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Review

Recruitment of RNA molecules by connexin RNA-binding motifs: Implication in RNA and DNA transport through microvesicles and exosomes

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ABSTRACT

Connexins (Cxs) are integral membrane proteins that form high-conductance plasma membrane channels, allowing communication from cell to cell (via gap junctions) and from cells to the extracellular environment (via hemichannels). Initially described for their role in joining excitable cells (nerve and muscle), gap junctions (GJs) are found between virtually all cells in solid tissues and are essential for functional coordination by enabling the direct transfer of small signalling molecules, metabolites, ions, and electrical signals from cell to cell. Several studies have revealed diverse channel-independent functions of Cxs, which include the control of cell growth and tumourigenicity. Connexin43 (Cx43) is the most widespread Cx in the human body. The myriad roles of Cx43 and its implication in the development of disorders such as cancer, inflammation, osteoarthritis and Alzheimer's disease have given rise to many novel questions. Several RNA- and DNA-binding motifs were predicted in the Cx43 and Cx26 sequences using different computational methods. This review provides insights into new, groundbreaking functions of Cxs, highlighting important areas for future work such as transfer of genetic information through extracellular vesicles. We discuss the implication of potential RNA- and DNA-binding domains in the Cx43 and Cx26 sequences in the cellular communication and control of signalling pathways.

1. Introduction

Connexins (Cxs) and pannexins in vertebrates and innexins in invertebrates are a family of proteins involved in cell to cell and cell to the extracellular space communication [1,2]. In humans, 21 isoforms of the Cx multigene family have been identified [3]. Cxs show cell-type-specific but overlapping patterns of expression, as well as shared topology and functions, such that some Cxs can functionally replace others [4]. However, genetically modified animal models have demonstrated that distinct properties and functions of Cxs [5] make them largely non-interchangeable [4,6].

Cx hemichannels (connexons) consist of a hexameric unit of six Cxs. Hemichannels allow the direct exchange of molecules and metabolites with the extracellular matrix. Gap junction (GJ) channels are formed by the apposition of connexons from adjacent cells and allow direct exchange between contacting cells. Cxs are known to play a role in cellular functions such as cell guidance, cellular adhesion and cell growth, in both gap junction-dependent and gap junction-independent manners. Cx43 is the most completely characterized Cx isoform in terms of its channel gating properties, identified phosphorylation sites, protein interactions and channel assembly and turnover. Cx43 consists of 382 amino acids (Fig. 1A) and has versatile functional properties reflected by its distribution in multiple tissues and cell types [7,8]. Cx43 is a conditional tumour suppressor gene and participates in the synchronous contraction of muscle cells, bone remodelling, embryonic development

Abbreviations: Cxs, connexins; Cx26, connexin26; Cx43, connexin43; CL, cytoplasmic loop; CTD, C-terminal domain; EGFRvIII, epidermal growth factor receptor variant III; E1-E2, extracellular loops; GJs, gap junctions; GJIC, gap junction intercellular communication; MVs, microvesicles; mtDNA, mitochondrial DNA; NMR, nuclear magnetic resonance spectroscopy; NTD, N-terminus; M1-M4, transmembrane domains; RPBs, RNA-binding proteins.

