevented heptanol –

Table 1
Reported interactions between gap junction proteins in preparations which endogenously express them.

Protein	Via its	And	Motif	Main approaches[a]	Cells or tissues	References
Cx26		Cx30		cl ci	Mouse cochlea	[247]
				cl ci	Mouse cochlea	[248]
		Cx32		bt ci	Guinea pig liver	[249]
				ep	Mouse hepatocytes	[250]
				cl ci	Mouse mammary gland	[251]
				cl em	Rat liver	[252]
				gt cl	Mouse astrocyte–oligodendrocyte junctions	[246]
				bt td	Rodent hepatocytes	[253]
		Cx43		ci	Rat Sertoli cells	[224]
Cx32		Cx47		gt cl	Mouse oligodendrocytes	[246]
Cx33		Cx43		cl ci	Rat Sertoli cells	[177]
Cx40		Cx43		ci ep	A7r5 cells	[14]
				at bt nmr		[26]
				ep	Human mesenchymal stem cells, canine ventricular myocytes	[254]
		Cx43, possibly Cx37		ep	Rat basilar artery smooth muscle	[255]
		Cx43, Cx45		cl ep	Rat ventricular myocytes	[256]
Cx46		Cx50		ci	Ovine lens	[257]
Inx2	C-terminal	Inx3	C-terminal	cl ci dh at	Drosophila embryos	[33]
Inx2	C-terminal	Inx3	C-terminal	at		[154]

[a] : bt: biochemical techniques (cell-free assays, chimeras, truncated connexins, mimetic peptides, *etc.*); cl: colocalization; ci: co-immunoprecipitation; dh: double hybrid; at: affinity techniques (pull-down, affinity binding assays, surface plasmon resonance); em: electron microscopy immuno labeling; ep: electrophysiology; gt: gene targeting; td: tracer diffusion.

and low pH – induced uncoupling junction closure, and action potential propagation block [28,29], and peptides corresponding to either the last 10 CT aa of Cx43 or to the L2 region (aa 119–144 in the CL) altered the activity of Cx43 hemichannels [30], for example.

The dimerization of the Cx43CT was suggested to be one of the structural changes involved in the pH regulation of Cx43, increasing the Cx43CT/Cx43CL binding affinity to bring the channel to a closed state [31,32], being important for heteromeric channel regulation.

The binding of Inx2 and Inx3 via their CT was proposed to provide a mechanism for oligomerization of heteromeric channels [33]. An interaction between Panx1 and Panx2, initiated while these pannexins are still residents of the endoplasmic reticulum and consistent with the potential formation of mixed Panx1/2 channels, was observed in NRK and HEK 293 cells [34]. In this study, in contrast, Panx1/Panx3 physical interaction only occurred when Panx1 was not glycosylated or glycosylated to only the Gly1 species.

3. The protein partners of gap junctional proteins

Proteins are endowed with a capability of steric fitting with other proteins to form high molecular weight complexes with emergent functions. Protein–protein interactions often take place via special domains of the peptide chains [35] (see Box 1 for more details); among them, PDZ (Postsynaptic density-95/Disk large/Zonula occludens-1) motifs are of special interest since they often are found to bind C-terminal hydrophobic amino acids of transmembrane proteins. PDZ domains are 80–100-amino acid protein binding cassettes, identified initially in PSD-95, Dlg, and ZO-1, that can recognize the very C-terminal amino acid residues of their binding partners [36], but also have been implicated in homodimerization, heterodimerization and binding other regions of proteins (see below).

PDZ proteins are classified according to their modular organization (for review, see [44]). Proteins in the MAGUK (membrane-associated guanylate kinase) family are characterized by multiple PDZ domains, one SH3 domain, and one GUK-like domain (for review see [45]). The Src homology 3 (SH3) domains can interact with proline-rich motifs whereas Src homology 2 (SH2) domains associate with phosphotyrosine-containing sequences. The GUK-like domain may also allow for multiprotein interactions [46]. MAGUK proteins have emerged as key elements in the organization of protein complexes in specialized membrane regions (*e.g.* synapses or intercellular junctions). Via their multiple protein–protein interaction domains, they interact with a wide range of membrane receptors and ionic channels. Several proteins have been reported to be able to interact (Table 2) or only to co-localize (not quoted) with different GJ proteins.

Box 1
Protein modules and Cx43 signaling.

Many genes that have different functions and lack significant overall homology at the nucleic acid sequence level, encode proteins with structurally similar domains. The X-ray diffraction/crystallography technique revealed that the homology in primary sequence is even more pronounced in the three-dimensional structure. Because of their predictive value in signal transduction, the domains directly involved in Cx43 gap junctions are briefly introduced. For more details on protein–protein interaction domains involved in signal transduction, the reader is referred to Pawson and Scott [35] and Sudol [37]. The conformation of the proteins participating in protein–protein interactions is crucial. One way to alter these conformations is by (de-)phosphorylation.

PSD95/Disk large/ZO-1 homology domain (PDZ)
PDZ (80–90 amino acids) domains have a hydrophobic pocket that binds the very C-terminal residues of their target proteins [38]. Target proteins include multi-spanning transmembrane proteins such as receptors and ion channels. Two classes of PDZ-binding consensus sequences have now been identified: Class 1: -x-S/T-x-V/I/L-COOH; Class 2: -x-V/I/L-x-V/I/L-COOH [36]. Recent evidence shows that binding to internal hydrophobic sequences can occur, and that PDZ domains can form heterodomain interactions [39]. One of the best described examples of clustering signal and effector proteins via PDZ domains is InaD, which forms a peripheral membrane signaling complex at rhodopsin receptors (see [40] for details).

Src Homology 2 domain (SH2)
SH2 domains characteristically bind phospho-tyrosine residues in a hydrophobic pocket. An additional pocket confers substrate specificity by binding amino acids more C-terminally located. Upon tyrosine phosphorylation of the substrate, SH2 modules associate with their substrates. One well-documented example of specific SH2-tyrosine binding is the activated PDGF receptor [41].

Src Homology 3 domain (SH3)
The other well-known Src-homology domain, the SH3 domain, binds proline-rich sequences. Aliphatic prolines residues of the substrate located on one side of an alpha-helix bind separately to hydrophobic pockets in the SH3 domain. As for the other protein domains, the flanking amino acids of the consensus sequence determine binding specificity. For example, the v-Src SH3 domain has been shown to bind to a specific proline-rich stretch in Cx43 [42].
For a comprehensive overview of other domains implicated in connexin interactions, see [43].

3.1. Cytoskeletal proteins

In contrast with tight junctions and adherens junctions, known for a long time to interact with the actin cytoskeleton, gap junctions were only recently reported to associate, frequently via adaptors, with the actin cytoskeleton. Actin has been reported to directly interact with Panx1 (preferentially F-actin; [47]) and with Cx30 [48], in contrast with other connexins, Cx43 for example, of which binding to actin is thought to occur indirectly. Using a combination of atomic force microscopy and immunocytochemistry, Yamane et al. [49] suggested an association between Cx43 and the tip of actin filaments in embryonic rat astrocytes. Among the F-actin-binding proteins which connect extra- and intracellular stimuli to cytoskeleton modeling and reorganization, Drebrin ("Developmentally regulated brain protein"), was identified as a binding partner of the C-tail Cx43 in mouse brain homogenates [50] and cortactin co-immunoprecipitated and co-localized with Cx43 and F-actin in C2C12 cells [51]. Cx43 was shown to bind to microtubules [52]; α-tubulin and β-tubulin appeared equally bound [53].

3.2. Scaffold proteins (Zonula Occludens proteins)

In the different junctional complexes, adaptor proteins, which possess a modular organization with several protein–protein interaction domains, usually bind to the cytoplasmic C-terminal tail of transmembrane proteins and connect them to the actin cytoskeleton directly or indirectly by recruiting other proteins, creating and maintaining multiprotein complexes. Zonula Occludens (ZO) proteins, in

Table 2
Reported interactions of junctional proteins with membrane-associated proteins.

Partner protein	Via its	Cx	Cx motif	Main approaches[a]	Cell types[b]	References
Calmodulin		Cx32	NT (aa 1–21); CT (aa 216–230)	bt		[93]
			M3, C-terminal	bt ci	*COS-7 cells*	[152]
		Cx35/Cx36/Cx34.7	First residues (10 to 30) of the CT	at		[258]
		Cx43	Cytoplasmic loop (aa 136–158)	at nmr		[143]
		Cx44	Cytoplasmic loop (aa 129–150)	bt nmr		[143]
		Cx50	Cytoplasmic loop and C-terminal	cl ci	*HeLa cells*	[94]
			Cytoplasmic loop (aa 141–166)	bt nmr		[142]
ZO-1	PDZ-2	Cx30		cl ci at	Mouse brain and spinal cord	[259]
	PDZ-2	Cx31.9	The most C-terminal residues	cl ci	*HEK 293 cells*	[130]
		Cx32		cl ci	Cultured rat hepatocytes	[68]
	PDZ-1	Cx36	Four C-terminal residues (SAYV)	cl ci em at	Mouse brain, *HeLa cells*	[136]
	PDZ-1		A 14-residue C-terminal fragment	cl ci at	*HeLa cells*, β TC-3, mouse pancreas and adrenal gland cells	[135]
				cl ci	P C12 cells	[260]
	PDZ-1			cl ci	Mouse retina, *HeLa cells*	[163]
	PDZ-1	Cx35 (orth. hCx36)	Four C-terminal residues (SAYV)	cl ci at	Goldfish brain sect ions, *HeLa cells*	[244]
		C40		cl ci	Porcine vascular endothelial cells	[71]
	PDZs	Cx43	C-terminal 5 residues	cl ci at	*HEK 293 cells*, rat cardiomyocytes	[59]
	PDZ-2		Extreme C-terminal	cl ci dh	*COS-7*, Rat -1, mink lung epithelial cells	[60]
	PDZ-2		C-terminal 5 residues	cl ci em	Rat adult ventricular myocytes	[194]
				cl ci	42GP A9 cells, rat test is lysates	[261]
	PDZ-2		C-terminal	ci at	C57B16 mouse cortical astrocytes	[101]
	PDZ-2		Last 19 C-terminal residues	nmr		[129]
	PDZ-2		CTl (residues at the -3 position)	cl ci dh	*MDCK cells*	[128]
	PDZ-2			cl ci at	Mouse brain and spinal cord	[259]
	PDZ-2		C-terminal (amino acids)	cl at	*HeLa cells*, rat cardiomyocytes	[62]
				cl ci	Porcine vascular endothelial cells	[71]
	PDZ-2		C-terminus	cl ci	*NRK and HEK 293 cells*	[262]
				cl ci at	Human trophoblastic cells	[216]
				cl ci	*HeLa cells*	[162]
		Cx43, Cx45		cl ci at	*ROS 17/2.8 cells*	[140]
	PDZs	Cx45	C-terminal 4 residues	cl ci dh	*MDCK cells*	[131]
			12 most C-terminal residues	cl ci at	*ROS 17/2.8 cells*	[189,263]
	PDZ-2			cl ci at em	*HeLa cells*	[163]
	PDZ-2	Cx46, Cx50	Most C-terminal residues	cl ci	Mouse lens	[132]
	PDZ-2		Most C-terminal residues	cl ci em at	Mouse lens	[134]
	PDZ-2	Cx47		cl ci em at	Mouse brain, *HeLa cells*	[133]
ZO-2		Cx43	C-terminal end	at	NRK cells	[264]
	PDZ-2		C-terminal end	cl ci dh	NRK, *HEK 293T* cells, heart tissues	[64]
	PDZ-1	Cx36	Four C-terminal residues (SAYV)	cl ci at	*HeLa and β TC-3 cells*	[223]
ZO-3	PDZs	Cx45	C-terminal 4 residues	cl ci dh	*MDCK cells*	[131]
	PDZ-1	Cx36	Four C-terminal residues (SAYV)	cl ci at	*HeLa and β TC-3 cells*	[223]
Occludin		Cx26		at	Human intestinal cell line T 84	[69]
		Cx40, Cx43		cl ci	Porcine vascular endothelial cells	[71]
		Cx32		cl ci	*CHST8 cells*	[67]
				cl ci	Cultured rat hepatocytes	[68]
Claudin-1		Cx32		cl ci	Cultured rat hepatocytes	[68]
Claudin-5		Cx40, Cx43		cl ci	Porcine vascular endothelial cells	[71]
MUPP-1	PDZ	Cx47	C-terminus	cl ci	Mouse brain and sciatic nerve	[72]
β-catenin		Cx43		cl ci	Neonatal rat cardiomyocyte, *N2A cells*	[77]
				ci	Rat Sertoli cells	[205]
p120		Cx43		cl ci	NIH 3T3 cells	[75]
N-Cadherin		Cx43		cl ci	NIH 3T3 cells	[75]
				ci	Rat Sertoli cells	[205]
Plakophilin-2		Cx43		ci	Rat Sertoli cells	[205]
Ankyrin-G		Cx43		ci	Rat adult heart lysates	[181]
Integrin α5/β 1		Cx43		cl ci	NIH 3T3 cells	[265]
Crumps		Inx2		cl ci dh at	Drosophila embryos	[80]
AQP0	C-ter	Cx45.6, Cx56		cl ci at	Embryonic chick lens	[266]
	C-ter	Cx45.6	Cytoplasmic loop	ci at	Embryonic chick lens	[82]
	C-ter	Cx50	Cytoplasmic loop	cl at	*Chick lens*	[83]
Nav 1.5		Cx43		cl ci	Mouse myocardium	[81]
mAChR		Cx43		cl ci	Rat myocardium	[84]
$P2X_7$		Cx43		cl at	Mouse macrophages, J774 cells	[87]
		Panx1		ci	*HEK 293 cells*	[88]

