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Review

Biological Functions of Connexin43 Beyond Intercellular Communication

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Connexin43 (Cx43) is commonly associated with direct cell–cell communication through gap junctions (GJs). However, recent groundbreaking studies have challenged this dogma, implicating Cx43 in other biological processes, such as transcription, metabolism, autophagy, and ion channel trafficking. How Cx43 participates in these processes remains largely unknown, although its high turnover rate, capacity to bind to myriad proteins, and the discovery of truncated isoforms of Cx43, ascribe to this protein unanticipated roles in chief processes that require fine-tuned regulation. Accordingly, Cx43 can be regarded as a central integrative hub to which diverse cues converge to be processed in a concerted manner. In this review, we examine the noncanonical roles of Cx43 and discuss the implications of these functions in human diseases and future therapeutic strategies.

Highlights

Proteomic analyses ascribe regulatory roles to Cx43 in transcription, metabolism, autophagy. and ion channel trafficking that go beyond direct cell–cell communication.

Cx43 participates in long-distance extracellular vesicle-mediated communication.

Cx43 localizes to the nucleus, where it can regulate gene expression.

Cx43 localizes to the mitochondria, where it modulates respiratory capacity, ATP production, and redox balance, with important roles in cardioprotection mediated by ischemic preconditioning.

N-terminally truncated isoforms of Cx43 emerge as novel players in mitochondrial homeostasis, nuclear transcription, and trafficking of newly synthesized Cx43.

A Second Life for an Old-Fashioned Protein: The Role of Cx43 Beyond Gap Junction Communication

Channel-forming connexin proteins are canonically associated with gap junction (GJ)-mediated communication between adjacent cells, ensuring both metabolic and electrical coupling, fundamental for tissue and organ homeostasis (Box 1). As a result, GJ-mediated intercellular communication (GJIC) has been the main focus in the connexin field over the past 30 years. However, emerging evidence has ascribed unanticipated biological roles to connexins that go beyond direct intercellular communication, pointing towards broader functions of these membrane proteins. For example, unbiased proteomic approaches of Connexin43 (Cx43), have revealed a long list of binding proteins related to various biological processes and mechanisms. Moreover, the presence of Cx43 in intracellular compartments, including the nucleus and mitochondria, has been reported. Several studies have unveiled these noncanonical functions in detail, associating Cx43 with features such as gene transcription, development, mitochondrial homeostasis, **autophagy** (see Glossary) regulation, intracellular trafficking, and long-distance communication mediated by **extracellular vesicles** (**EVs**).

Here, we present an integrated overview of the recent literature that has revealed unforeseen functions for connexins, with a particular focus on Cx43. In fact, Cx43 is the most ubiquitously distributed family member, expressed in multiple cell types across almost all tissues and organs. This has favored its study as a model for other connexins. Most of the differential features attributed to each connexin family member have been related to the size and variability of their intracellular loop and C terminus [1]. In agreement, many of the noncanonical roles of Cx43 have been ascribed to its cytosolic C-terminal tail constituting a preferential platform to accommodate diverse protein–protein interactions. Given its unusual versatility and short half-life, it is conceivable that the evolutionary selection of Cx43 as the predominant connexin isoform in mammals makes it a preferential protein to exhibit biological functions beyond GJIC.

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Box 1. Canonical Functions of Connexins: GJ and Hemichannels

Connexins are a homogeneous family of proteins expressed in almost every tissue or cell type and typically assemble into tightly packed clusters of intercellular channels, called GJs (reviewed in [50]). So far, 20–21 different connexin genes have been identified in the mammalian genome, and are commonly classified according to sequence homology and divided into five subfamilies (alpha, beta, gamma, delta, and epsilon) [77]. For example, members of the alpha subfamily are named *GJA* followed by a number in order of their discovery (e.g., *GJA1*) [77]. Nevertheless, connexin proteins are named after their specific molecular weight in kDa (e.g., Cx43). The general structure of connexins comprises four α-helical transmembrane domains and two extracellular loops that are highly conserved among the family members. The N and C terminals, as well as the intracellular loop (IL), all located in the cytoplasm, differ considerably in both length and composition and are characteristic of each connexin type (Figure 1). Connexins assemble into homomeric or heteromeric hexamers (called connexons or hemichannels) in the endoplasmic reticulum (ER) or Golgi apparatus, which then traffic to the cell surface along microtubules [78]. Newly formed connexons tend to move laterally towards existing GJ channels, where they can dock with their counterparts in neighboring cells, forming large GJ plaques by accretion [79]. Depending on the cell type and the connexin expressed, connexons can function as hemichannels, providing a pathway for autocrine or paracrine signaling, whereas GJ channels allow direct passage of ions, metabolites, second messengers, and even some small noncoding RNAs and peptides between adjacent cells, without exposure to the extracellular environment. Both communication pathways are believed to be essential for healthy development and tissue homeostasis and have been shown to have a major role in diverse pathological processes [1,80].

Most connexins have a short life span of only a few hours [65]. The connexin interactome comprising dozens of binding proteins is known to have critical roles in GJ assembly and stability, in the regulation of channel function, as well as in connexin degradation [3]. During turnover, one of the two adjacent cells sharing a GJ internalizes the entire GJ plaque or fragments into a unique double-membrane structure called a connexosome or annular GJ (Figure 1). Although interactions of Cx43 with both clathrin and caveolins have been demonstrated, the detailed molecular mechanisms of GJ endocytosis remain obscure (reviewed in [81]). Nevertheless, it is accepted that internalized GJs ultimately fuse with lysosomes to be degraded [78].

Given the clear link between abnormal Cx43 expression and multiple human diseases, we anticipate that these noncanonical functions of Cx43 will bring new perspectives for the development of successful therapeutic strategies.