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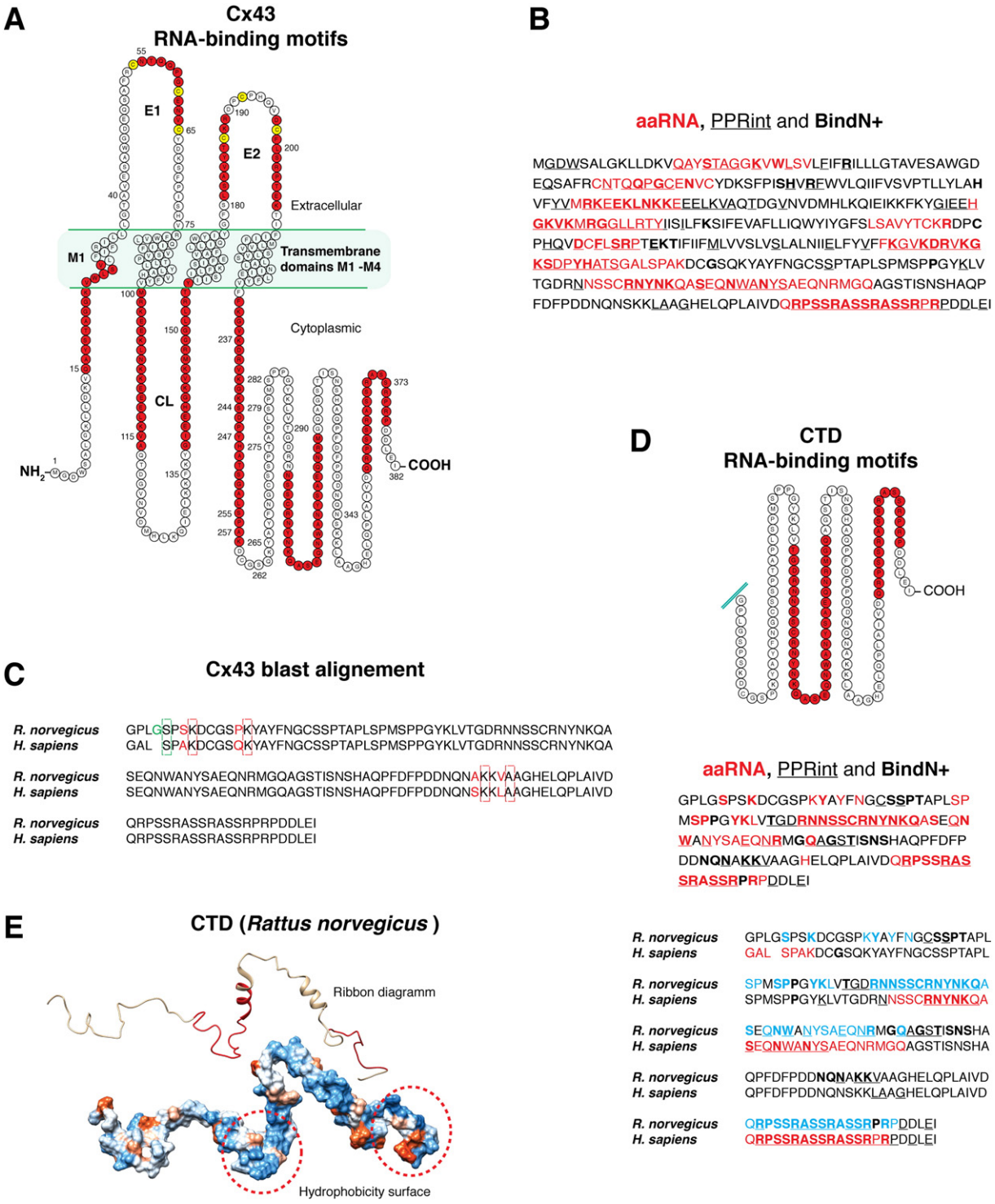

Fig. 1. Schematic representation of Connexin43 and the predicted RNA-binding domains. (A) Topological diagram of the Cx43 structure, with an N-terminal end, four transmembrane domains (M1–M4), two extracellular loops (E1, E2), an intracellular loop (CL) and the C-terminal domain (CTD). The membrane region is shown in light green. Yellow circles represent extracellular cysteine residues. Amino acid sequences in which the three computational methods coincide to predict RNA binding propensity are shown in red. (B) Amino acid sequence of Cx43 with the predicted representation of three computational methods. aaRNA in red (highest score), PPRint (SVM threshold: −0.2) underlined and BindN+ (specificity: 85%) in bold. (C) Amino acid residue alignment between the CTD of Cx43 of *Rattus norvegicus* and *Homo sapiens*. Amino acid substitutions are shown in red. Gaps are shown in green. (D) Topological diagram of the CTD of Cx43 of *R. norvegicus*. RNA binding propensity (amino acid sequence) predicted by the three computational methods is shown in red. Below, sequence alignment between part of the CTD of rat and human with the predicted representation of three computational methods. aaRNA in blue and red, PPRint underlined and BindN+ in bold (E) Ribbon model of the CTD structure (top) and hydrophobicity surface (bottom) according to the Kyte and Doolittle scale with values ranging from blue (hydrophilic) to orange red (hydrophobic) [115]. The red dashed circles highlight the predicted RNA-binding domains.

and homeostasis in tissues, among other functions. A hallmark of the broad functional spectrum of Cx43 is the rare disease oculodentodigital dysplasia, caused by mutations in the Cx43 gene (GJA1), which affects the shape and function of many different parts of the body [9].