[a] : bt: biochemical techniques (cell-free assays, chimeras, truncated connexins, mimetic peptides, oligomerization assays, chemical cross-linking tests, …*etc.*); cl: colocalization; ci: co-immunoprecipitation; fs: co-fractionation or co-sedimentation; dh: double hybrid; em: electron microscopy immuno labeling; at: affinity techniques (pull-down, affinity binding assays, surface plasmon resonance); nmr: nuclear magnetic resonance.

[b] : in roman characters, cells where Cxs were endogenously expressed; in italics, cells where Cxs were exogenously expressed, surexpressed or mutated.

addition to the characteristic modules of the MAGUK protein family (PDZ, SH3 and GUK domains), have a distinctive C-terminus comprising acidic- and proline-rich regions. ZO-1, a 220 kDa peripheral membrane protein, tethers transmembrane proteins either directly (*e.g.* connexins, occludin, claudins, tricellulin, CAR (coxsackievirus and adenovirus receptor) and JAMs) or via their adapter proteins, *e.g.* α-catenin or afadin, to the actin cytoskeleton [54–57]. All Cxs explored so far have been found to be able to associate with ZO proteins

(Table 2), thus ZO proteins may play a general role in the formation, distribution and turnover of gap junctions and/or in recruiting signaling molecules that regulate intercellular communication. Moreover, ZO proteins also exhibit several nuclear localization (NLS) and nuclear export signals (NES), which may enable them to shuttle between the cytoplasm and the nucleus (for recent review, see for example [58]), to transduce signals leading to altered gene expression and cell behavior.

Cx43 was the first connexin found to interact with a ZO protein [59,60], and this interaction has been subsequently observed in a variety of cell types (Table 2). Cx43-ZO-1 associations do not necessarily overlap directly with whole junctional plaques, and ZO-1 has frequently been observed only at the plaque perimeter [61–63]. The finding that the C-terminus of Cx43 also binds to ZO-2 [64] presents the intriguing prospect that these ZO proteins, interacting with the Cx43-PDZ-binding motif, compete to influence gap junctional patterning. Conversely, a competition between Cx-partners of ZOs or even between Cxs and proteins belonging to other classes of junctional complexes, tight junctions (ZO-1 was the first TJ-associated protein to be identified) (see [57]) or anchoring junctions (see [56]), still widens the possibilities of adaptations of intercellular junctions to the cell needs. However the fact that functional GJs are formed even when the connexin/ZO-1 interaction is prevented (see [65]) indicates that there are other mechanisms for plaque formation as well.

3.3. Tight junction associated proteins

Tight junctions are strands of transmembrane domain proteins of apposed cells that interact across the extracellular space to provide both barrier and fence functions, thereby limiting the flow through the extracellular space. Moreover, tight junction proteins help to polarize membrane compartments, leading to separated apical and basolateral domains (for review, see [66]). Tight junctions are formed by complexes of proteins including transmembrane proteins (including claudins and occludins), cytoplasmic scaffolds (including ZO proteins) that allow association with cytoskeletal elements and signaling molecules (see [57]).

Occludin, a 65 kDa transmembrane phosphoprotein with a topology similar to Cxs', was found to interact with Cx32 in immortalized mouse hepatocytes [67] and cultured rat hepatocytes [68]. Nusrat et al. [69], using a bait interaction system, noted that Cx26 interacted with a coiled-coil domain of occludin. Claudins form a multigene family (20 members; see [70]) of $\approx$22 kDa transmembrane proteins of the macromolecular tight junction complex, with also a topology similar to Cxs'. Claudin-1 was found co-localized with Cx32, occludin and ZO-1 at cell borders of rat hepatocytes lines. Association of Cx32 with the tight-junction proteins was demonstrated by co-immunoprecipitation [68]. Moreover, occludin, claudin-5 and ZO-1 were shown to co-localize and to coprecipitate with Cx40 and Cx43 in porcine blood–brain barrier endothelial cells [71]. Multi-PDZ domain protein 1 (MUPP-1), a tight junction-related protein often co-localized with ZO-1 at tight junctions, was found co-associated with Cx47 in homogenates of mouse brain and sciatic nerve [72].

Thus, if gap junctions and tight junctions represent different types of specialized plasma membrane microdomains, they however share common adaptor molecules, particularly Zonula Occludens proteins, and frequently present intermingled relationships where the different proteins co-assemble into macromolecular complexes.

3.4. Anchoring junctions-associated proteins

The adhesive interactions between a cell and its surroundings play a central role in regulating normal cell functions such as cell division, migration, and differentiation by their coupling to signaling pathways and to the cytoskeleton (for review, see *e.g.* [73]). During organ formation, wound healing, tissue reorganization, and metastasis, cell contacts are the subject of constant remodeling. Integrins, cadherins and cell adhesion molecules (*e.g.* selectins) are transmembrane proteins which mediate cell–cell and cell–matrix interaction and communication.

Cadherins comprise an important family of transmembrane glycoproteins that mediate calcium-dependent cell–cell adhesion and are linked to the actin cytoskeleton via catenins (see [74]). In NIH3T3 cells, Cx43, N-cadherin and different N-cadherin-associated proteins were co-localized and co-immunoprecipitated, suggesting that Cx43 and N-cadherin are co-assembled in a multiprotein complex containing various N-cadherin-associated proteins [75]. The fact that GJ formation was disrupted in the presence of peptides that compromise homophilic cadherin interactions suggests that GJ might form preferentially between cell types that express the same type of cadherins [76].

Catenins are multifunctional proteins which include α-catenin and armadillo catenins, β-catenin, plakoglobin (γ-catenin, a primary component of desmosomes and fascia adherens) and p120 catenin. Plasma membrane-associated β-catenin acts as a component of cell-adhesive junctions, and it was found co-localized and co-immunoprecipitated with Cx43 in neonatal rat cardiomyocytes [77,78]. In the latter study, β-catenin was suggested to associate with α-catenin, ZO-1 and Cx43 during gap junction development. In mouse neural crest cells, p120 catenin was found co-localized with Cx43 and N-cadherin and, in preliminary studies in NIH3T3 cells, Cx43 co-immunoprecipitated with one of the two p120 catenin bands present [79].

In the epithelia of embryonic *Drosophila*, Inx2 interacts with core proteins of adherens and septate junctions [80].

3.5. Membrane channels and receptors

Sodium channel complexes at intercalated discs (IDs) of ventricular myocytes are composed of tetrodotoxin-resistant $Na_v1.5$ channels closely associated with both N-cadherin and Cx43 [81]. Aquaporins are water channel proteins belonging to the major intrinsic protein (MIP) superfamily of membrane proteins; aquaporin-0 (AQP0), the most abundant membrane protein in lens fibers, was found to interact with two binding sites within the intracellular loop region of chick Cx45.6 [82] or of its human ortholog Cx50 [83].

In rat myocardium, Cx43 can interact (maybe indirectly) with M2/M3 subtypes of acetylcholine seven transmembrane-domain muscarinic receptors, and ischemia was found to specifically impair this association [84]. Combining confocal microscopy and freeze-fracture replica immunogold labeling, Rash et al. [85] observed, in “mixed” (chemical plus electrical) synapses of goldfish and rat brain, a close proximity between Cx35 and a NMDA glutamate receptor subunit (NR1).

Receptor protein tyrosine phosphatases (RPTPs) form a family of cell-surface receptors characterized by the diversity of their extracellular domains, resembling some features of those present in several cell adhesion molecules. However, in contrast to most of them, RPTPs also contain one or two intracellular phosphatase catalytic domains. RPTPs can bind to themselves (homophilic interactions) as well as to other proteins (heterophilic interactions). The first catalytic domain of RPTPμ has been found to interact with the C-tail on Cx43 in diverse cell systems; such interaction hypothetically might keep Cx43 in a non-tyrosine phosphorylated state, thereby preventing Src-mediated channel closure [86].

Extracellular ATP binds to a plethora of ionotropic (P2X) and metabotropic (P2Y) receptors, which mediate its action. The P2Y receptors (at least ten subtypes) are classical 7-transmembrane domain metabotropic receptors coupled to G proteins. Upon ATP-binding, P2X receptors (classified $P2X_1$ to $P2X_7$) undergo millisecond time-scale transitions that lead to the open I_1 state, allowing the passage

of Na^+, K^+, and Ca^{2+} through the channel pore. In $P2X_2$, $P2X_4$ and $P2X_7$ receptors, the gradual opening over seconds to minutes of a larger pore that allows passage of molecules up to 900 Da is observed. This second open state is referred to as open I_2 state but the mechanisms that underlie its opening still remain unclear. In one model, the I_2 state is proposed to be an intrinsic P2X property whereas in a second one, it is thought to be due to the opening of junctional hemichannels built of connexins or pannexins. In mouse macrophages and J774 cells, Cx43 and $P2X_7$ receptors were found to co-localize and to co-immunoprecipitate with the anti-Cx43 antibody pulling down $P2X_7$ [87]. Panx1 was seen to be co-immunoprecipitated with $P2X_7$ receptors into HEK 293 cells [88] and cultured cortices of embryonic rat [89].

3.6. Calmodulin

Calmodulin (CaM) now appears to be a constitutive or dissociable Ca^{2+}-sensing subunit of a surprising variety of ion channels found in a wide range of species from Homo to Paramecium (for review, see [90]). The ability of a non-specific CaM antagonist (trifluoperazine) to prevent CO_2-induced uncoupling of Xenopus embryonic cells led to the suggestion that CaM may participate in channel gating [91]. This hypothesis was strengthened by evidence for CaM binding to Cx32 in gel overlays [92] and that a fluorescent CaM derivative bound to Cx32 and Cx32 fragments *in vitro* [93]. With inhibition of CaM expression, the slow gating and CO_2-sensitive gating virtually disappeared whereas the expression of a CaM mutant (the N-terminal EF hand pair replaced by a copy of the C-terminal pair) dramatically increased the chemical gating sensitivity of gap junction channels composed of Cx32 and decreased their sensitivity to transjunctional voltage [20], suggesting a CaM participation in this mechanism.

Surface plasmon resonance assays with peptides corresponding to different parts of another Cx, the Cx43, did not reveal molecular binding to CaM, but the authors suggested that interactions might occur via other regions of the Cx43CT or indirectly [22]. In immunoprecipitation assays, CaM has been found to interact with Cx50 but neither with a truncated Cx50 (lacking the C-terminus) nor with C-terminus, suggesting that CaM would not bind directly to C-terminus, but would require the presence of some other part of Cx50trunc, presumably the cytoplasmic loop (CL), to form a CaM, CT and CL protein complex [94]. As emphasized by Harris [10], calcium has a long and contentious history as a modulator of GJIC and might have an indirect action via its interaction with calmodulin (as is the case for different membrane channels; see [10]).