Proteomic Analyses Feed the Mystery

It has been recognized that the nature and dynamics of the proteins that interact with connexins likely determines the noncanonical functions of connexins. Therefore, increasing attention has been given to the large-scale identification and characterization of the Cx43 **interactome** under specific pathophysiological conditions, and this has been used as a paradigmatic example for other connexin family members. According to this concept, three major unbiased proteomic studies, using rat glial cell lines, human primary articular chondrocytes, and rat hearts, enabled the identification of over 60 confirmed interactors of Cx43 as well as a large number of putative interactors that await further verification [2–4]. Remarkably, besides proteins involved in **ubiquitin conjugation**, **clathrin-mediated endocytosis**, and **intercellular junctions**, associated with the regulation of GJIC, these works identified unanticipated associations of Cx43 with proteins related to biological processes, such as mitochondrial metabolism, RNA transport, and nuclear functions. In addition, specific proteomic analysis of the Cx43 interactome in mitochondria of mouse hearts unveiled interactors involved in oxidative phosphorylation and redox balance [5]. Although proteomic analyses may have overestimated the number of Cx43 binding partners, many of these noncanonical interactors have been validated, strengthening the involvement of Cx43 beyond GJIC [4,6].

The identification of cell type-specific Cx43 interactors points to GJ-independent mechanisms by which connexins regulate tissue homeostasis. These noncanonical functions of Cx43 may also contribute to the etiology of human diseases, such as osteoarthritis and cardiac **ischemia/reperfusion injury** [7].

The Role of Cx43 in Gene Transcription

Ample evidence has demonstrated that both the genetic deletion and transient knockdown of Cx43 profoundly affect the cellular **transcriptome**, altering the expression of genes involved in

Glossary

Alternative translation: generation of multiple proteins from the same mRNA molecule, driven by recognition of alternative translation start codons.
Autophagosome: double-membrane vesicle that encloses damaged organelles and/or long-lived proteins targeted for degradation by macroautophagy.
Autophagy: degradation of cellular components within lysosomes, which can be further divided into three main pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy.
Caveolae: inverted omega-shaped membrane invaginations comprising caveolins, involved in signal transduction and endocytic trafficking.
Clathrin-mediated endocytosis: process whereby transmembrane proteins, metabolites, hormones, or viruses are internalized by inward budding of the plasma membrane.
Cx43 mimetic peptide: short amino acid sequence mimicking a target domain of Cx43, designed to prevent GJ formation or modulate hemichannel and/or connexin function.
Epithelial to mesenchymal transition (EMT): process whereby a polarized epithelial cell assumes a mesenchymal phenotype, including enhanced migratory capacity, invasiveness, apoptosis resistance, and increased production of extracellular matrix.
Extracellular vesicles (EVs): lipid bilayer particles released from cells. Based on their subcellular origin, mechanisms of biogenesis, size, and content, communicating EVs can be divided into: (i) exosomes (50–200 nm), released after fusion of multivesicular bodies with the cell surface; and (ii) microvesicles (100–1000 nm), directly shed from the plasma membrane. However, this nomenclature constitutes a simplistic and limited view of the complexity and heterogeneity of this population of bilayered nanosized vesicles, favoring the indiscriminate use of the term 'EV'.
Interactome: full range of molecular interactions with a particular protein (bait) that occurs within a biological sample or cell.
Intercalated discs: specialized membrane regions at the longitudinal termini of the cardiomyocytes, comprising intercellular junctions that

diverse biological processes, including cell adhesion and migration, cytoskeleton dynamics, and cell signaling, proliferation, and differentiation [8,9]. Remarkably, the Cx43-dependent gene expression profile was shown to be dramatically affected by the genetic background of the mice used for transcriptomic analyses (C57BL/6 versus 129/SvEv) [10]. In this respect, it is plausible that disease phenotype variability associated with mutations in Cx43, for example those causing **oculodentodigital dysplasia (ODDD)**, may depend on modifier genes.

Whether these effects are related to, or dependent on, the localization of Cx43 in the nucleus has been a matter of intense debate (Figure 1). The presence of nuclear Cx43 has been demonstrated in human gliomas, colorectal tumors, and lung tumors, where it was associated with poor overall survival [11–13]. The occurrence of Cx43 in the nucleus has also been described in normal cells, including primary chondrocytes, pointing to a biological role for nuclear Cx43 under physiological conditions [4]. When ectopically expressed, the soluble C terminus of Cx43 was shown to localize to the nucleus and to inhibit cell proliferation [14]. Consistent with a direct function of Cx43 in the control of gene expression and/or chromatin structure, bioinformatic analyses predicted multiple DNA- and RNA-binding motifs in the Cx43 sequence [15].

Intriguingly, the existence of a **nuclear localization signal (NLS)** in Cx43 has never been clearly proven despite ample evidence demonstrating the presence of Cx43 in the nucleus [4]. In fact, the identity of the domain(s) required for the translocation of both the full-length and the truncated forms of Cx43 into the nucleus remains obscure (see Emerging Roles of Truncated Forms of Cx43). Indeed, it is unclear whether Cx43 can reach the nucleus after cotranslational insertion into endoplasmic reticulum (ER) membranes or whether it originates from the plasma membrane. Studies carried out with multiple cell surface receptors and channels have implicated both pathways in the transport of membrane proteins to the nucleus (Box 2) and may serve as suitable models to unveil the mechanisms underlying the trafficking of Cx43. Consistent with this notion, the interactions with nuclear-associated proteins, including the GTP-binding protein Ran, importin-β, and several molecules with transcription factor activity identified in proteomic analyses, may provide some clues [3,4]. Recent evidence demonstrates that basic transcription factor-3 (BTF3) can directly bind to the Cx43 C terminus and translocate it into the nucleus [16]. In a similar manner, full-length Cx43 has been described to translocate to the nucleus in lung cancer cells during the G1 phase of mitosis, through an interaction with A-kinase anchoring protein 95 (AKAP95) [17]. Specific signaling pathways, including Wnt signaling, have also been shown to cause increased nuclear translocation of Cx43, concomitant with reduced cytosolic and membrane-associated Cx43 [18].