2. Horizontal transfer through membrane vesicles

The transmission of information between cells in the body occurs through several mechanisms of communication, such as the secretion

of factors including small molecules, nucleotides and bioactive lipids, cell to cell contacts mediated by GJs, tunnelling nanotubes that connect cells, and membrane transfer by the secretion of membrane vesicles generally referred to as microvesicles (MVs) (e.g., microparticles, apoptotic blebs which can also contain cytosolic organelles and nuclear fragments, and exosomes) [10].

The biogenesis and properties of microvesicles (see Fig. 3B) include a diverse group of membrane vesicles that share common characteristics and participate in intercellular communication as membrane-derived particles [11]. These MVs increase the complexity of cell signalling by transmitting direct information via the proteins, lipids, nucleic acids and other components that they contain, which can directly alter signalling in the recipient cell. Horizontal transfer to recipient cells occurs through the fusion or internalization of MVs [12–14]. However, the transfer of compounds also occurs through membrane adhesion and transport by "sticky" vesicles and GJs [15–19].

MVs are highly effective delivery vehicles and may contain genetic material in the form of mRNAs or microRNAs that are transmitted by intercellular communication, including those involved in tumourigenesis, growth, division, differentiation or stress responses. MVs are known to play a role in pathology by promoting tumour growth [20], stimulating the autoimmune response in rheumatoid arthritis [21], transporting proteins and RNA such as EGFRvIII mRNA in clinically distinct subtypes of glioblastoma [22], and spreading viruses or prions [23–26] among dozens of other functions such as reprogramming target cells and tissue repair [27].

Thousands of mRNAs and hundreds of miRNAs have been found inside exosomes. Some studies have suggested that there is a selective loading of specific mRNA molecules into exosomes [28]. In addition, mitochondrial DNA (mtDNA) has been found in the exosomes of astrocytes, myoblasts and glioblastoma cells [29–31]. Interestingly, exosome-delivered RNAs mediate de novo transcriptional and translational changes in recipient cells [32]. In a report published in 2007, it was demonstrated that exosome-mediated transfer can even occur between mouse exosomes containing mRNA and human mast cells, resulting in the expression of mouse proteins in human cells [32].

A recent report has demonstrated that Cx43 controls the interaction between exosomes containing Cx43 and recipient cells, favouring the internalization of the vesicles [18]. This study also demonstrated that exosomes and cells are able to exchange material through Cx channels, similar to the ability of contacting cells to communicate through GJ channels [18]. These results clearly suggest that Cx43 has pivotal and differential roles in cellular communication mechanisms that occur through membrane particles.

The horizontal transfer of coding and non-coding RNAs offers new perspectives on intercellular communication, and has been already suggested to have potential therapeutic applications in gene delivery. The presence of Cxs on MVs, their ability to target cells and the increased efficacy of Cx43-expressing delivery particles [16,18] highlight the importance of this mechanism. It is important to note that tunnelling nanotubes also provide cytoplasmic bridges between cells that may allow the transfer of annular gap junctions (double membrane vesicles) and MVs containing Cxs.

3. Structural organization of connexins

All Cxs share the same structural arrangement consisting of four transmembrane domains (M1–M4), two extracellular loops (E1–E2), and three intracellular domains: one cytoplasmic loop (CL), the N-terminus (NTD) and the C terminal domain (CTD) [5] (Fig. 1A). These structural motifs were confirmed by solving the crystal structure of the domains of Cx43 [33–35], human connexin26 (Cx26) and Cx26 hexameric GJ channels [36–39]. The X-ray structure of Cx26 solved by Maeda et al. revealed structural details of different domains as well as potential interactions between them [38]. These authors have described the diameter of the complete structure including the diameter of hydrophilic GJ channels at the cytoplasmic surface.