3.7. Enzymes

The C-tail of several connexins (*e.g.* Cx43) contains consensus phosphorylation sites for several protein, and several connexins have been shown to be substrates of several phosphorylation/dephosphorylation catalyzers, including tyrosine and serine/threonine PKs (*e.g.* Src, Protein Kinases A (PKA), C (PKC) and G (PKG), Mitogen Activated Protein Kinase (MAPK), cdc2 and casein kinase 1 and 2 (CK1 and 2)) and protein phosphatases (see for reviews [95–98]). More stable interactions between some enzymes and connexins have also been reported (see Table 3).

3.7.1. Protein kinases

3.7.1.1. Src protein tyrosine kinases. Regulation of Cx43 by Src has been well documented. *In vitro*, Cx43 binds the SH3 domain of v-Src, but not of c-Src, and mutations in the SH3 and SH2 domains of v-Src as well as in the proline-rich region or tyrosine 265 of Cx43, reduced interactions between v-Src and Cx43 *in vivo* [42]. In v-Src-transformed fibroblasts, Cx43 appeared as an endogenous substrate of $pp60^{v\text{-}Src}$; this enzyme and Cx43 were partially co-localized in regions of the plasma membrane and co-immunoprecipitated, showing that the two proteins were

Table 3
Reported interactions of connexins with enzymes.

Partner protein	Via its	Cx	Cx motif	Main approaches[a]	Cell types[b]	References
$pp60^{c\text{-}src}$	SH3	Cx40	A CT-proline rich domain	nmr		[148]
	SH3			at bt nmr		[26]
		Cx43	Y265	ci	Rat-1 cells, *COS-7*, *A431*, *HeLa cells*	[100]
	SH2		C-terminal	at	Neonatal rat cardiomyocytes, *HEK 293*	[65]
	SH3			ci at	C57B16 mouse cortical astrocytes	[101]
	SH3		CT (Lys-264–Lys-287 residues)	nmr		[129]
				ci	Mouse cortical astrocytes	[107]
	SH3		CT, near P ro 280	nmr		[148]
	SH3			at bt nmr		[26]
				ci	Rat Sertoli cells	[205]
$pp60^{v\text{-}src}$	SH3	Cx43	A CT-proline-rich mot if	at ci	*Rat-1 fibroblasts*, *HEK 293 cells*,	[42]
	SH2		Tyrosine 265		*v-Src-transformed fibroblasts*	
			C-terminal	cl ci	*v-Src-transformed fibroblasts*	[99]
PKC_ε		Cx43		cl ci	Neonatal rat cardiomyocyte in culture	[102]
PKC_α and PKC_ε					Human cardiomyocytes	[103]
PKC_γ				cl ci fs	*N/N1003A cells*, bovine lens cells	[116]
		Cx46 and Cx50		cl ci	Rat lens	[104]
$PKC\zeta$		Cx43		cl ci at	TtT/GF cells	[105]
CK1		Cx43		ci	NRK cells	[267]
DMPK		Cx43		cl fs	Rat adult rat	[106]
ERK1/2		Cx43		ci	Mouse cortical astrocytes	[107]
CaMKII		Cx36	Cytoplasmic loop and CT sites	cl bt at	Mouse inferior olive	[108]
		Cx35 (orth. Cx36)		cl ci at	Goldfish hindbrain	[109]
PP1/PP2A		Cx43		cl ci fs	Rabbit and human heart t issues	[111]
PP2A					TtT/GF cells	[105]
RPTPμ		Cx43	C-terminal	ci	Rat-1 cells, *COS-7*, *A431*, *HeLa cells*	[86]
Nedd4	WWs	Cx43	P Y motif at t he CT	cl ci at nmr	WB-F344 cells	[112]
TSG101		Cx45, Cx43, Cx31	C-terminal	ci at bt	HM1 embryonic stem cells	[113]

[a] : bt: biochemical techniques (cell-free assays, chimeras, truncated connexins, mimetic peptides, oligomerization assays, chemical cross-linking tests, …*etc.*); cl: colocalization; ci: co-immunoprecipitation; fs: co-fractionation or co-sedimentation; dh: double hybrid; em: electron microscopy immuno labeling; at: affinity techniques (pull-down, affinity binding assays, surface plasmon resonance); nmr: nuclear magnetic resonance.
[b] : in roman characters, cells where Cxs were endogenously expressed; in italics, cells where Cxs were exogenously expressed, surexpressed or mutated.

associated in a stable complex [99]. Two different hypotheses for Cx43 binding to c-Src have been put forward. Firstly, in cardiac myocytes, constitutively active c-Src (Y527F) was able to phosphorylate Y-265 of Cx43-CT, and this phosphorylated residue behaved as a binding site for the c-Src SH2 domain, in parallel with the disruption of the interaction of Cx43 with ZO-1 [65]. Similarly, in other cell systems, the phosphorylation of Cx43 by c-Src on Y-265 leads to loss of gap junctional communication, which also could be rescued by the Cx43Y265F mutant [100]. Secondly, an interaction between c-Src SH3 domain and polyproline sequences of the C-terminus of Cx43 was suggested to occur under conditions of cellular stress (*e.g.* intracellular acidification) in mouse astrocytes, reversing ZO-1/Cx43CT binding and facilitating Cx43 internalization [101].

3.7.1.2. Protein kinase C. Two PKC isoforms (PKCα and PKCε) are known to localize to plasma membrane sites; in non-stimulated myocytes of new-born rat, PKCε displayed a discontinuous pattern of localization at intercellular contact sites and partial co-localization with Cx43. Treatment with FGF-2 or phorbol 12-myristate 13-acetate (PMA, or 12-O-TetradecanoylPhorbol 13-Acetate, TPA) induced a more continuous pattern of PKCε distribution, and the anti-Cx43 staining appeared to overlap extensively with that of PKCε [102]. In myocardium, both PKCα and PKCε co-localized with Cx43 [103]. In rat cardiomyocytes, PKCε but not PKCα co-precipitated with Cx43 [102] whereas in human cardiomyocytes PKCα was also present in the co-immunoprecipitate complex with either Cx43 or PKCε antibodies [103]. In the latter study, recombinant PKCε or α increased kinase activity significantly in the co-immunoprecipitated Cx43 complexes, but phosphorylation of purified human Cx43 by recombinant PKCα or ε resulted in only PKCε-mediated Cx43 phosphorylation. The TPA-induced activation of PKCγ caused its translocation into membrane fractions, where it phosphorylated Cx50 at serine and threonine residues and Cx46 only at threonine residues; PKCγ, Cx46 and Cx50 then co-immunoprecipitated with caveolin-1 [104]. In TtT/GF cells (a pituitary folliculo-stellate-like cell line), contrarily to PKC-α, the atypical isoform ζ did interact with Cx43 under basal conditions [105].

3.7.1.3. Other protein kinases. Myotonic dystrophy (DM) is a dominantly inherited disease involving skeletal muscles, heart, endocrine organs, lens and the central nervous system. The molecular basis of this autosomal disorder has been identified as the expansion of a CTG repeat in the 3′ untranslated region of a gene encoding a PK, Myotonic Dystrophy Protein Kinase (DMPK). The pathophysiology of the disease and the role of DMPK are still obscure. The main localization sites of this enzyme are neuromuscular and myotendinous junctions, terminal cisternae of the sarcoplasmic reticulum in the skeletal muscle and at IDs in the cardiac muscle. In the last site, DMPK was found co-localized with Cx43 both in the tissue and after immunoblot analysis of partially purified GJ [106].

The disruption of Cx43 function is known to result from the activation of the downstream mitogen-activated protein (MAP) kinase (see for example [97,98]); although the MAP kinases ERK1/2 did not interact appreciably with Cx43 in control mouse cortical astrocytes, they bound strongly (particularly ERK2) to Cx43 during chemical ischemia/hypoxia [107].

Cx36, the most common Cx at electrical synapses, interacts with and is phosphorylated by the calcium/calmodulin (CaM)-dependent protein kinase CaMKII [108], and Cx35, its fish ortholog, is also associated with CaMKII [109].

3.7.2. Protein phosphatases

These enzymes dephosphorylate phosphoproteins in order to regulate their function, thus the role of protein phosphatases is going to be connexin isoform specific due to the fact that not all connexins are phosphoproteins. The major phosphoconnexin is Cx43, dephosphorylable by two protein phosphatases, PP1 and PP2A (see [110]), which co-localize and co-immunoprecipitate with Cx43 in several cardiac preparations, such as normal and an arrhythmogenic rabbit model of non-ischemic heart failure as well as human heart preparations [111]. Other protein phosphatases are also involved in reported Cx interactions with RPTPs (see Section 3.5 above).

3.7.3. Other enzymes

3.7.3.1. Ligases. The ubiquitin protein ligase Nedd4 was shown by pull-down assays to bind to the C-terminus of Cx43 and this observation was confirmed *in vivo* by co-immunoprecipitation and co-localization experiments [112]. Tumor susceptibility gene 101 (TSG101), a human cellular protein belonging to a group of apparently inactive homologs of ubiquitin-conjugating enzymes, was found associated with the C-tail of Cx45, -30.2, -36, and -43 in pull down analyses and Cx31, -43 and -45 co-precipitated with endogenous TSG101 protein in lysates from HM1 embryonic stem cells [113].

3.7.3.2. Synthase. Cx37 binds via its CT to a region of endothelial nitric oxide synthase (eNOS), an enzyme of fundamental importance to vascular biology and disease [114].

3.8. Proteins involved in intracellular protein trafficking

Caveolins-1, -2 and -3 are a multi-gene family of 21–24 kDa integral membrane proteins; they act as scaffolding proteins to cluster lipids and signaling molecules within the caveolae and sometimes to regulate those proteins (including various membrane channels) that are targeted to the caveolae. They are present in specialized lipid raft domains, cholesterol-sphingolipid-rich microdomains that function as platforms for membrane trafficking and signal transduction. Caveolin-1 was found to associate with Cx43 [115,116]. In the former study, some other connexins (Cx32, Cx36, and Cx46) were also targeted to lipid rafts, while Cx26 and Cx50 were specifically excluded from these membrane microdomains.

PKCγ, as other conventional PKC isoforms, is cytoplasmic when inactive; its activation by growth factors (*e.g.* IGF-1) or TPA can translocate it from the cytosol to the membrane and allows it to interact with and to phosphorylate Cx43. This leads Cx43, Cav-1 and PKCγ to redistribute within the lipid rafts, resulting in a decrease in gap junction plaques [116]. In rat epidermal keratinocytes, newly synthesized Cx43 interacts with Cav-1 and Cav-2 in the Golgi complex, and they seem to traffic together to the plasma membrane in lipid rafts; once at the cell surface, the majority of Cx43 dissociates from Cavs before gap junction plaque formation [117].

3.9. Other protein partners of gap junction proteins

Additional protein partners of junctional proteins have been identified (see Table 4). A 150-kDa protein, named Cx43 Interacting Protein (CIP150), which associates with the juxtamembrane region in the C-terminus tail of Cx43 was for example identified in different cell types (HEK 293, HeLa, COS7 and NRK cells; [118]), a 85-kDa protein (CIP85) in HEK cells [119] and a 75-kDa protein (CIP75) in HeLa cells [120]. In the latter case, it was noticed that Cx43 that immunoprecipitated with CIP75 was not ubiquitinated [120].

Organ of Corti Proteins 1 and 2 (OCP1 and OCP2) are relatively small acidic proteins predominantly expressed in the vertebrate auditory system. OCPs (accounting for approximately 10% of the total proteins found in the OC) are components of OC-specific ubiquitin ligases, which presumably bind selected protein targets, positioning them for ubiquitination. Cx26 can be co-immunoprecipitated from extracts of the rat organ of Corti by immobilized anti-OCP1, implying that OCP1 and Cx26 may associate *in vivo* [121] but no evidence was

Table 4
Reported interactions of gap junctional proteins with other partner proteins.