There is also evidence that Cx43 can regulate gene transcription indirectly, namely through its channel function. Expression of wound healing-related genes in human gingival fibroblasts was shown to be modulated by the **Cx43 mimetic peptide** Gap19 (Box 3), which is widely used to block hemichannel activity, with the concomitant impairment of ATP signaling and activation of the extracellular signal-regulated kinase (ERK)-1/2 pathway [19]. Nevertheless, it is possible that the effects of Gap19 extend beyond classical hemichannel inhibition and likely impact the intracellular pool of Cx43, namely by affecting its translocation to the nucleus. Studies in a lung cancer cell line suggested that Cx43 modulates gene expression by acting as a histone deacetylase inhibitor [20]. Additional insight is emerging from other connexins. For example, a recent report implicated Cx26 in the stabilization of pluripotency transcription factor NANOG, through the formation of a complex with focal adhesion kinase (FAK), which is required to drive cancer stem cell renewal specifically in triple-negative breast cancer, but not luminal breast cancer [21]. Given that Cx26 and NANOG have been identified in pluripotent stem cells of epithelial tissues, this mechanism may also have a role in physiological stem cell renewal [22]. Further

ensure mechanical and electrical coupling.
Intercellular junctions: specialized structures providing adhesion and communication between cells or with the extracellular matrix, comprising tight junctions, adherens junctions, and GJs.
Ischemia/reperfusion injury: tissue damage consequent to the restoration of blood supply after a period of oxygen and nutrient deprivation of the myocardium (ischemia), caused by excessive oxidative stress and inflammation.
Ischemic preconditioning: brief episodes of ischemia and reperfusion preceding a period of sustained ischemia, often associated with cardioprotection.
Mitochondrial targeting sequence (MTS): sequences rich in highly hydrophobic and basic amino acids, present in transmembrane domains and flanking regions of some membrane proteins targeted to the mitochondria.
Nuclear localization signal (NLS): short mono- or bipartite sequences of basic amino acids that enable recognition by importins for transport of cargo into the nucleus.
Nuclear matrix: filamentous protein network inside the nucleus that supports chromatin organization.
Nuclear pore complexes (NPCs): basket-like multiprotein structures, formed by nucleoporins, that provide a route for nucleocytoplasmic shuttling of soluble proteins and RNAs.
Oculodentodigital dysplasia (ODDD): rare genetic syndrome characterized by defects in the eyes, teeth, and fingers, caused by more than 70 mutations in the gene encoding Cx43.
Transcriptome: entire collection of gene readouts of a cell, including mRNAs, which are translated into proteins, and noncoding RNAs that can regulate gene expression.
Ubiquitin conjugation: formation of an isopeptide bond between the C terminal glycine in ubiquitin and the ε-amino group of lysine residues on target proteins.