It is predicted that the overall structure solved for the Cx26 protein and Cx26 GJ channels are representative of the structures of all Cxs and GJ channels since Cxs share sequence and topological homology. However, the CTDs are the least conserved domains among Cxs and have resisted crystallization, which suggests that CTDs are probably unstructured and highly flexible [40].

3.1. Identified RNA-binding motifs in Cx43 and Cx26

The experimental determination of protein-RNA complexes is a major challenge in structural biology due to difficulties associated with their crystallization or resolution by nuclear magnetic resonance spectroscopy (NMR). To solve this problem, many computational methods have been developed and successfully used for predicting protein-RNA binding sites, using protein sequences and/or structural features. Here we present several RNA-binding motifs predicted in the Cx43 and Cx26 sequence using different computational methods. Our analysis utilized three different programs with different criteria and prediction accuracy and confirms the presence of at least ten potential RNA-binding motifs in the sequence of human Cx43 (Fig. 1A–B and Supp. Fig. 1A–C), including a motif in the N-terminal domain (NT), one in the transmembrane domain M1, sequences located next to the cysteine residues of each extracellular loop (E1 and E2), two separated sequences in the cytoplasmic loop (CL) and three motifs in the CTD. Fig. 1A–B and Supp. Fig. 1A show the homology modelling structure depicting structural features predicted for the Cx43 protein and the identified putative RNA-binding sites (aaRNA).

The aaRNA web server (https://sysimm.ifrec.osaka-u.ac.jp/aarna/) uses a method that out-performs sequence or structure predictors by combining in-line homology modelling with the features of the SRCPred method and hidden Markov model (HMM)-based evolutionary conservation scores for conservation evaluation, local relative accessible surface area (rASA) and Laplacian norm (LN) coordinates to represent molecular structure [41]. More than ten motifs (red) were identified using the validated aaRNA method (Fig. 1B), out of which ten motifs were selected based on their overall score, threshold and specificity. Fig. 1A–B represent the Cx43 sequence and structure, highlighting the common motifs that were independently predicted by the aaRNA (red) and PPRint (underlined) (http://www.imtech.res.in/raghava/pprint/) and BindN+ (bold) (http://bioinfo.ggc.org/bindn±/) programs [42,43]. BindN+ applies a machine learning method (SVM) to the sequence-based prediction of DNA- or RNA-binding residues from biochemical features (molecular mass, hydrophobicity, side chain pKa values, among others) and evolutionary information. SVM is a widely used algorithm for binary classification by means of supervised learning. The BindN+ system performs a three-iteration PSI-BLAST search against the UniProtKB database to derive evolutionary information. Similarly, PPRint also accepts amino acid sequences as input and automatically generates the evolutionary profile by running PSI-BLAST. Like BindN+, it generates SVM patterns from the PSSM profile and predicts RNA interacting residues using an SVM model.

Out of all the domains of Cx43, only part of the structure of the CTD (from rat) has been resolved to an atomic level [33,35,44,45]. An analysis using the amino acid sequence and the structure of the rat CTD, which has 98% sequence homology to human CTD (Fig. 1C), confirms the same predicted RNA-binding motifs that we previously identified for the human CTD sequence (Fig. 1B and D). The ribbon structure and the hydrophilic (blue) and hydrophobic (red) faces are shown in Fig. 1E, with the predicted RNA-binding motifs indicated by dashed circles.