Partner protein	Via its	Cx	Cx motif	Main approaches[a]	Cell types[b]	References
eNOS	aa 843–854	Cx37	C-terminal	bt ci at	*Neuro2A cells*	[114]
α- or β-tubulin		Cx43	35 amino-acid domain juxta-membrane region CT	cl fs at	Rat-1 cells, *COS-7*, A431, *MDCK*, *HEK 293*, T51B and human fibroblasts	[52,53]
β-Tubulin		Cx43		ci bt	HL-1 cardiomyocytes, *HeLa cells*	[217]
			C-loop and C-terminal	at ci	*HeLa cells*	[268]
F-Actin		Cx30		cl ci	*HeLa cells*	[48]
		Cx43		afm	Embryonic rat astrocytes	[49]
				cl ci	*C2C12 cells.*	[51]
		Panx1	C-terminal end	ci fs	BICR-M1Rk	[47]
β-Actin		Cx43		ci	Mouse cortical astrocytes	[107]
Drebrin		Cx43	C-terminal	ci at	*Vero cells*, mouse astrocytes	[50]
Cortactin		Cx43		cl ci	*C2C12 cells.*	[51]
				cl ci	Mouse seminiferous tubules	[269]
Consortin		Cx43		ci at	*HeLa cells*	[157]
Vinculin		Cx43		cl ci	NIH 3 T 3 cells	[79]
αII-s pectrin-SH3i		Cx43		cl ci	Rat and mouse myocardium	[180]
Caveolin-1	aa 82–101, aa 135–178	Cx43		cl ci fs	NIH 3 T 3, *HEK 293T and COS-7 cells*	[115]
		Cx26, -32, -36, -46		ci		
		Cx43		cl ci fs	*N/N1003A cells*, bovine lens	[116]
				cl ci fs	*U251 cells*	[270]
Cav-1, Cav-2			C-terminal	cl ci	Rat epidermal keratinocyte, *HEK*	[117]
Caveolin-3		Cx43		cl ci dh bt	Mouse heart tissue, *HeLa cells*	[271]
β-Arrestin		Cx43	C-terminal S^{368}	bt cl ci	*Osteoblastic cell*	[207]
Ubiquitin		Cx43		ci	E36, BWEM cells	[201]
				ci	IAR20 cells	[202,203]
				cl ci	IAR20 cells	[204]
CIP75	UBA domain	Cx43	PY motif at the CT (residues 264–302)	cl ci	*HEK 293*, *HeLa*, *MDCK,S180*, mouse fibroblasts	[150]
				cl ci at	*HeLa cells*	[120]
CIP85	SH3	Cx43	CT (a Proline rich region, P^{253} LSP^{256})	ci dh	*HEK 293 cells*	[119]
CIP150		Cx43	CT (residues 227–242)	dh at	*HEK 293*, *HeLa*, COS7 and NRK cells	[118]
OCP-1		Cx26		ci at	Rat cochlea	[121]
NOV		Cx43	C-terminal	cl ci at	*C6 cells*, mouse astrocytes and different human glioma cell lines	[124]
			CT (residues 257–382)	ci	*Jeg3 cells*, *HEK 293T cells*	[123]
Dlgh1	SH3/Hook	Cx32	C-terminal and cytoplasmic loop	cl ci dh at	Mouse liver, *SKHep cells*	[125]
BDIF-1	SH3	Cx43	C-terminal (a Proline rich region)	cl ci	*HEK 293 cells*	[149]
Tom20, Hs p90		Cx43		ci	Pig cardiac mitochondria	[126]
ANT				cl ci		
Kvβ3		Panx1		cl dh	*Neuro2A cells*	[272]

[a] : bt: biochemical techniques (cell-free assays, chimeras, truncated connexins, mimetic peptides, oligomerization assays, chemical cross-linking tests, ...*etc.*); cl: colocalization; ci: co-immunoprecipitation; fs: co-fractionation or co-sedimentation; dh: double hybrid; em: electron microscopy immuno labeling; afm: atomic force microscopy; at: affinity techniques (pull-down, affinity binding assays, surface plasmon resonance); nmr: nuclear magnetic resonance.
[b] : in roman characters, cells where Cxs were endogenously expressed; in italics, cells where Cxs were exogenously expressed, surexpressed or mutated.

found of Cx26-OCP1 (also called Fbx2) similar association in the mouse organ of Corti [122].

Nephroblastoma OVerexpressed (NOV) is a member of the CCN (for CeflO/Cyr61 and Nov) family, a group of structurally related, secreted, extracellular matrix-associated proteins involved in diverse biological processes like proliferation, differentiation, adhesion, migration and angiogenesis. NOV/CCN3, which may be involved in cell cycle control (see [123]), was found to interact with the C terminus of Cx43 [123,124].

Disks large homolog 1 (Dlgh1), the mammalian homolog of the *Drosophila* disks large, is another MAGUK protein family which has been suggested (see [125]) to down regulate cell proliferation. Cx32 was found to interact via its cytoplasmic loop and carboxyl tail with the SH3/HOOK domain of Dlgh1 [125].

Cx43 was co-purified when pig cardiac mitochondria were subjected to immunoprecipitation for Tom20 (Translocase of the outer membrane 20), with Hsp90 (Heat shock protein 90) and with ANT (Adenine Nucleotide Transporter). This led to the suggestion that Cx43 interacted with the regular mitochondrial protein import machinery [126].

4. Possible structural domains of junctional proteins involved in interactions with partner proteins

4.1. Structural domains of connexins

Cx43 is the most ubiquitous and the most extensively studied connexin; its large C-terminal domain, making a cytoplasmic fuzz visible under electron microscopy, is likely to be relatively flexible. This carboxyl terminus is not required for formation of functional channels but is critical for the regulation of GJIC. Intramolecular particle–receptor interaction between the CT domain and the cytoplasmic loop, whereby amino acid residues of the cytoplasmic loop act as receptor to which the CT can bind ("ball-and-chain" closure mechanism, see Section 2), have been put forward for the regulation of GJIC upon acidification [22] or voltage difference (*see* [127]). In the "cork-type" gating model proposed by Peracchia et al. [20], a CaM lobe would physically obstruct the cytoplasmic mouth of the channel pore. Apart from these intramolecular interactions, the CT of Cx43 interacts with several partner proteins (Fig. 1).

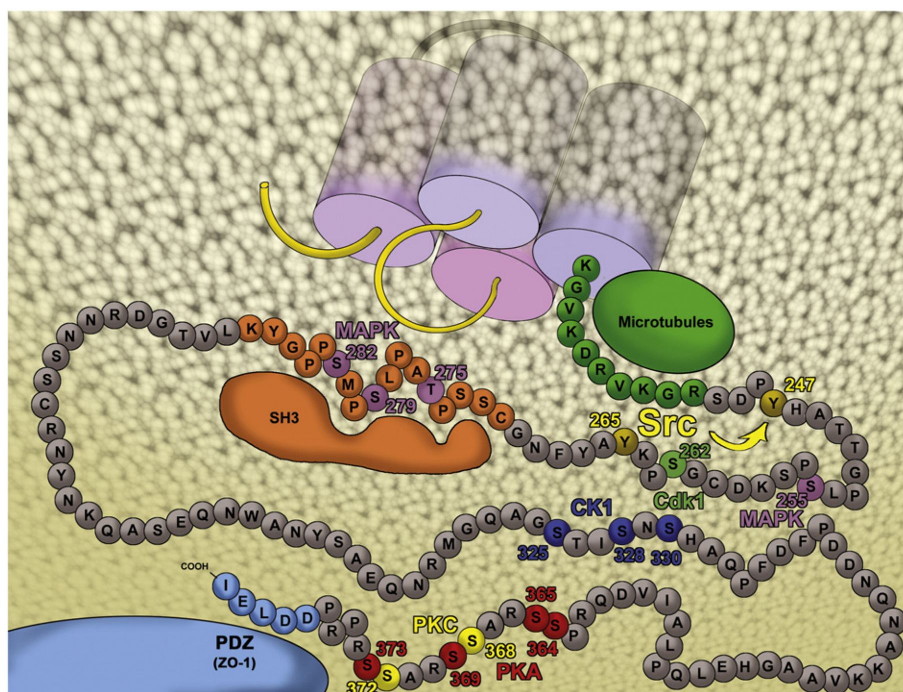

Fig. 1. Possible domains of Cx43 involved in interactions with partner proteins. Several domains of the C-terminal tail of the protein have been found involved in protein–protein interaction with partner proteins.

4.1.1. ZOs

Except Cx36 (and its fish ortholog, teleost Cx35), which interacts with PDZ-1 of ZO-1, Cxs (Cx30, -31.9, -43, -46, -47 and -50) mainly interact with PDZ-2 of ZO-1 and, to a lesser extent, ZO-2; only Cx36 and Cx45 have been found to interact with ZO-3 (see Table 2). Cx43 associates with ZO-1 via its extreme CT; Jin et al. [128] suggested that this interaction would take place through a typical Class II PDZ binding domain. NMR titration experiments determined that the ZO-1 PDZ-2 domain affected the last 19 amino acid residues of the CT of Cx43 [129]. Jin et al. [128] emphasized the fact that Cx31.9, Cx43, Cx46 and Cx50, in contrast with Cx32, exhibited similar PDZ binding motifs (D-L-X-I) in their CT. Cx31.9 [130], Cx45 [131], Cx46 [132], Cx47 [133] and Cx50 [134] also can interact with ZO-1 via their C-terminus whereas Cx36 binds PDZ-1 of ZO-1 [135,136]. Cx36 indeed, in contrast with the first connexin group, contains the C-terminus amino acids Y-V, the binding motif domain present in the C-terminus of most of the claudins and reported to be responsible for their interaction with the PDZ-1 domain of ZOs [137]. A domain of 14 C-terminus amino acids (and particularly the last 4 ones) of the Cx36 sequence appeared to be required for Cx36 interaction with the PDZ-1 domain of ZO-1 since a peptide corresponding to this region showed binding capacity to the PDZ-1 domain of ZO-1 behaved as a competitive inhibitor of Cx36/ZO-1 interaction *in vitro* [135]. Sequence analysis and immunocytochemical data suggested that Cx36 might directly interact with ZO-2 at mouse retinal gap junctions, but an indirect association, via a partner protein (*e.g.* ZO-1) remained however possible [138].

ZO-1 binding by Cx43 was lost when phenylalanine residues were substituted for ^{375}Pro and ^{377}Pro, showing that Cx43 localization to a gap junction implies a specific structural conformation at residues ^{374}Arg–^{379}Asp that is also important for ZO-1 binding [139].

As ZO-1 was able to bind to a truncated Cx45 protein lacking the canonical PDZ binding domain-binding present at the C-tail, Laing et al. [140] suggested that Cx45 might have a large and complex binding site for ZO-l, comprising the residues between amino acid 357 and the Cx45 C-terminus, an alternative possibility being the existence of 2 distinct binding sites, one involving the C-tail and the second the amino acids proximal to amino acid 360. The authors however did not exclude either the possibility of an artifactual ZO-1/Cx45 binding or of an indirect interaction (*e.g.* an association of Cx45 with Cx43 bound to ZO-1).

4.1.2. Calmodulin

To transduce changes in $[Ca^{2+}]_i$ into diverse activities and function, CaM exhibit different specificities on binding to a wide range of target proteins. According to Zhou et al. [141], subtle changes in $[Ca^{2+}]_i$ would be sensed by the "high-affinity" C-domain of CaM, enabling it to preferentially interact with the intracellular loop of Cx proteins whereas higher $[Ca^{2+}]_i$ elevations would trigger the fully open conformation able to completely inhibit GJIC, via a mechanism not yet elucidated. Moreover, the molecular scenario of CaM effects seems different for the α-class (Cx43, -44, -50) and the β-class (Cx32), probably due to the fact that the α-class Cxs lack the NT glycine hinge and possess longer intracellular loops and CT than the β-class Cxs [142]. According to these authors, Ca^{2+}-CaM interaction with α-class Cxs would occur via a direct association with their intracellular loop region. Török et al. [93] identified a high-affinity CaM-binding region in the NT of Cx32 that is absent in the lens Cxs (Cx43, -46 and -50). In these Cxs, high-affinity CaM-binding sites have been identified, encompassing residues 138–157 for Cx43 [143], 132–153 for human Cx46 [141] and 141–166 in Cx50 [142]. These sequences, with conserved hydrophobic residues, are similar to CaM-binding motifs found in different other proteins (*e.g.* calcineurin, adenylate cyclase, *etc.*, see [142]).