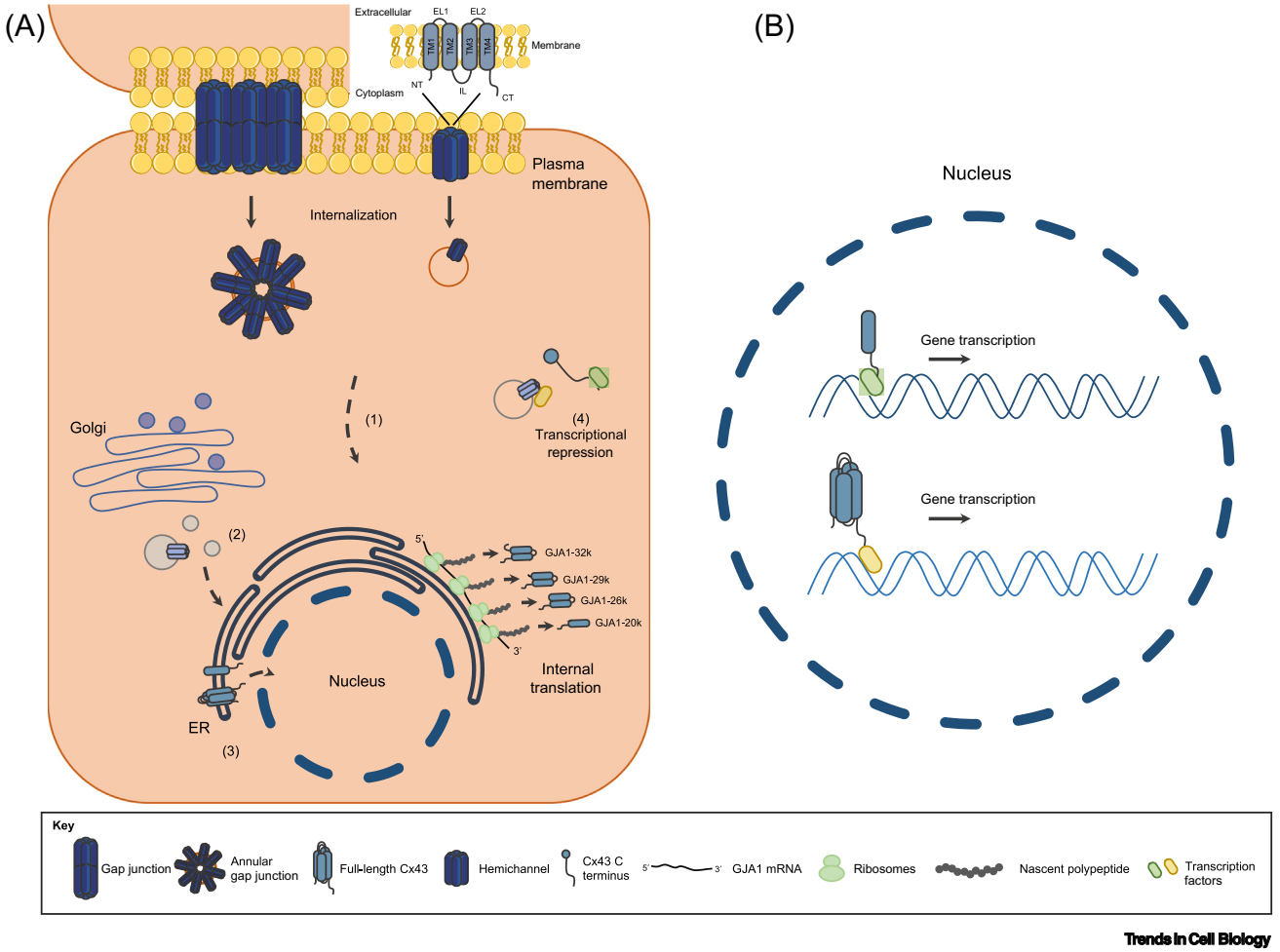

Figure 1. The Role of Connexin 43 (Cx43) in Gene Transcription. Proposed models for Cx43-mediated modulation of gene transcription (A), involving either trafficking of full-length Cx43 to the nucleus from the plasma membrane (1), Golgi (2) or endoplasmic reticulum (ER) (3), or by sequestration of transcription factors (4) in the cytosol. Internal translation of the coding region of *GJA1* mRNA gives rise to N-terminally truncated proteins with different sizes and variable endogenous expression levels, including GJA1-32k, GJA1-29k, GJA1-26k, and GJA1-20k. Once at the nucleus, both full-length Cx43 and GJA1-20k (B) can directly bind to DNA or to transcription factors, affecting gene expression. Inset depicts the structure of a Cx43 monomer, with four transmembrane domains (TM1–TM4), two extracellular loops (EL1 and EL2), one intracellular loop (IL), and cytoplasmic N (NT) and C terminals (CT). Gap junction (GJ) channels are formed after docking of Cx43 hemichannels between apposed cells, enabling the exchange of small molecules. Abbreviation: INM, inner nuclear membrane.

insight into the direct and indirect regulation of gene transcription by connexins is deemed particularly critical to elucidate the context-dependent and connexin isoform-specific differences underlying both pro- and antitumorigenic features of connexins (reviewed in [1]).

The Role of Cx43 in Development

Given the importance of fine-tuned gene expression regulation during embryogenesis and development, transcription-related roles of Cx43 may assume particular relevance in these biological processes. Indeed, it has been long recognized that Cx43 has crucial functions during central nervous system development that are independent of GJIC. Radial migration of developing neurons depends on the establishment of Cx43-mediated dynamic adhesive contacts, via interaction between Cx43 and the actin cytoskeleton [23]. In agreement, the association between Cx43 and adherens junction proteins, including N-cadherin, was demonstrated to drive neural crest cell

Box 2. Trafficking of Cell Surface Proteins to the Nucleus

Multiple cell surface proteins can be localized at the nucleus, including receptor tyrosine-protein kinase ErbB (ErbB)-2, epidermal growth factor receptor (EGFR), fibroblast growth factor receptors (FGFRs), or insulin-like growth factor receptor 1 (IGF-1R) (reviewed in [82]). Although the mechanistic details remain elusive, delivery of cell surface-localized ErbB-2 to the nucleus relies on the formation of a complex between importin-β and players involved in clathrin-mediated endocytosis [83]. Localization of EGFR and ErbB-2 at the inner nuclear membrane (INM) may result from the 'integral trafficking from the ER to the nuclear envelope transport' (INTERNET) pathway, involving recognition of NLS by ER-associated importin-β and interaction with glycoproteins across the **nuclear pore complexes (NPCs)** [84,85]. Moreover, the interaction of translocon Sec61β with EGFR and ErbB-2 at the ER and INM likely mediates the final translocation of membrane-embedded receptors into the nucleoplasm [85]. In addition, EGF-induced internalization of EGFR promotes retrograde Golgi-to-ER transport *en route* to the nucleus [86].

Interestingly, FGFR1 and IGF-1R can be mainly found as **nuclear matrix**-associated proteins, where they directly bind to chromatin, regulating gene transcription [87]. Despite FGFR1 and IGF-1R lacking canonical NLS, trafficking of FGFR1 to the nucleus is coupled with adherens junction internalization, whereas colocalization between IGF-1R with early endosome antigen (EEA)-1 was detected at the nucleus, likely as a result of either nucleocytoplasmic shuttling of the endocytic machinery or fusion of receptor-containing endocytic vesicles with the nuclear membrane [88,89]. Furthermore, internalization into **caveolae** preceded nuclear localization of interferon (IFN)γ receptor (IFNGR)-1, which is likely chaperoned via interaction with the NLS motif of the ligand, IFNγ [90]. TGF-β receptor (TβRI) also lacks a classical NLS motif but undergoes Ran GTPase-dependent nuclear import in basal and ligand-induced conditions, which depends upon interaction with importin-β, probably using nucleolin or Smad2/3 as adaptors [91]. Alternatively, receptors can be directly routed to the nucleus after being newly synthesized. Consistent with this hypothesis, nuclear FGFR1 was found in its nonglycosylated form, suggesting that translocation of FGFR1 from the ER to the nucleus occurs before Golgi processing (reviewed in [92]). It is conceivable that Cx43 trafficking to the nucleus involves: (i) the recycling pathway, through the Golgi to ER retrograde transport, implying that plasma membrane-residing Cx43 can reach the nucleus; and/or (ii) the direct backward transport of Cx43/ GJA1-20k from the ER to the nucleus after protein synthesis through the network of contiguous membranes that link the ER and the nuclear membrane.

motility (reviewed in [24]). Furthermore, multiple studies reported that Cx43 expression is required to maintain undifferentiated progenitors during early developmental stages, thus preventing premature neuronal differentiation (reviewed in [25]). Recently, knockdown of Cx43 in *in vitro* models of human neurogenesis was shown to increase the number of differentiated neurons, at the expense of impaired glial cell differentiation. This is likely through a mechanism that involves the sequestering of β-catenin at the plasma membrane, which negatively impacts β-catenin transcriptional activity [26]. Exogenous expression of the cytoplasmic C terminus of Cx43 was