In contrast to Cx43, the structure of Cx26, the Cx26 connexon (hemichannel) and the Cx26 GJ were resolved by cryo-electron microscopy and X-ray crystallography to an atomic resolution [37,38,46–48], providing valuable information for the prediction of RNA-binding sites. Fig. 2A and Supp. Fig.1D represent the Cx26 topology and

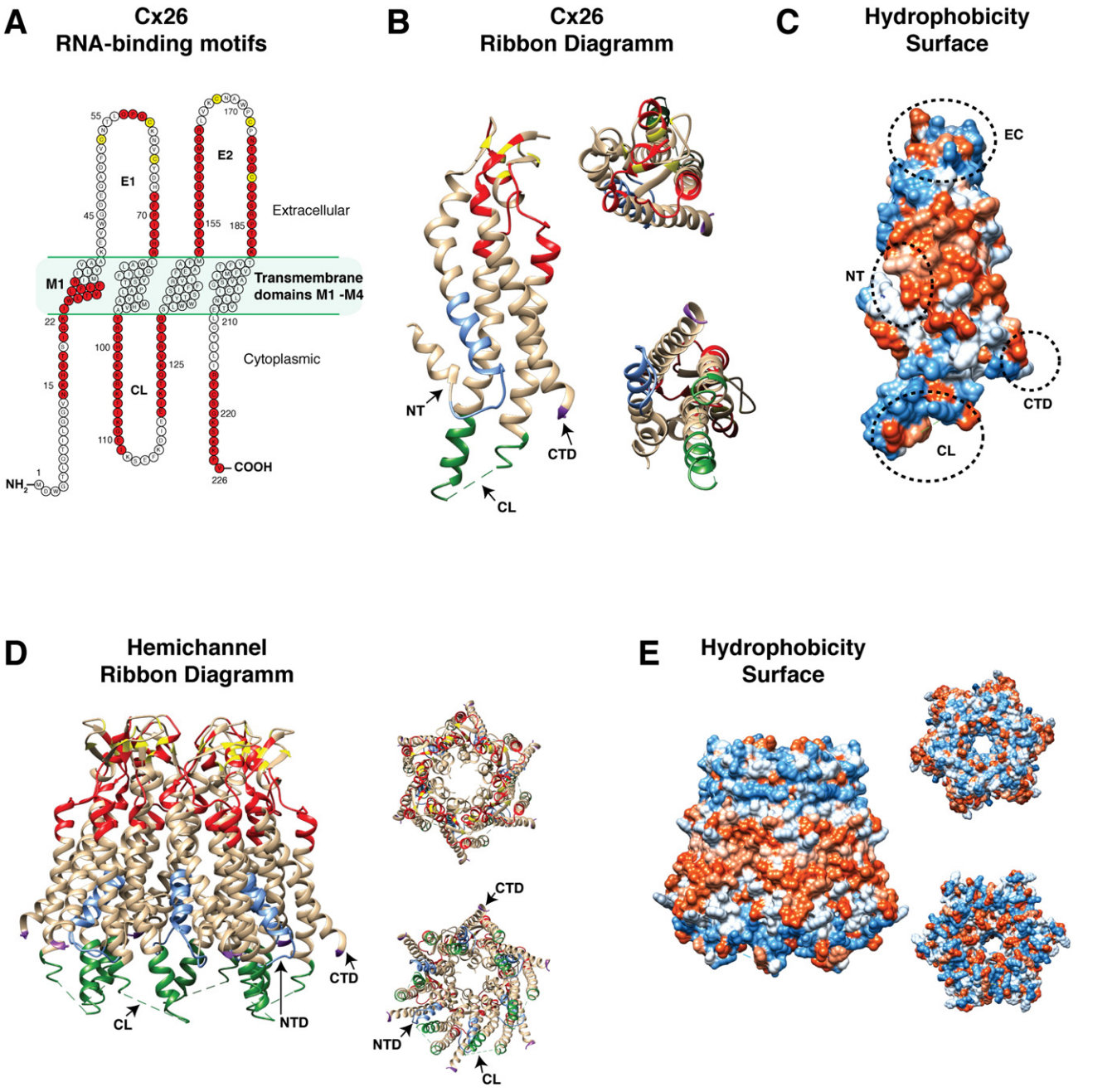

Fig. 2. Predicted RNA-binding motifs in the Cx26 sequence. (A) Topological diagram of Cx26 amino acids and structure. Yellow circles represent extracellular cysteine residues. The amino acid domains predicted to have RNA binding propensity by the three computational methods are shown in red. (B) Ribbon model of Cx26 structure with the predicted RNA-binding motifs in different colours. Light blue represents the NT–M1 domains; green, CL; red E1 and E2; yellow, cysteine residues. The motif predicted in the CTD is shown in purple. The extracellular (above) and cytoplasmic (below) axial views are shown on the right. (C) Ribbon model of the hemichannel (connexon) structure formed by Cx26. The axial views for extracellular (above) and cytoplasmic (below) are shown on the right. (D) Molecular surface showing the hydrophobicity values according to the Kyte and Doolittle scale [115]. The black dashed circles show the location of the predicted RNA-binding motifs. (E) Density of the overall hydrophobicity on the molecular surface of the hemichannel [115].