4.1.3. Caveolin

Cx43 appears to interact with two distinct caveolin-1 domains, *i.e.* the caveolin-scaffolding domain (residues 82–101) and the CT domain (135–178); a mutational analysis of Cx43 revealed that the hypothesized PDZ- and presumptive SH2/SH3-binding domains within the Cx43-CT were not required for this targeting event or for its stable interaction with caveolin-1 [115]. Lin et al. [116] noticed that Cx43 has in residues 25–32 of the first transmembrane sequence a perfect consensus sequence for binding to a Cav-1 scaffolding domain. The region surrounding S364 in the C-terminus was suggested to be involved in the interaction of Cx43 with intracellular trafficking proteins [144].

4.1.4. Protein kinases

Cx43/v-Src association is mediated by interactions between the SH3 domain of v-Src and a proline-rich region of Cx43 and by the SH2 domain of v-Src and tyrosine 265 of Cx43 [42,145], and it was suggested that such interaction might induce a structural change in the C-terminus region of Cx43, thereby hindering the interaction between Cx43 and the ZO-1 PDZ-2 domain [65]. SH3 domains (50–70 amino acids long) possess three shallow pockets where the peptide ligands bind. Two of the pockets are 2.5 nm long and 1 nm wide, large enough to accommodate each of the prolines in the PxxP motif, accompanied by a hydrophobic residue (*i.e.*, A, I, L, V, and P). Proline is indeed unique among the 20 common amino acids in having the side-chain cyclized onto the backbone nitrogen atom. This means that the conformation of proline itself is limited, with backbone angles of ~− 65°. It also restricts the conformation of the residue preceding the proline because of the bulk of the N-substituent and results in a strong preference for a β-sheet conformation. As a consequence, polyproline sequences tend to adopt the PP II helix, which is an extended structure with three residues per turn. This implies that the two prolines in the SH3 domain ligand core, PxxP, are on the same face of the helix and are thus well placed to interact with the protein (see [146]). Such sequence-specific ability of proline-rich regions to interact with other proteins might explain how a peptide of the same sequence as amino acids 271 to 287 of Cx43 (CSSPTAPLSPMSPPGYK) can prevent pH gating of Cx43-expressing oocytes [147]. As the Cx40-CT sequence does not content a consensus PXXP binding motif, the SH3 domain of Src was proposed to interact with domains rich in proline and/or positively charged residues of Cx40-CT [148].

Alev et al. [108] identified 2 juxtamembrane cytoplasmic sequences of Cx36 that serve as potential binding sites for CaMKII and proposed a model of mechanistic framework for CaMKII/Cx36 interaction. Upon local Ca^{2+} elevation, Ca^{2+}/CaM would bind to the autoinhibitory domains of CaMKII, allowing the cytoplasmic loop binding site of Cx36 to interact with the catalytic site of the kinase.

4.1.5. Other protein partners

The Cx43 35-amino acid juxtamembrane region is necessary and sufficient for tubulin binding. This motif (^{234}KGVKDRVKGK^{243}, residues typically found in tubulin binding domains are underlined) is unique for Cx43 and was not found in the sequence of other connexin family members [52]. CIP150 was shown to associate with the region corresponding to 16 amino acids in the juxtamembrane region in the CT tail of Cx43 (amino acids 227–242; [118]) whereas CIP85–Cx43 interaction would depend on the SH3 domain of CIP85 and a proline-rich region of Cx43 (^{253}PLSP^{256}; [119]). The proline-rich regions of Cx43 (^{253}PLSP^{256}, ^{274}PTAPLSPMSP^{283}) also appear to exhibit particular affinity and selectivity toward SH3 domains, and Cx43 was found to interact with BDIF-1 (Brain-Derived Integrating Factor-1; [149]). CIP75, which contains an ubiquitin-associated (UBA) domain at its C terminus and an ubiquitin-like (UBL) domain at its N terminus, was found to interact with Cx43 via its UBA domain [150].

The growth regulator NOV (CCN3) directly interacts with the C-terminus of Cx43 [124] in the region between amino acids 257 and 382 [123]. The interaction of Cx43 with Nedd4 involves all three WW domains of the latter; furthermore, whereas WW1 and WW2 domains mainly interact with the unphosphorylated form of Cx43, WW3 binds phosphorylated and unphosphorylated forms equally. In addition, the surface plasmon resonance approach revealed that only the WW2 domain binds to the PY motif located at the C-terminus of Cx43 [112]. A peptidic sequence homologous to a region of endothelial nitric oxide synthase (eNOS), an enzyme of fundamental importance to vascular biology and disease binds to Cx43-CT [114].

In the dual model proposed by Alev and co-workers [108] for CaMKII-Cx36 interaction, the binding of Ca^{2+}/CaM to CaMKII would allow the interaction of CaMKII with the cytoplasmic loop of Cx36, triggering CaMKII autophosphorylation, leading to binding of the activated kinase to a CT-site of Cx36.

Truncation experiments of Cx32 suggested that a crucial gap-junction targeting sequence resides between amino acid residues 207 and 219 on the cytoplasmic C-tail [151]. In hepatocytes from Cx32-deficient mice transfected with Cx32, Cx26, or Cx43 cDNAs, endogenous occludin was in part localized at cell borders, co-localized with Cx32, whereas neither was detected in parental cells nor in Cx26 or Cx43-transfected cells. Several amino acid sequences in Cx32 were proposed as CaM-binding motifs, including residues 1–21 (NT) and 216–230 (CT) [93] and parts of the third transmembrane domain and of the CT-tail [152]. ^{175}Asparagine of Cx32 was proposed to be a critical residue for docking and forming functional heterotypic gap junction channels with Cx26 [153].

4.2. Structural domains of innexins

Inx2 and Inx3 form heterodimers *in vivo* in *Drosophila* embryos, with a binding of CT of Inx2 to the CT of Inx3 [33]. Moreover, in contrast to the selective binding behavior of the Inx2 C terminus, the CT of Inx3 has the potential to interact more broadly (*e.g.* to heteromerically interact with the cytoplasmic loop domain of Inx2). RNA aptamers capable of binding to a proximal epitope on the CT of Inx2 specifically inhibited the heterologous interaction of Inx2 and Inx3 CTs *in vitro* [154].

5. Functional importance of protein–protein interactions

5.1. Channel functions

A model for gap junction formation has progressively emerged from biochemical and cell biological studies. The constituent proteins of gap junctions are, as many other integral plasma membrane proteins (for example other channels, other intercellular junctions or different membrane receptors) renewed several times a day (for review, see [155]). Potential interacting partners (including cytoskeletal proteins, scaffold proteins, enzymes, *etc.*) play important roles in correct subcellular localization of gap junction channels and in functional regulation of gap junction-mediated intercellular communication:

5.1.1. Synthesis, transport to the plasma membrane and insertion of proteins

Connexins enter the secretory pathway and are delivered to the plasma membrane by way of the endoplasmic reticulum and the Golgi complex; evidence have however been brought that Cx26 can reach the cell surface via a Golgi-independent pathway (see for example [156]). del Castillo et al. [157] identified consortin as the first potential trans-Golgi network cargo receptor for the sorting and cell surface targeting of transmembrane proteins, able to simultaneously interact with the connexins to be sorted and with the adaptor machinery.

Microtubules are highly dynamic cytoskeleton structures formed through the polymerization of α- and β-tubulin dimers elongating existing microtubules and have a well-studied role in the targeted Cx43 delivery at the cell membrane [76,158–160]. Individual microtubules typically emanate from the microtubule organizing centers (MTOCs, *e.g.* centrioles or basal bodies), anchored via their “minus” ends. The microtubule polarity is important for protein transport since the motor proteins kinesin and dynein typically slide preferentially in either the “plus” or “minus” direction, respectively, along the microtubule, allowing vesicles to be directed to or from the endoplasmic reticulum and Golgi apparatus. Shaw et al. [76] proposed a model of direct targeting of hemichannels to cell–cell junctions in which the microtubule plus-end-tracking protein EB1 (able to co-immunoprecipitate with Cx43) binds, via its C-terminus, to

p150[GLUED], a subunit of the dynein/dynactin complex, which localizes with adherens junctions through direct binding with β-catenin. In *in vitro* assays using isolated Cx32-containing vesicles from rat hepatocytes able, when stimulated by the addition of ATP, to move along the microtubules, Cx32 and kinesin motor proteins were found in the same vesicles [161]. In the model proposed by Rhett et al. [162], adherens junctions nearby to GJs might act as sites of preferential delivery of connexons to nonjunctional plasma membrane proximal to GJs.

ZO proteins are at the center of a network of protein interactions, linked to the actin cytoskeleton via their CT, to Cxs via their NT. In the mouse retina for example, ZO-1, interacting with Cx45 via its PDZ-2 domain and with Cx36 via its PDZ-1 domain, provides for co-scaffolding of Cx45 with Cx36, forming "bihomotypic" gap junctions, with Cx45 structurally coupling to Cx45 and Cx36 coupling to Cx36 [163]. The critical role of ZO-1 in mediating this association by tethering the two Cxs within gap junctions was indicated by abolition of Cx36/Cx45 co-IP after removal of the C-terminal 4 aa residues of Cx36, required for the interaction of Cx36 with the PDZ-1 domain of ZO-1 [163]. The fact that ZO-1 and ZO-2 seem to compete throughout the cell cycle for association with Cx43 led Singh et al. [64] to suggest that the ratio of ZO-1 and ZO-2 interacting with Cx43 may influence the stability and/or stabilization of gap junctions. As ZO-1 can target to the periphery of Cx43 junctional plaque independently of PDZ-2-mediated interactions, Hunter and Gourdie [164] put forward a targeting sequence that would initially involve ZO-1 bound to junctional complexes (possibly N-cadherin-based) adjacent to GJs, followed by a transfer of ZO-1 and its direct engagement with Cx43 at GJ edges. In Cx43-HeLa cells, actin co-immunoprecipitated with ZO-1 only in the Triton-insoluble fraction, suggesting that ZO-1, actin and Cx43 were present in the same multiprotein complex only when connexons were aggregated in detergent-resistant GJs [162].

5.1.2. Cellular selectivity of gap junction formation

The *de novo* formation of a GJ plaque, its growth and disassembly are still poorly understood. A connexon present in the plasma membrane docks with a connexon in the contiguous cell membranes to form an intercellular channel and a GJ plaque is formed when several intercellular channels cluster.

The extracellular domains of connexons may also dock with connexons of adjoining cells and thereby link adjacent cells independently of channel formation, through a mechanism similar to cell adhesion molecules (CAMs) [165–168].

Nevertheless, a close proximity between the plasma membranes of neighboring cells mediated by anchoring junctions facilitates GJ formation and an intimate linkage between gap junction and adherens junction formation has frequently been reported (for review, see [169]). Cadherins are generally regarded as the main driving force of self-assembly. In human squamous carcinoma cells, E-cadherin-mediated cell–cell adhesion facilitated the growth and assembly of only preformed GJs but was not sufficient to trigger the assembly of GJs *de novo* [170]. In rat liver epithelial cells, antagonist effects of E-cadherin and N-cadherin on the assembly of Cx43 into GJs (with E-Cadherin facilitating and N-Cadherin inhibiting the assembly) were observed [171].

However, full lateral overlap of cadherin transdimers requires a distance of 20–25 nm between contiguous plasma membranes [172] whereas connexons only protrude very slightly (see [173]) from the membrane surface, the intercellular space being 2 to 3 nm wide. In lens fibers, interaction between the extracellular loop domains of two apposing AQP0 molecules were proposed to allow the formation of 11–13 nm thin junctions [174].

Liu et al. [83] observed that AQP0 interaction with Cx50 (the human ortholog of chick Cx46.5) enhanced gap junction coupling and hypothesized that the cell-to-cell adhesion function of AQP0 might serve to increase the proximity of adjacent cells, enhancing the probability of connexon docking.