Box 3. Therapeutic Strategies Targeting Cx43

Given their incontestable importance in human physiology and their putative roles in various disease processes, connexins have emerged as druggable targets, particularly Cx43 [93]. Cx43 channel function can be altered by a broad spectrum of nonspecific drugs with many off-target effects. However, it is particularly attractive to develop therapeutic strategies intervening with various aspects of the Cx43 life cycle, including mRNA levels, hemichannel opening, and GJ size or channel function. Indeed, transient knockdown of Cx43 or blocking Cx43 hemichannels after wounding of the skin has been shown to reduce edema, inhibit inflammation, improve epithelial recovery, and reduce scar formation (reviewed in [94]). Two formulas have been tested in Phase II clinical trials to target Cx43 in epidermal wound healing, in particular in chronic diabetic or nonhealing ulcers. Nexagon is a 30-mer antisense oligonucleotide (AsODN) that decreases Cx43 expression, and αCT-1 is a peptide that mimics the last nine amino acids of Cx43 preceded by an internalization sequence, enabling the peptide to penetrate the cell cytoplasm, which decreases Cx43-ZO1 interaction and exerts effects on Cx43 phosphorylation [93]. Additional future applications and (pre)-clinical trials for these compounds include postsurgical scarring, nonhealing corneal wounds, and (diabetic) retinopathy. Over the past decade, several other Cx43 targeting peptides have been developed; these include peptides mimicking regions of extracellular loops of Cx43 (Gap26, Gap27, and Peptide 5, which likely block GJIC by preventing connexon docking, while the mechanism of their hemichannel block still needs to be resolved [93]. By binding to the Cx43-C terminus, another class of peptides (L2 and Gap19) prevent intracellular loop–C-terminal interactions, thereby keeping hemichannels in a state that is refractive to opening [95]. Finally, the dipeptide ZP1609 (danegaptide) has antiarrhythmic properties and has been shown to reduce infarct size following ischemia/reperfusion in pigs [96]. Nevertheless, this GJ coupling-modulating peptide failed to improve myocardial salvage in patients with ST-elevation myocardial infarction (STEMI) in a Phase II randomized clinical trial [97]. It is promising that multiple tools interfering with Cx43 expression, its interactome, or channel function have appeared in recent years, yet many questions still need to be addressed regarding their exact working mechanisms as well as their specificity and stability in organisms.

sufficient to rescue the wild-type phenotype, suggesting that this Cx43 domain integrates signal transduction pathways involved in neurogenesis [27]. Similarly, the C terminus of Cx50 participates in lens fiber cell proliferation and differentiation [28].

The Role of Cx43 as a Scaffolding Complex

Several studies have identified Cx43 as a scaffolding protein that can modulate the function of the proteins it interacts with, and/or determine the noncanonical roles of Cx43 itself [4]. Besides a direct impact on gene expression in the nucleus, Cx43 may hijack transcription factors, hindering their nuclear translocation (Figure 1). In agreement, Cx43 interaction prevents heat shock-induced nuclear import of heat shock cognate protein (Hsc)70. By competing with cyclin D1 for Hsc70 binding, Cx43 impedes nuclear accumulation of p27 through the control of nuclear translocation of the cyclin D1-CDK4-p27 complex, ultimately blocking cell cycle progression [29]. Moreover, the sequestration of the transcription factor specificity protein (Sp)-1 in the cytoplasm of bone marrow stromal cells after expression of Cx43 and Cx45, was demonstrated to impact C-X-C motif chemokine (CXCL)-12 synthesis [30]. Along these lines, hijack of the nuclear factor kappa B (NF-κB) transcription factor by Cx40 has been shown to inhibit its nuclear translocation and initiation of proinflammatory signaling, thus ascribing to Cx40 a vital role upon endothelial homeostasis [31]. In addition, interaction of Cx32 with the scaffolding protein Discs Large homolog 1 (Dlgh1) at the plasma membrane was suggested to block cell cycle progression in hepatocytes [32].

Besides a repressive effect, Cx43 can positively induce transcription factor activity. Notably, Cx43 is implicated in transforming growth factor (TGF)-β signaling by competing with Smad2/3 for binding to microtubules, leading to the release of Smad2/3, which can translocate to the nucleus to regulate transcription [33].

Aside from transcription factors, Cx43 can also regulate the dynamics of proteins at the plasma membrane. It was demonstrated that downregulation of Cx43 decreases the surface expression and function of voltage-gated sodium channels (Nav1.5) at the cardiac **intercalated discs**, implicating Cx43 in the regulation of Nav1.5 membrane localization [34]. Mechanistic studies further revealed that the C terminus of Cx43 is required to assist in microtubule plus-end delivery of Nav1.5 to the intercalated discs [35]. This finding indicates that, in addition to its role as a main constituent of low-resistance channels required for efficient cardiac electrical impulse conductance, Cx43 is also an important regulatory hub involved in the function, trafficking, and distribution of other cardiac proteins [35].

Additionally, a sequestering effect of Cx43 has been implicated in autophagy regulation, through the modulation of **autophagosome** formation. In this case, Cx43 localized at the plasma membrane directly interacts with autophagy-related proteins (Atgs) involved in the initial steps of autophagosome formation, namely Atg16. Thus, it acts as a negative regulator of basal autophagy by hijacking components of the initiation complex [36]. Interestingly, upon nutrient starvation (a classical autophagy inducer), binding of Atg14 to Cx43 at the plasma membrane triggers internalization of Cx43, relieving its inhibitory effect on autophagy [36]. Given the importance of autophagy in several pathological contexts, including cardiovascular disorders and cancer, Cx43-mediated downregulation of autophagy may constitute an important regulatory module for the development of disease, rendering it a potential therapeutic target.

Emerging Roles of Truncated Forms of Cx43

Recent studies demonstrated the existence of Cx43 fragments generated by endogenous **alternative translation**. The translation of the coding region of *GJA1* mRNA, which encodes