sequence, highlighting the domains that were consistently predicted by the aaRNA (red), PPRint (underlined) and BindN + (bold) computational approaches (Supp. Fig. 1D). Concordantly with the predictions obtained for Cx43, the predicted RNA-binding motifs include those in the NTD, M1, E1 and E2 loops next to cysteine residues, two separated sequences in the CL and one motif in the CTD (Fig. 2A). The ribbon structure of Cx26 indicating the position of the predicted RNA-binding motifs is shown in Fig. 2B. Despite the accuracy of the prediction methods, these putative RNA-binding motifs will require experimental validation to confirm and understand such interactions.

An understanding of the sequence specificities of such interactions and the identification of the RNA species bound to those domains will provide essential information and new models of regulatory processes and human diseases. Interestingly, the RNA-binding motifs found in the C-loop (MRKEEKLNKKEEELKVA and GIEEHGKVKMRGGLLRTY) and in the CTD of Cx43 (FKGVKDRVKGKSDPYHATSGALSPAK and QRPSSRASSRASSRPR) are rich in arginines and lysines. Lysine- and arginine-rich motifs have the highest propensity to bind with RNA. It has been reported that individual arginine residues govern binding to an RNA ligand, and the inherent flexibility of the peptide backbone may make it possible for specific recognition of RNAs [49,50].

These sequences play many critical roles in Cx43 function. For example, QRPSSRASSRASSRPR sequence is a pivotal site of protein-protein interactions [51]. This protein-RNA binding site (RPSSRASSRASSR) was

independently predicted by the three computational methods (Fig. 1B). Notably, the sequence contains several serine residues that are phosphorylated (e.g. S364, Ser365, S368, Ser369, S372, S373) by different kinases (e.g. protein kinase C, protein kinase A, Ca + 2/calmodulin-dependent kinase II) [52,53]. The phosphorylation and dephosphorylation of Cx43 can induce conformational changes and regulate the kinetics of Cx channels assembly, gating and turnover [8,54]. Modification of protein properties by post-translational modifications influences in the protein activity, stability, subcellular localization and ability to interact with other molecules (e.g. RNA or DNA) and proteins. The heterogeneous nuclear ribonucleoproteins A2B1 protein (hnRNPA2B1) has been demonstrated to control the loading of certain miRNAs into exosomes through recognition of specific short motifs [55]. These authors have demonstrated that SUMO conjugation controls the interaction of hnRNAPA2B1 with RNA molecules [55]. Sumoylation and phosphorylation are associated events. There are many examples in the literature where the sumoylation enables the interaction of kinases resulting in the phosphorylation and activation/inhibition of the protein. These and other evidences suggest that the RNA-binding site RPSSRASSRASSR may play an important role in the loading of RNA/DNA/protein complexes acting as a platform through which connexin-binding partners could interact. Besides, there are two matrix metalloproteinase-7 (MMP-7) cleaved sites at the A357-I358 and D379-L380 residues [56]. These two cut sites in the Cx43 protein (LAIVD*RPSSRASSRASSRPR*PDDL) would generate a free C-terminal peptide containing the DNA- and RNA-binding motifs (Figs. 1A and 4A).

3.2. Hydrophobicity of connexin domains

The members of the connexin family have similar topologies, and each domain has differential functional roles. For example, the EC loops facilitate the binding of one connexon to a connexon on an adjacent cell to form a GJ channel [57]. The NTD is a key component in gating [58]. The CL and CTD regulate channel activity depending on environmental factors such as cytoplasmic pH or calcium concentration [59,60]. The CTD is also a critical regulatory region essential for channel-dependent and channel-independent functions of the protein [61–64].