5.1.3. Opening of intercellular channels

The mechanisms of connexon assemble to form intercellular channels are also still poorly understood and involve interactions between the extracellular loops. Unapposed hemichannels, able to open under certain conditions, must remain closed most of the time to maintain the plasma membrane permeability barrier. The opening of the "loop gates" when hemichannels dock allows a transition to the junctional configuration. The second extracellular loop would be a particularly important determinant of heterotypic compatibility [10,175].

Ahn et al. [176] observed that if deleting most of its CT did not prevent Cx32 from forming functional channels, to substitute the intracellular loop and/or tail of Cx32 with those of Cx29 prevented Cx32 from forming functional gap junctions. Cx33, a Cx specifically expressed in the testis, was seen to form with Cx43 a complex that was sequestered in the intracellular compartment within early endosomes, causing a marked inhibition of GJIC between Sertoli cells [177].

5.1.4. Localization of gap junction plaques

Cytoplasmic scaffold proteins appear to play key roles in the assembly of membrane specialized areas (*e.g.* cellular junctions, channel or receptor clusters, *etc.*), organizing membrane proteins into specialized membrane domains. Cytoskeletal-based perimeter fences were for example seen to selectively corral a membrane-protein subpopulation of potassium channels (Kv2.1 channels) to generate stable 1–3 μ^2 clusters [178]. These authors noticed that despite the stability of these microdomains, the channels retained within the cluster perimeter were surprisingly mobile, showing that the clustering did not result from a static scaffolding-based structure. Connexin channels clustered in gap junctional plaques share these characteristics, where ZO-1 is preferentially localized at the periphery of the Cx43 [61–63], or Cx50 [179] plaques, suggesting that a ZO-1-actin perimeter fence could selectively corral gap junction channels. ZO-1 might act as part of a dynamic mechanism for apportioning connexons between free hemichannels and GJs [162].

In some cell types, gap junctions are preferentially located in specific membrane areas; in adult heart for example, Cx43 gap junctions localized to the IDs of myocytes.

The link between Cx43 and α-spectrin, via ZO-1, was suggested to serve to localize Cx43 at the cardiac IDs [59]. Ursitti et al. [180] observed an interaction between Cx43 and a particular alternatively spliced isoform of αII-spectrin containing a 20-amino-acid insert adjacent to its SH3 domain and suggested that the localization of Cx43 to gap junctions would require the αII-SH3i form of αII-spectrin and that this activity would be regulated by the JNK pathway. The cytoskeletal adaptor protein Ankyrin-G (AnkG) might act as organizing center of subcellular microdomains, clustering protein complexes of complementary function, as, at cardiac IDs, Nav1.5, the desmosomal component PKP2, and Cx43 [181].

A molecular mechanism of Cx43 lateralization was for example proposed by Kieken et al. [182], where intracellular acidification caused by coronary occlusion led to Phospho-c-Src translocation to the ID, allowing its interaction via its SH3 domain with the PDZ-2 domain of ZO-1, as well as 2 additional lower-affinity Cx43CT domains, causing displacement of the Cx43CT from ZO-1. This enabled Cx43 to move in the membrane lipid bilayer from the ID to the lateral membrane, causing decreased conduction velocity and formation of an arrhythmogenic substrate.

5.1.5. Properties of gap junctional channels

The formation of GJ channels by two or more Cxs enables cells to produce channels that have unique permeability and gating characteristics that could not be obtained using a single Cx [127,183,184].

Under experimental conditions, many Cxs showed the ability to form heterotypic channels with different connexins (see for example [10]), but the reported cases in native cells are still in limited number (see Table 1).

5.1.6. Modulation of junctional channel activity

Ca^{2+}, the first identified agent able to cause a "decoupling of the highly permeable intercellular bridges" [185] was proposed to act via CaM, through an interaction between Ca^{2+}-bound CaM and one or more intracellular domains of Cxs [20,91,186]. How CaM controls the chemical gating of hemichannels or intercellular channels is not yet understood, but Dodd et al. [186] proposed a CaM-Cx32 model of interaction via a *trans*-domain or *trans*-subunit bridging. A "cork-type" gating was also proposed for Ca^{2+}-CaM-induced inhibition of GJIC mediated by the lens Cx43 [143], Cx44 [141] and Cx50 [142].

Reversible and dynamic protein phosphorylation is a pivotal mechanism in the regulation of essentially all cellular functions, including GJIC, but the precise mechanisms remain unclear. The anchoring of involved enzymes at specific subcellular sites is critical for their functioning. The fact that junctional channel activity is well preserved during intracellular cell dialysis (in conventional whole cell mode of the patch-clamp technique (see [187])) and even after membrane excision (when the membrane of one of the cells is mechanically ruptured whereas the second cell is investigated; [188]) shows that enzymes important in the regulation of the channel permeability are firmly anchored in the channel vicinity. In the scaffolding model proposed by Laing et al. [189], the different domains of ZO-1 serve as docking modules for enzymes (*e.g.* PKs and PPs), localizing them in the vicinity of junctional channels. The balance between Cx43/c-Src and Cx43/ZO-1 associations for example may influence the activity of junctional channels.

However, this GJIC modulation is much more complex than direct connexin phosphorylation and increasing evidence indicates that protein partners are involved. This view is supported by a lack of correlation between the degree of junctional coupling and the pattern of Cx43 or Cx45 phosphorylation, suggesting that the participation of additional regulatory component is required (for review, see [96]). However, several associations of Cxs with protein partners were found to depend on the phosphorylation status of Cx43, as the binding of c-Src to Cx43 (blocked by inhibition of Cx43 dephosphorylation; [107]) or the interaction between Cx43 and the rat Nedd4 domain WW3 and possibly that of domain WW1 (prevented by the specific phosphorylation of Cx43 at S279 and S282; [112]).

Several stimuli (for example ischemia, oxidative stress, activation of tyrosine kinases, of different PKC isotypes, of the MAP kinase signaling pathway, *etc.*) frequently lead to an interruption of the GJIC very likely by affecting the association between connexins and their protein partners, with consequences on the strength of intercellular coupling (for detailed review, see for example [190]). More recently, Cx43 interaction with the atypical PKC-ζ was found in TtT/GF cells to be reduced by acute treatment with TNF-α, causing a transient intercellular uncoupling, showing that TNF-α maintained the enzyme apart from Cx43 to prevent the phosphorylation of Ser368 [105].

Consequences of the binding of the c-Src SH3 domain to Cx43 and to Cx40 differ on the interactions of these Cxs with ZO-1. It can indeed disrupt the Cx43/ZO-1 interaction, leading to down-regulation of GJIC [65,101,129] whereas it did not affect the Cx40-CT/ZO-1 PDZ-2 domain complex [148].

Cx43-based cell-to-cell communication between Rat-1 fibroblasts was seen to be inhibited by depletion of phosphatidylinositol 4,5-bisphosphate (commonly known as PIP_2), a compound functioning as an intermediate in the inositol 1,4,5-triphosphate and diacylglycerol (IP3/DAG) pathway, which is initiated by ligands binding to G protein-coupled receptors activating the $G\alpha_q$ subunit. PIP_2 is a substrate for hydrolysis by phospholipase C (PLC). Inhibiting PIP_2 hydrolysis kept Cx43 channels open after receptor activation [191]. As ZO-1 binds directly to the very C terminus of PLCβ3 via its third PDZ domain, it was proposed to be a key intermediate in assembling Cx43 and PLCβ3 into a complex, thereby facilitating regulation of Cx43 channel function via PIP_2 hydrolysis upon receptor activation [191].

Compared to cells where only one pannexin is expressed, the combination of Panx1 and Panx2 resulted in a reduced dye uptake in 293T cells [34].

5.1.7. Degradation of gap junctions

The strength of cell-to-cell communication is regulated by the gating of the channel pore but largely depends also on the number of intercellular channels present in gap junction plaques. Newly formed channels are constantly being added to the periphery of existing plaques whereas older portions are internalized from the center of the plaque (see [192]). Several of connexin partners have been reported to contribute to the degradation of junctional plaques, particularly:

5.1.7.1. ZO-1. Earliest observations had revealed that gap junction fragments were degraded by internalization of a double-membrane structure called "annular junction" into one of the two cells, where subsequent lysosomal or proteasomal degradation occurred, except in some cases where the connexons were recycled. Elements of the actin cytoskeleton were seen closely associated with annular junctions [193]. ZO-1 was suggested to confer specificity to actin-based contractile processes via its ability to simultaneously interact with Cx43 and cytoskeleton [194]. In 42GPA9 Sertoli cells, Gilleron et al. [195] noticed that if ZO-1 was usually localized on both sides of the gap junction plaque, its presence became restricted to one side of the junctional plaque during the endocytic GJ internalization and the formation of annular junctions, suggesting that the dissociation of ZO-1 from Cx43 – preferentially on one side of the junctional plaque – had a prominent role in GJ endocytosis. In HeLa cells stably expressing tagged Cx43 (Cx43-YFP, Cx43 tagging being known to prevent ZO-1 binding), GJ internalization did not occur in response to inflammatory mediators, showing that ZO-1-Cx43 interaction is required for GJ internalization [196].

c-Src appears involved (via modifications in the Cx43/c-Scr/ZO-1 complex) not only in the modulation of the functional state of junctional channels [101,107] but also in the stability of GJ plaques, facilitating the GJ internalization [101,107,195] and subsequent degradation of annular junctions [197]. According to Akoyev and Takemoto [198], the close proximity of the ^{368}Ser/PKCγ phosphorylation site to the PDZ/ZO-1-binding domain on the Cx43-CT suggests that ZO-1/Cx43 effects and PKCγ effects may be connected, ZO1 being able to modulate the interaction of PKCγ with Cx43 and, thus, the PKCγ-driven gap junction disassembly of Cx43.

5.1.7.2. Ubiquitin and/or partners. Attachment of ubiquitin to cellular proteins is a major post-translational modification that can have profound effects on protein stability, localization or interaction pattern (for references, see [192]). Conjugation of ubiquitin is a complex enzymatic process that leads to the formation of an isopeptide bond between the C-terminal Gly of ubiquitin and the ε-amino group of a Lys residue of the substrate protein [199]. Due to the presence of internal Lys residues, ubiquitin can repeatedly be attached to itself, forming chains with various topologies and functions. Attachment of a single ubiquitin marks substrate proteins for degradation in lysosomes whereas polyubiquitination targets the substrate to the proteasomal pathway [200]. The mechanism of proteasomal degradation of connexins is still poorly understood; adaptor proteins might bind both Cx43 and a proteasomal component (see for example [150]).

Sequential immunoprecipitation using anti-Cx43 and anti-ubiquitin antibodies demonstrated polyubiquitination of Cx43 in cultured E36 hamster cells [201]. To discriminate whether Cx43 was mono- or polyubiquitinated in response to TPA treatment, Leithe and Rivedal [202] used antibodies that differentiate between different

forms of ubiquitinated proteins and observed that, in IAR20 cells, Cx43 was monoubiquitinated rather than polyubiquitinated in response to PKC activation.

CIP75, able to interact with Cx43 (via its UBA domain) and with subunits of the proteasome complex (through its UBL domain) had been proposed to function as an adaptor or shuttle factor in the degradation of Cx43 via the proteasomal pathway [150]. However, in subsequent observations from the same group, CIP75, despite the fact it is an ubiquitin-binding protein, did not appear to interact with ubiquitinated Cx43, as if Cx43 was one of the rare cases of non-ubiquitinated proteins targeted for proteasomal degradation [120]. In the model proposed by these authors, CIP75 would interact with non-ubiquitinated Cx43 in the ER and then acts as a shuttle to transport Cx43 from the ER to the proteasome for degradation. TSG101 might also be involved in the proteasomal degradation of connexins [113]. The CIP85-Cx43 interaction was reported to stimulate the degradation of Cx43 through a currently unknown mechanism involving the lysosomal pathway [119]. The EGF- and TPA-induced degradation of Cx43 was found to be preceded by Cx43 ubiquitylation [203–272]. Ubiquitin recruitment was particularly pronounced in gap junction regions that were in the process of internalizing into one of the adjacent cells [204]. After PKC activation, entire gap junction plaques were, in IAR20 cells, sometimes coated with ubiquitin, suggesting that internalization of junctional plaques would not only concern the center of the plaque as previously observed [158], but also its periphery [204].