Cx43, produces not only the full-length protein, but also at least four different amino terminal truncated forms with different sizes (Figure 1). The 20-kDa isoform (GJA1-20k), comprising a portion of the fourth transmembrane domain together with the C terminus, is the most abundantly expressed in various cell types *in vitro* and *in vivo* [37–40]. GJA1-20k has been shown to interact with full-length Cx43, thereby regulating its subcellular distribution, formation of GJs, and intercellular communication [37,38]. The biological relevance of GJA1-20k has been highlighted in studies showing that translation of GJA1-20k is increased upon inhibition of important signal transduction pathways, namely the PI3K/Akt/mammalian target of rapamycin (mTOR) and the mitogen-activated protein kinase-interacting serine/threonine-protein kinases (Mnk)-1/2 pathways [37,40]. Both are frequently activated in cancer and loss of GJA1-20k, but not full-length Cx43 expression, was reported in low-grade human breast cancers [41]. Therefore, it is conceivable that downregulation of Cx43-GJs during TGF-β-mediated **epithelial to mesenchymal transition (EMT)**, implicated in development and diseases such as cancer, is associated with the downregulation of GJA1-20k and defective Cx43 hemichannel oligomerization at the Golgi [39]. However, as discussed earlier, Cx43 can potentiate TGF-β/EMT signaling through the release and translocation of Smad2/3 transcription factors [33]. GJA1-20k can also participate in the regulation of EMT through its binding to BTF3 and translocation to the nucleus of neural crest cells *in vivo*, where they form a complex with polymerase II that directly regulates N-cadherin transcription and collective cell migration [16]. These seemingly contradictory studies demonstrate that a complex interplay between Cx43 and GJA1-20k occurs, and that subtle context-dependent pathways may regulate the timing, expression, and even the role of GJA1-20k to allow fine-tuned cellular behavior during EMT. This example further illustrates the urgent need for the development of specific tools to enable investigation of the distinct contribution of full-length Cx43 and its smaller isoforms to tissue homeostasis and physiology.

In the heart, GJA1-20k stabilizes actin filaments, which are required for microtubule-mediated Cx43 hemichannel delivery to the intercalated discs and preserves Cx43 at the plasma membrane under pathological conditions, including myocardial ischemia [38]. Further studies carried out in the heart demonstrated that, in response to cellular stresses, such as ischemia/reperfusion, endogenous GJA1-20k is upregulated and translocated to the outer mitochondrial membrane (OMM; Figure 2) [38]. The presence of GJA1-20k on mitochondria has been shown to modulate mitochondrial biology, facilitating its microtubule-dependent transport and rescue of mitochondrial network upon stress [42,43]. Additionally, increased GJA1-20k expression reduced ischemia-induced cardiac damage by promoting mitochondrial biogenesis and a protective mitochondrial phenotype, similar to that observed during **ischemic preconditioning**, with a reduction in membrane potential, respiration, and reactive oxygen species (ROS) production [43]. Although certain pathophysiological conditions, including ischemia, can induce GJA1-20k expression *in vivo*, we have only scarce mechanistic and functional knowledge of these events [43]. It becomes increasingly clear that GJA1-20k, similar to Cx43, has multimodal functions due, in part, to its context-dependent subcellular localization (to microtubules, the Golgi, mitochondria, or the nucleus), which is an intriguing feature that deserves further exploration.

The Role of Cx43 in Mitochondria

Full-length Cx43 has also been detected in mitochondria of multiple cell types, including astrocytes, endothelial cells, and cardiomyocytes [44,45]. Various studies demonstrate that mitochondrial Cx43 is present mainly in the inner mitochondrial membrane (IMM), to where it is translocated by heat shock protein (HSP)90-dependent translocase of the outer membrane (TOM) complex pathway, with the C terminus oriented to the intermembrane space (Figure 2) [46–48]. Although the mechanistic details and signals that drive transport of Cx43 into the mitochondria remain mostly unknown, in-depth studies performed on other cell surface proteins and receptors