The structure and amino acid sequences of Cxs are highly conserved, but the cytoplasmic domains vary considerably between different isoforms, differing mainly in the sequence and lengths of the CL and CTD. For example, Cx26 contains a shorter CTD compared to Cx43. The overall ribbon structure of the Cx26 monomer and the hexameric hemichannel are shown in Fig. 2B and D, respectively. The different colours show the RNA-binding motifs located in different protein domains. The RNA-binding motifs located in the CL are represented in green, while those in the CTD are represented in purple. Note that the studies of Maeda *et al.* only describe the structure of a small sequence of the Cx26 CTD [38]. The hydrophobicity of the Cx26 monomer and the hemichannel are shown in Fig. 2C and E. As expected, the extracellular and cytoplasmic axial views show that hydrophilic residues (blue) are oriented towards the central axial pore, which allows the transfer of ions and small signalling molecules with relatively low specificity. Hydrophilic areas are also concentrated in the cytoplasmic and extracellular domains. The predicted RNA-binding domains coincide with some of these hydrophilic regions (dashed circles) (Fig. 2C), suggesting that the positively charged amino acid residues could form ionic bonds with negatively charged phosphate groups. Other types of interactions such as electrostatic interactions may be involved in this binding.

3.3. Functional cooperation between RNA-binding motifs

Most RNA-binding proteins contain several domains, including different types of RNA-binding motifs, which seem to be highly versatile and structurally diverse. Many proteins containing different RNA-binding motifs do not interact with each other and appear to bind RNA independently. However, other proteins recognize RNA sequences with a specific affinity that would not be possible for a single domain or without the cooperation of multiple domains. For example, multiple domains are capable interacting simultaneously with different sequences of the same RNA (long RNAs) separated by many nucleotides. The tethering of domains (polypeptides) to create a larger binding interface usually improves the attachment of RNA to the protein [65].

Interestingly, some Cx domains with predicted RNA-binding motifs structurally interact with each other. For example, the cytoplasmic NTD has been described to play a critical role in channel gating as part of the transjunctional voltage sensor [66–68] and interacts with transmembrane domain M1 (Supp. Fig. 3A). Electron cryo-crystallography, X-ray diffraction [38] and cryo-electron microscopy [37] demonstrated the presence of a plug created by the folding of the NTD fold into the channel (M1 domain). The presence of a plug has been suggested to physically block the channel within the membrane. The structural interaction between the NTD and M1 changes conformation depending on gating, but nevertheless occurs whether the channel is open or closed [69] (Supp. Fig. 3A). On the other hand, the M1 domain of Cx26 is required for hexamer formation and channel function [70]. Deafness-associated Cx26 mutations in the M1 domain affect the assembly and functioning of hemichannels and GJs [70]. It is possible that the binding of RNA to the NTD (and/or M1) could be implicated in the attachment or sequestering of long RNAs to the membrane and/or in the transport of small RNAs, such as microRNAs, through the pore [71,72].

An interaction between the cytoplasmic loop (CL) and plug loop of Cx26 has been described to regulate the complex gating mechanism of these channels [48,73,74]. Therefore, it cannot be ruled out that structural interactions described between the NTD, the M1 and the CL could simultaneously mediate or enhance the interaction with RNA sequences.

E1 and E2 are located on the extracellular face of connexons and may thus theoretically interact with extracellular small RNAs that are not encapsulated within vesicles but are protected against RNase digestion by association with other proteins [75] (Fig. 3 and Supp. Fig. 3B). The interaction of these RNAs (represented as fuchsia and blue lines) with the extracellular loops of the connexon could be implicated in intercellular communication by the transference of the small RNA (fuchsia line) directly into the cell through the pore of the channel [71,76]. However, these interactions may also be involved in intercellular communication by the binding and recruitment of longer extracellular RNAs (blue lines) to membrane vesicles, such as exosomes, while these vesicles travel through the extracellular space (Fig. 3B). Several other species of intracellular RNA that interact with the cytoplasmic domains (CL and CTD) may also form part of this complex intercellular communication through single membrane or double membrane vesicles (Fig. 3A–B). The intramolecular interaction between the CL and CTD affects the activity of the channels [77,78] and, together or individually, may also be implicated in the recruitment of different species of RNA.