5.2. Importance in protein partner activities

Several gap junction proteins, besides their role as pore-forming subunits, mediate gap junction- and hemichannel-independent effects in cell growth, differentiation, tumorigenicity, injury, and apoptosis, but the mechanistic aspects of such actions still remain largely unknown. One plausible mechanism would be regulating effects of GJ proteins through their interactions with partner proteins (for detailed review, see for example [190]).

5.2.1. Importance in expression, localization and activity of partner proteins

The organization of metazoa is based on the formation of tissues made of individual cells connected by different structures (intercellular junctions) made of densely packed transmembrane proteins linked to proteins anchored on cytoskeleton filaments. The different intercellular junctions frequently present intermingled relationships where the different proteins co-assemble into macromolecular complexes and their expressions are co-ordinately regulated (see [169]). The connexon-mediated cell adhesion may play in microtissue self-assembly a role comparable to that of cadherins, and cells expressing chimeric connexins capable of docking, but not channel formation, show no change in their ability to migrate or adhere (see [168]). In the rat embryonic cerebral cortex for example, gap junctions do not mediate neuronal migration via their channel properties but provide dynamic adhesive contacts that interact with the internal cytoskeleton to enable leading process stabilization along radial fibers as well as the subsequent translocation of the soma [166].

Cx43 and plakophilin-2 (PKP2) form a protein complex within the desmosome-like junction to regulate cell adhesion at the blood-testis barrier (BTB) formed by adjacent Sertoli cells. BTB is regarded as one of the tightest blood–tissue barriers in mammals but must however undergo extensive restructuring to allow the transit of primary spermatocytes (see [205]). The knockdown of both Cx43 and PKP2 caused redistribution of occludin and ZO-1 from the cell-cell interface and perturbation of the barrier function of the Sertoli cell tight junction [205]. Cx43 was suggested to be responsible for the necessary cross-talk between different junction complexes to maintain their adhesive function and to coordinate them to maintain the immunological barrier integrity during the spermatocyte transit [206]. Downregulation of Cx37 by Cx37 antisense leads to an increased nitric oxide (NO) production [114].

The inter-dependency of junctional multiprotein complexes is for instance illustrated by the fact that subcellular targeting of MUPP-1 as well as Cx32 and ZONAB (ZO-1-associated Nucleic Acid Binding protein) to mouse oligodendrocyte gap junctions required the presence of Cx47, which is also necessary for assembly and maintenance of Cx43 at oligodendrocyte/astrocyte gap junctions [72].

Systemic elevation of parathyroid hormone (PTH) leads to a potent bone anabolic effect, except in mice lacking Cx43, the most abundant Cx expressed in bone cells (see [207]). Cx43 expressed in osteoblasts was proposed to sequester a pool of β-arrestin by interaction with its phosphorylated S^{368}, allowing PTH-dependent pro-survival signaling downstream of cAMP. PTH would induce Cx43 dephosphorylation (likely by activating protein phosphatases), leading to the release of β-arrestin. Free β-arrestin would then bind to the PTH Receptor-1 (PTHR1), causing inhibition of cAMP production, and internalization of PTHR1. In Cx43-deficient osteoblasts, a larger pool of β-arrestin is available to bind to PTHR1, thus blunting cAMP accumulation, transcription of cAMP target genes, and survival signaling induced by PTH [207].

Purinergic signaling plays particularly important roles in paracrine signaling, which allows multiple cells within a tissue to actively respond to environmental stresses (as metabolic inhibition, mechanical shear or microbial invasion for example) sensed by only a few cells at the site of environmental insult or stimulation. Panx1 appears as the molecular substrate of a large pore activated by either metabotropic P2Y [208] or ionotropic $P2X_7$ [88,209–212] receptors, allowing the diffusion of molecules up to 900 Da.

In cardiac IDs, according to Sato et al. [181], desmosomes, gap junctions and sodium channels would not be independent but rather act as a “functional triad” where changes in composition of one could affect the function and integrity of the other, with significant consequences to electric cardiac synchrony.

5.2.2. Transcription regulation

ZOs, associated in junctional multiprotein complexes, are also known to be engaged in the transmission of signals from the plasma membrane to the nucleus to regulate gene expression. ZO-1 is associated with the transcription factor ZONAB [213], ZO-2 with the transcription factors Jun, Fos and C/EBP [214]. The binding of transcription factors to ZO-1 results in their membrane sequestration at intercellular junction level and hence the inhibition of their transcriptional activity.

Haplodeficient mice expressing a CT-truncated Cx43, incompetent to interact with ZO-1, formed GJs at cardiac IDs that were larger than those observed in wild-type litter mates [215]. siRNAs used to knock down ZO-1 levels caused an unchecked accretion of undocked connexons at the plaque periphery, resulting in larger GJs and fewer hemichannels [162]. Knocked down expression of ZO-1 was also seen to reduce Cx43 expression in human trophoblasts (with resulting lessened cell–cell fusion and differentiation [216] and GJ formation in human squamous carcinoma A431D cells [170]).

Smads are intracellular proteins that transduce extracellular signals from transforming growth factor-β (TGF-β) ligands to the nucleus where they activate downstream TGF-β gene transcription. Smad2/3 binds to β-tubulin; Cx43 competing with Smad2/3 for binding to microtubules would induce Smad2/3 release from microtubules, causing, via the formation of a complex with a co-mediator Smad (Smad4), a translocation to the nucleus, where Smad2/3 and Smad4 accumulate, leading to activation of the transcription of target genes [217].

In mice cortical astrocytes, Iacobas et al. [218] observed that genes encoding gap junction protein and purinergic receptors were coordinately expressed and were also coordinately expressed with a

network of additional genes encoding cytoskeletal proteins and kinases. Immunoprecipitation assays and antibody array studies confirm interactions between certain of these participants, allowing these authors to propose a model of supramolecular complex where GJ and purinergic P2 receptor form functional units.

6. Roles of protein–protein interactions

The interactions between gap junction proteins and their protein partners (*e.g.* scaffolding proteins, cytoskeletal proteins, protein kinases and phosphatases), membrane receptors strongly influence the subcellular localization of junctional components and their functional state.

6.1. Cellular growth control and cancer

Besides the transmembrane diffusion of small molecules and metabolites that support tissue homeostasis and electrical and chemical signaling, gap junction proteins are, via their selective protein–protein interactions, involved in the control of cell growth, proliferation, apoptosis, and would serve as tumor suppressors. In the model proposed by Solan and Lampe [219] for example in normal rat kidney cells during their progression from G0/G1 through S phase and into mitosis, Cx43 is at first found predominantly at the plasma membrane, assembled into "typical" gap junction plaques, associated with both ZO-1 and ZO-2, at the plasma membrane. Then the assembly of Cx43 into plaques becomes less efficient, accompanied by a shift in subcellular Cx43 localization and a decreased co-localization with ZO-1 while the ZO-2 interaction was maintained. When cells enter mitosis, GJIC is interrupted and Cx43 is predominantly intracytoplasmic [219].

Heteromerization of *Drosophila* Inx2 and -3 is crucial for epithelial organization and polarity of the embryonic epidermis, and Inx3 is mislocalized to the cytoplasm in Inx2 mutants and is recruited into ectopic expression domains defined by Inx2 misexpression [33].

Cancer was the first pathology associated with gap-junction defect [220] but, in spite of the evidence accumulated since then, the molecular involvement of gap junctions and their structural proteins in cancer has not been elucidated. The lack of a satisfying explanation may come from the complexity of this disease, evolving through various stages during tumor progression, with cancer cells exhibiting different phenotypes (for recent review, see for example [221]). Several Armadillo catenins known as connexin partners are highly implicated in tumor progression both by their malfunction as adapter proteins (linking cadherins to the belt of actin filaments in adherens junctions but also by their role in regulating gene expression (see for example [222])). As emphasized by Duffy et al. [125], there is increasing evidence of connexin involvement in the modulation of transcriptional activity via two pathways, either a translocation of a part of Cx molecules (*e.g.* their CT) to the nucleus or their interaction with proteins with transcriptional activity. The latter mechanism has for example been proposed for Cx43 interaction with transcription regulators as NOV [123,124] or transcription factors as ZONAB, Jun, Fos or C/EBP (see for example [223]).

6.2. Cell motility

Gap junctional proteins, associated with scaffolding proteins anchored to the cytoskeleton form multiprotein complexes, providing dynamic intercellular adhesive contacts particularly important for processes such as cell migration, recently reviewed by Olk et al. [224]. Dynamic adhesive interactions of connexins with the internal cytoskeleton may then play in cell migration a role more important than to provide an aqueous channel for cell-to-cell communication. In the rat embryonic cerebral cortex for example, such interactions would enable leading process stabilization along radial fibers as well as the subsequent translocation of the soma [166].

6.3. Immune response

The molecular mechanism(s) governing inflammasome (the multiprotein complex involved in innate immunity) signaling still remains poorly defined; the Panx1/$P2X_7$ complex has been suggested to represent a crucial component of the inflammasome (see for example [89,225]). Neutrophil activation was abolished and innate host responses to bacterial infection were impaired by inhibiting or silencing panx1 hemichannels or P2Y2 receptors [226]. T-cell receptor/CD28 stimulation was seen to rapidly (within 5 min) cause translocation of Panx1 to the immune synapse [227].

6.4. Skin, hearing and vision

A large variety of connexins is present in the epidermis (8 Cxs are for example expressed in mouse epidermis with overlapping expression patterns in the different epidermal layers; [228]). Some of the mutations in Cx26, Cx30 and Cx31 are linked not only with deafness, but also with skin disorders; Rouan et al. [229] observed in carriers of the Cx26 mutation ΔE42 (where the amino acid residue 42 is eliminated), focal Cx26/Cx43 co-localizations at gap junctional plaques in affected skin tissues. When expressed in oocytes, Cx26 mutants impaired GJIC, possibly by hindering Cx43 channel formation or, alternatively, by their gained ability to interact with Cx43, locking up Cx43 into nonfunctional channels. Changing the amino acids within the first extracellular domain might, as suggested by Thomas et al. [230], alter the compatibility for oligomerization partners. More than 97% of mice in which the Cx43-CT had been truncated (Cx43K258stop) die shortly after birth due to a defect of the epidermal barrier [228,231]. The authors suggested that the deletion of the last 125 aa residues of the CT not only impairs channel closure by intramolecular protein–protein interaction but might also prevent the interactions of Cx43 with partner proteins.

Gap junctions are important in maintaining lens transparency and metabolic homeostasis, and the appropriate heteromeric assembly of Cx46 and Cx50 appears crucial for the clarity and growth of the lens (see [232]). These proteins have mutually exclusive developmental patterns and function as well as differential protein–protein interactions, Cx46 segregating in lipids rafts and interacting with caveolin-1 within the lipid rafts whereas Cx50 was excluded from lipid rafts and did not show any interaction with caveolin-1 [115]. A TPA-induced activation of PKCγ was found to decrease the density of Cx50 assembled with Cx46 in gap junction plaques [104]. According to Yu et al. [82] direct interaction of AQP0 with the intracellular loop domain of Cx45.6 would not affect gap junction-mediated intercellular communication.

6.5. Cardiovascular health and disease

GJ channels play essential roles in the normal function of the cardiovascular system, mediating the spread of the electrical impulse that triggers synchronized contraction of the cardiac chambers and contributing to the coordination of activities between cells of the arterial wall. In mammalian hearts, cells most prominently express junctional channels built of Cx40, Cx43 and Cx45, each of them having a characteristic developmental and regional expression pattern in the heart (see [110]). In the major arteries, endothelial gap junctions may simultaneously express Cx37, -40 and -43 whereas underlying medial smooth muscle predominantly expresses Cx43, with Cx45 additionally expressed at restricted sites (see [233]). Heteromeric connexin channels form between all the heart Cxs, although there is still little functional data concerning their impact on cardiac conduction. Heterotypic channels display asymmetric voltage

sensitivities which may function as a "gate" to promote directional impulse propagation (in the AV nodal region, from atria to ventricles, for example).