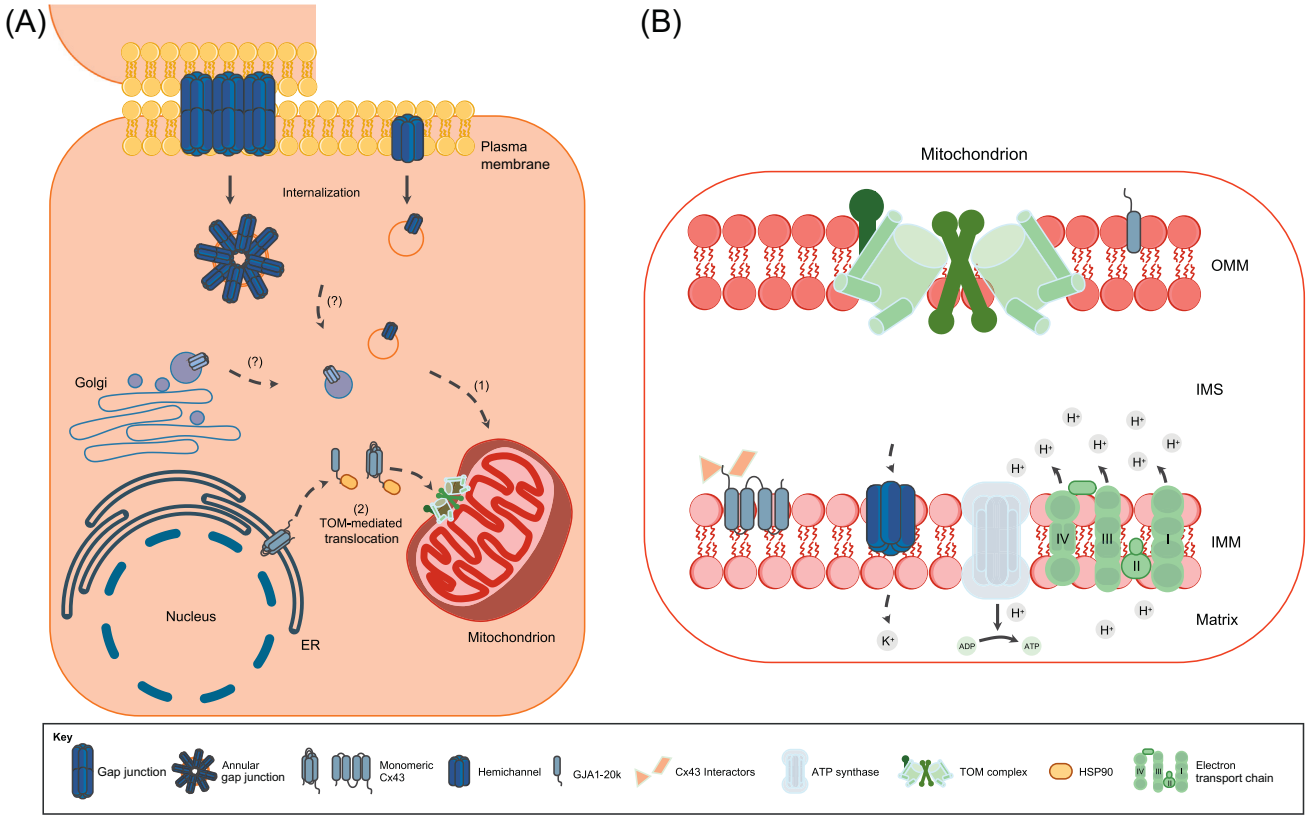

Figure 2. The Role of Connexin 43 (Cx43) in Mitochondria. Proposed models for the translocation of Cx43 into the mitochondria, involving either (1) fusion of Cx43-containing vesicles with mitochondria or (2) Translocase of the outer membrane (TOM)-mediated translocation (A). Once at the mitochondria (B), full-length Cx43 localizes at the inner mitochondrial membrane (IMM), where it impacts mitochondrial metabolism, either by acting as a K^+ channel, through the modulation of reactive oxygen species (ROS) and ATP production, or via protein–protein interactions. In addition, GJA1-20k localizes to the outer mitochondrial membrane (OMM), with important roles in mitochondrial biogenesis and trafficking. Abbreviations: HSP, heat shock protein; IMS, intermembrane space.

may provide important insights into such trafficking and can serve as models for future research (Box 4).

The role of mitochondrial Cx43 remains controversial but a substantial amount of data indicates that it modulates ROS generation, ATP levels, potassium (K^+) influx to the mitochondrial matrix, and respiration, being important in pathophysiological contexts such as ischemia/reperfusion injury ([48], reviewed in [49]). However, the exact mechanisms by which Cx43 regulates these mitochondrial processes, either in the form of a monomer, interacting with various players and participating in signal transduction pathways, or functioning as a mitochondrial channel, needs clarification. Nevertheless, the concept of Cx43 as a mitochondrial channel has been supported by results showing sensitivity to classical Cx43 channel blockers, including 18-α glycyrrhetinic acid (18αGA), carbenoxolone, and Gap19 (reviewed in [49]).

In cardiomyocytes, where most of the studies focusing on mitochondrial Cx43 have been performed, Cx43 is present exclusively in subsarcolemmal mitochondria, which have a prominent role in superoxide production, and is absent from interfibrillar mitochondria, characterized by a higher calcium (Ca^{2+}) retention, respiratory capacity, and antioxidant regeneration [46]. The impairment of mitochondrial Cx43, either by pharmacological inhibition or genetic ablation, was

Box 4. Trafficking of Cell Surface Proteins into Mitochondria

Most multispanning mitochondrial membrane proteins, including channels involved in electron transport and ATP synthesis, are encoded by nuclear genes. According to currently accepted models, mitochondrial precursor proteins are maintained in an unfolded or loosely folded conformation in the cytosol, stabilized by chaperones that facilitate further association with the mitochondrial import machinery, such as the TOM complex. Submitochondrial cargo sorting, either to the OMM or the IMM, is determined by different targeting signals that allow interactions with TOM proteins and small chaperones of the translocase of the inner membrane (TIM) family (reviewed in [98]). Additionally, in the ER surface-mediated protein targeting (ER-SURF) pathway recently described in yeast and likely conserved in higher eukaryotes, membrane proteins can be retrieved and rerouted directly from the ER to the mitochondria via the chaperone Djp1 [99].

Analogous to what was described for the nucleus, cell surface receptors can also be localized within mitochondrial membranes, including ErbB-2, EGFR, and nerve growth factor (NGF) family members, where they can affect cellular metabolism and chemoresistance [100,101]. Mitochondrial import of ErbB-2 relies on a **mitochondrial targeting sequence (MTS)** that facilitates the interaction with mitochondrial (mt)HSP70 [101]. Similarly, a functional MTS was identified in EGFR, being able to signal mitochondrial import of the full-length receptor [102]. EGF stimulation increased EGFR translocation to the mitochondria through a mechanism that depends on association with Tid1 short form (Tid1-S), an alternatively spliced isoform of Tid1 (also known as mtHSP40) that bridges further interaction with mtHSP70 [100]. Interestingly, clathrin-mediated endocytosis of EGFR can contribute to the transport of EGFR to the mitochondria in response to EGF [102]. It has been hypothesized that endosome-mitochondrial fusion or the action of TOM complexes underlies the insertion of EGFR into mitochondrial membranes.

Sorting of G-protein-coupled receptor kinase 2 (GRK2) into the OMM was also described, particularly under oxidative stress conditions [103–105]. ERK-mediated phosphorylation of GRK2 promoted binding to HSP90, which further targeted GRK2 to the mitochondria, where it was reported to participate in cell death signaling [105].

shown to result in reduced ADP-stimulated complex 1 respiration, mitochondrial K^+ influx, oxygen consumption, and ROS production, with the concomitant loss of ischemic conditioning-mediated cardioprotection (reviewed in [49,50]). Although uncontrolled excessive ROS generation is associated with injury during reperfusion, low mitochondrial ROS levels present signaling properties that are essential for the protective effect of ischemia pre- or post conditioning. Importantly, overexpression of Cx32 in a Cx43-deficient model did not rescue the cardioprotective effect of preconditioning, suggesting that this effect is specific of Cx43 [48,51]. Inversely, mitochondrial Cx43 channels have been implicated in cell injury and/or death induced by mitochondrial Ca^{2+} overload, suggesting that Cx43 is important to regulate Ca^{2+} homeostasis in mitochondria [52]. In brown adipose tissue, the presence of Cx43 in mitochondria was also suggested to be required to maintain mitochondrial integrity and metabolic activity [53]. Subsequent to the early landmark discoveries around Cx43, other members of the connexin family have been found to regulate mitochondrial metabolism. For example, the closely related alpha-connexin Cx40 (*GJA5*) is expressed in mitochondria of coronary endothelial cells and its depletion leads to a decrease in mitochondrial Ca^{2+} uptake and ROS concentration [54].