In the vascular cell co culture model developed by Isakson and Duling [234], Cx37 for example was specifically excluded from myoendothelial junctions between endothelial cells and smooth muscle cells but was present into junctions interconnecting either two adjacent endothelial cells or two adjacent smooth muscle cells. This suggests that Cx hetero-oligomerization, based on the spatial organization of Cx distribution within the cells, allows to engender different classes of cell-to-cell interconnections, influencing intercellular signaling pathways.

In the heart, adherens junctions appear to nucleate GJs, and disruption of mechanical coupling has been suggested to lead, via the loss of GJIC, to different cardiomyopathies [235,236]. Mutations in genes encoding different desmosomal proteins (plakoglobin, desmoplakin, plakophilin 2 and desmoglein 2) have for example been identified in patients with arrhythmogenic right ventricular dysplasia/cardiomyopathy (see [237] for references). The gap junction remodeling observed in different cardiac pathologies has been suggested to result from alterations in Cx43/ZO-1 interaction [182,238]. ZO-1 forms with N-cadherin a multiprotein complex which, according to Palatinus et al. [239], would be a key determinant of stable localization of both adherens junctions and gap junctions at the ID. This is consistent with previous observations that cardiac-specific deletion of N-cadherin led to alteration in Cx40 and Cx43, disassembly of the ID structure and conduction slowing and arrhythmogenesis in adult mice [240,241]. Ferreira-Cornwell et al. [242] examined the effect of expression of either E-cadherin or N-cadherin in the heart of transgenic mice, and observed that misexpression of E-cadherin led to cardiomyopathy, with earlier onset and increased mortality compared with N-cadherin mice, with a dramatic decrease in Cx43. Silencing of plakophilin 2 expression resulted in cardiomyocytes of new-born rat in a drastic loss of Cx43 gap junction plaques, a significant redistribution of Cx43 and a decrease in intercellular dye coupling [243].

Smyth et al. [160] observed that microtubule plus-end tracking protein EB1 was displaced in ischemic human hearts, stressed mouse hearts, and isolated cells subjected to oxidative stress and shown that microtubule-dependent forward trafficking of Cx43 to the plasma membrane was a significant contributor to losses in cell–cell coupling during heart disease.

According to Vessey et al. [212], brief periods of ischemia/reperfusion would induce the formation of Panx1/$P2X_7$ channels, allowing the release of multiple cardioprotectants that mediate the protection of ischemic preconditioning and ischemic post-conditioning.

6.6. Nervous systems (CNS and PNS)

Cell interconnections in the central nervous system involve both chemical synapses and electrical synapses (gap junctions). In contrast with the regulation of chemical transmission, known to involve direct interactions of ligand-gated ion channels with different scaffold proteins, playing essential roles by facilitating channel insertion, anchoring, or removal from the plasma membrane (see for references [244]), little is still known concerning the importance of protein interactions in regulating electrical synapses.

At least 11 connexin subtypes coexist in the CNS (see for example [245]) and form channels which exhibit a great complexity in their composition and properties. In the mouse CNS, astrocyte Cx26 associates mainly with oligodendrocyte Cx32, whereas astrocyte Cx43 and Cx30 associate with oligodendrocyte Cx47 [246]. The significance of this diversity as well as the temporal expression patterns for the different connexins in neurons, astrocytes, oligodendrocytes, Schwann cells and glia have not yet been determined.

Drebrin, whose level is greatly decreased in brains of Alzheimer patients (see [50]), appears required for maintaining Cx43-made junctional channels in their functional state at the plasma membrane [50]. Flores et al. [244] observed that Cx35CT15/PDZ-1 interaction was of lower affinity and exhibited faster dissociation kinetics than Cx43CT20/PDZ-2 interaction and suggested that these characteristics could allow faster kinetics and more dynamic interactions between these two proteins, consistent with an involvement in synaptic plasticity.

Electrical synapses were for a long time considered to be less modifiable than chemical synapses, where activity-dependent plasticity has been ascribed to CaMKII activity, particularly in glutamatergic synapse, but this key enzyme now appears to undergo activity-dependent synaptic potentiation as well (see [108]). Cx36, the most common Cx at electrical synapses in mammals, was proposed to interact with and to be phosphorylated by CaMKII in a way similar to CaMKII phosphorylation of glutamate receptors [108].

7. Conclusions and perspectives

It progressively emerged in recent years that gap junction proteins do not operate as free-floating entities in the plasma membrane, but rather interact with a range of partner proteins that link them to the cytoskeleton and to signal transduction pathways (forming a macromolecular structure tentatively represented in Fig. 2). Such associations are likely to be important for correct targeting of channels to specific subcellular sites, for immobilization and clustering of the cell-to-cell channels, for the ability of channels to funnel ions and small molecules, and for modulation of the channel functions by kinases, phosphatases, and other regulatory proteins. Thus the elucidation of interactions with partner proteins promises to reveal a great deal about the function, regulation, and cell biology of gap junctional proteins. The latter may also, through their protein partners, be involved in cell functions very different from their tunnel-forming functions. Such structures, with six C-tails exposed per hemichannel (and thousands of C-tails per gap junction plaque) bind diverse signaling and structural proteins and thus form a frame crucial for development and tissue maintenance. The sequestration of transcription factors (*e.g.* ZONAB, Jun, Fos, *etc.*), via their interaction with ZOs, at the plasma membrane in the close vicinity of junctional structures suggests that gap junctional proteins might have additional functions in the regulation of gene expressions. Such interactions of gap junctional proteins with protein partners appear to have been conserved through evolution, very likely reflecting their functional importance.

It is however essential to keep in mind that the identification of partners of gap junctional proteins, as of partners of other membrane proteins (*e.g.* membrane receptors, transporters, and ion channels), protein which span the membrane multiple times and fold into special structures involving non-linear peptide loops, require rigorous biochemical evidence via multiple approaches. Co-localization observations in immunofluorescence microscopy need additional approaches (including for example *in vitro* binding assays, co-immunoprecipitation) to substantiate their interactions with the identified proteins. On the other hand, the screening of protein–protein interaction frequently results in identification of interactions which, although reproducible and specific, are not easily assessed. This is mainly due to the fact that proteins are misexpressed in a compartment from which they are typically excluded and therefore may be exposed to a repertoire of proteins that are not accessible in their natural environments. In addition, the fusion of a protein or a protein fragment to an effector protein (*e.g.* in two-hybrid or protein recruitment approaches) may result in misfolding of the protein and exposure of protein surfaces that are typically not accessible for association with other proteins. It is why the present survey was, for interactions between gap junction proteins, limited to observations made in systems where they were endogenously expressed.

In conclusion, gap junctional proteins increasingly appear as members of large signaling complexes including membrane

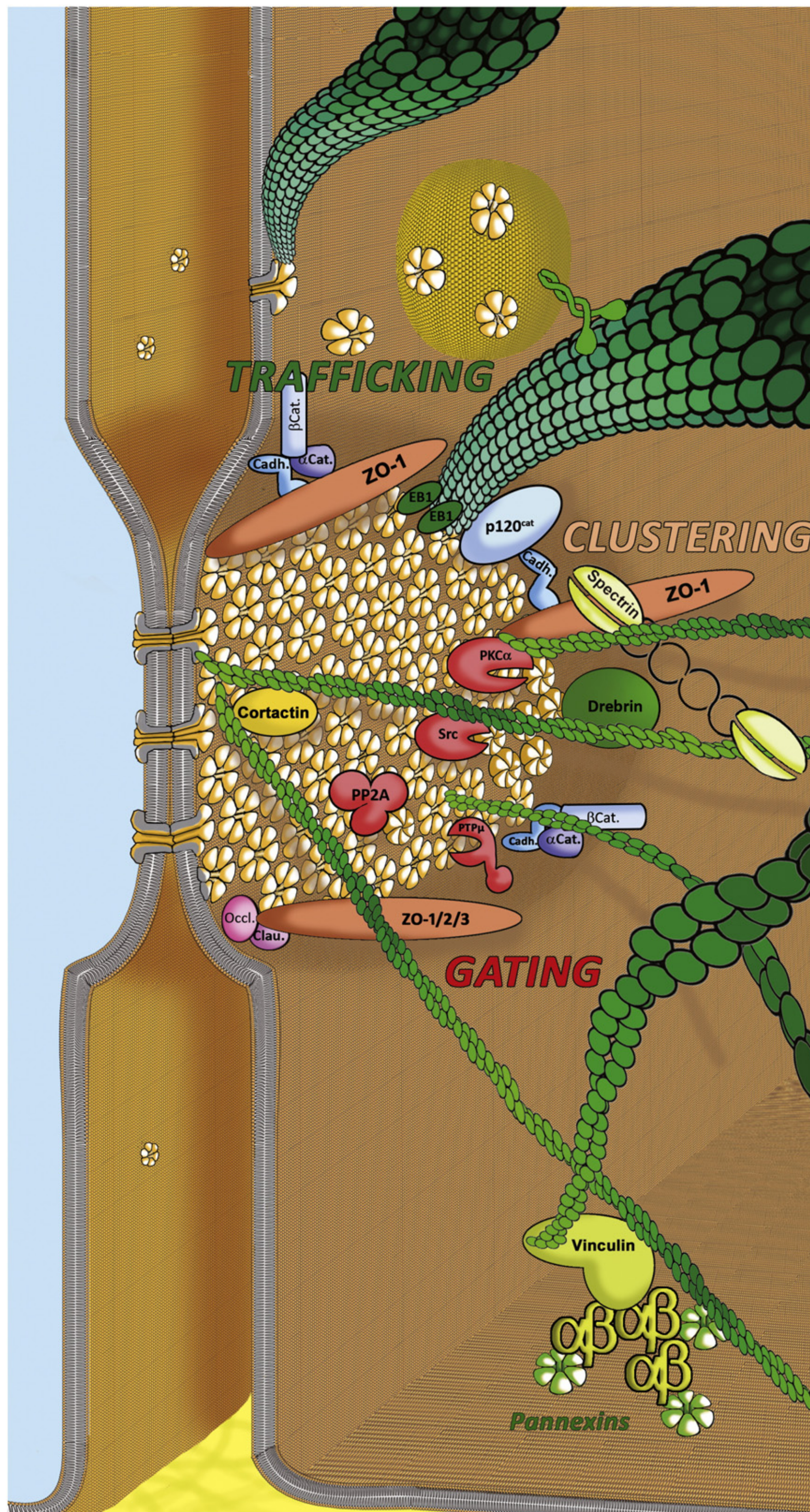

Fig. 2. Round about gap junctions. Artistic view of some of the cellular proteins found associated with Cx43- or Pannexins-made channels or in their close surrounding.

receptors, proteins of other cell junctions, enzymes (PKs, PPs, …), scaffolding proteins (*e.g.* ZOs), transcription factors and cytoskeleton. The importance of protein–protein interactions in the regulation of the trafficking, assembly and disassembly of junctional proteins, as well as the dynamic modulation of the opening state of junctional channels are progressively gaining support. Communication through such channels is sensitive to a variety of stimuli, including changes in the level of intracellular Ca^{2+}, pH, transjunctional applied voltage and phosphorylation/dephosphorylation processes. The finding that, in addition to their well-described function of transmembrane conduits, these structures participate in intracellular signaling pathways could provide an explanation for the number of genes that encode channel subunits. With heteromer formation, alternative splicing of mRNA and post-translational modifications, the potential number of

different channels that can be assembled from these genes is considerable. The specific linkage of different channel subfamilies to different signaling pathways could not only provide a *raison d'être* for a large number of channels that apparently have not very different properties, but might also help to explain the high degree of conservation in the sequences of different subfamilies of junctional channels throughout evolution. However, the identification of the cytoskeletal and signaling proteins that interact with gap junction proteins is only a first step in a long endeavor. The major challenges of the future are to quantify the stoichiometry of these protein–protein interactions, to picture their geometry by microscopic and structural biological approaches, to make out the hierarchy of organization, to reveal the dynamic regulation of gap junctional complexes and, perhaps most importantly, to understand the functional significance of these protein interactions in intercellular signalization in physiological conditions and associated with diseases.

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