Deregulation of cellular energetics and cell death are two central hallmarks of cancer in which mitochondria hold center stage. Indeed, Cx43 appears to modulate the B cell lymphoma 2 (Bcl2)/Bcl2-associated X protein (Bax) ratio, cytochrome-c release, and caspase-3 activation, thereby providing increased resistance to toxins, chemotherapeutic drugs, or ionizing radiation [55–57]. The exact mechanisms remain unclear, but the Cx43 C terminus has been shown to directly interact with, and activate, Bax via c-Jun N-terminal kinase (JNK) activation [58,59].

The Role of Cx43 in Long-Distance Cell Communication

Initially regarded as mere cell disposal vectors, EVs are now acknowledged as major vehicles for the exchange of information across distant cells and tissues, participating in myriad biological processes, from antigen presentation to cardiovascular diseases and cancer progression [60,61]. Mounting evidence has demonstrated that EV secretion is an evolutionarily conserved process, carried out by almost every cell type and detected in almost every biological fluid,

elucidating EVs as potential disease biomarkers and therapeutic vehicles [60,62]. In agreement, EVs can carry a large variety of active macromolecules, including proteins, metabolites, lipids, and nucleic acids, which can be selectively sorted during vesicle biogenesis [63,64].

Although proteomic studies identified the presence of several connexin family members, including Cx43, Cx45, and Cx32, in EVs, the first functional evidence for a role for Cx43 on EV-mediated communication was only reported in 2015 [65,66]. Since then, Cx43 has been reported in EVs released by epithelial cells, cardiomyocytes, endothelial cells, organotypic heart cultures, and circulating in human plasma [66–68]. It was demonstrated that hexameric Cx43 structures assemble at the EV surface, where they facilitate the rapid release of intraluminal contents directly into the cytoplasm of target cells, thus constituting an alternative mechanism of EV–cell interaction and information delivery to recipient cells [66]. According to the proposed model, Cx43 channels at the EV surface dock with unopposed hemichannels at the plasma membrane of target cells, forming a GJ-like structure that enables the transfer of small molecules [66,67].

Given the emerging importance of EVs in cancer and metastasis, the presence of Cx43 in EVs, and the capacity for Cx43 to interact with numerous RNA, DNA, and protein sequences, it will be critical to assess the complex pro- and antitumorigenic associations of connexins mediated through this pathway [69]. Remarkably, Cx43-containing vesicles have been explored as vehicles of therapeutic cargo, including small chemotherapeutics [67,70]. In fact, the use of cell-derived vesicles containing functional Cx43 channels to deliver doxorubicin dramatically increased its therapeutic effectiveness compared with liposomes or free drug [70]. Indeed, Cx43 not only preserves the chemotherapeutic properties of EVs loaded with doxorubicin, but can also diminish the cardiotoxicity of the treatment, suggesting that Cx43 confers target cell specificity to these vesicles [67,70]. Although the mechanisms underlying the reduced cardiotoxicity are undefined, communication between EV-Cx43 and tissues with reduced levels of free unopposed Cx43 hemichannels, such as the heart, could be impaired [65,67].

Nonetheless, given the ubiquitous expression of connexins, some strategies have been developed to modulate vesicle targeting to specific cell populations. Accordingly, engineered EVs containing the Cx43-S368A mutation that forms constitutively open channels, along with a lysosomal-associated membrane protein (Lamp2b) brain-targeting module, displayed increased delivery of cargo mRNAs to the brain of animal models of Parkinson's disease, which were able to mitigate neurotoxicity and neuroinflammation [71]. In addition, EV surface expression of single-domain antibodies against receptors targeting specific cell populations was demonstrated to confer increased selectivity of Cx43-mediated release of intraluminal contents [72].

A second mechanism of communication between nonadjacent cells is through actin-based cytoplasmic extensions comprising open-ended channels called tunneling nanotubes (TNTs), which mediate the passage of small molecules, protein aggregates, organelles, or viruses [73]. Classically, the presence of Cx43 in TNTs is associated with channel-mediated transfer of ions, small metabolites, or second messengers, such as Ca^{2+} and inositol trisphosphate (IP3), thereby participating in electrical and metabolic coupling between connected cells, which has been particularly associated with cancer development [65]. Indeed, Cx43-containing TNTs contribute to astrocytoma progression and radioresistance through the maintenance of Ca^{2+} homeostasis [74]. More recently, it was shown that Cx43 expression can regulate TNT-mediated transfer of mitochondria and the spread of HIV, thus anticipating channel-independent functions for TNT-Cx43 with implications for infection and immunity [75,76].

Concluding Remarks

The emerging concept of noncanonical functions of connexins is in its infancy. However, the biological and physiological impact of the results gathered over the past few years anticipate a promising future for this field. Although initially thought to solely underpin GJIC, it is now acknowledged that connexins participate in several other biological processes (see Outstanding Questions). Multiple reasons can explain this multifaceted nature of connexins. For example, it is conceivable that the unusual short half-life of Cx43 (1–3 h), which permits a precise regulation of protein levels by degradation, makes it a suitable candidate to participate in mechanisms requiring a fine-tuned regulation, such as cell cycle regulation, transcription, or development. Furthermore, connexins are emerging as regulatory hubs for different processes, owing to the presence of several protein–protein interaction domains that enable connexins to act as scaffolding proteins, modulating not only the subcellular localization and availability of their interactors, but also their function. Knowing the numerous pathophysiological processes in which connexins participate, this new way of looking at these proteins not only enhances our understanding of disease, but also raises new perspectives for the design of innovative therapeutic strategies.

Outstanding Questions

Which connexin domains are involved in its translocation to the nucleus?

How do connexins regulate gene transcription?

What is the physiological function and role of truncated connexin forms?

Will truncated forms of connexins provide insight into the complex disease spectra associated with connexin mutations?

How are connexins transported either to the IMM or OMM?

Is the function of mitochondrial connexins due mainly to channel-mediated transfer of ions, or do connexins act as scaffolding mitochondrial proteins?

How do EV-associated connexins regulate the docking of vesicles to the target cells?

Can connexins at the EV surface determine the selective targeting of the vesicles?

Can we use connexin-containing EVs to deliver drugs to specific cell types and/or tissues?

Can we perform pharmacological approaches specifically targeting the different intracellular pools of connexins?

What are the pathophysiological cues that determine the targeting of connexins to different organelles or cell structures?

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