Exosomes contain mostly the molecular constituents of their cell of origin. Nucleic acid binding is the most common function among proteins extracted from MVs/exosomes [79–81]. Each RNA- and DNA-binding protein has specific and differential functions. For example, the synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP) or hnRNPA2B1 have been demonstrated to control the loading of certain miRNAs into exosomes through recognition of specific short motifs [55,82]. However the molecular mechanisms of loading RNA and DNA molecules into exosomes remain elusive.

4. Membrane vesicles containing connexons

Cxs have high turnover rates, with half-lives ranging from 1.5 to 5 h [40,83–86]. When connexons of two opposing cells have docked to form double-membrane GJ channels and plaques, they are inseparable under physiological conditions [87,88]. The removal of these GJ channels from the plasma membrane occurs through the internalization of the channels (docked connexons) within one of the coupled cells through a

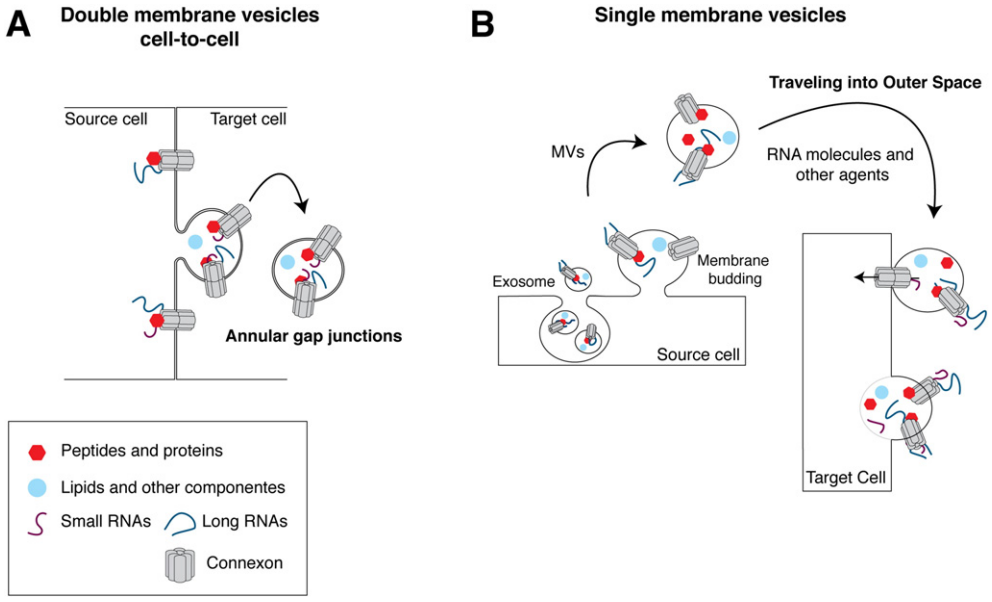

Fig. 3. Potential functional roles of the RNA-binding motifs. The image exemplifies the transfer of molecules from one cell to another via membrane vesicle trafficking. (A) Double-membrane vesicles (annular gap junctions) may allow the direct transfer of RNAs (purple and blue lines), proteins (red) and other molecules (blue circle) through mechanisms that involve the trafficking of vesicles from the donor to the contacting recipient cell. (B) Cellular communication via single membrane vesicles containing Cxs (connexons) may also enhance the transfer of several species of cytoplasmic and extracellular RNAs (purple and blue lines).

double membrane macrostructure called an annular gap junction vesicle or connexosome [87,89] (Fig. 3A). It has been widely accepted that these annular gap junction vesicles are targeted for degradation by autophagosomal and endo-lysosomal pathways [40,90]. However, this field remains controversial, and future studies are needed to define the biological importance of the direct uptake of double membrane structures containing connexin channels [91]. These hexameric connexons accommodate different molecules, including proteins attached to the cytoplasmic domains [92,93]. Through membrane vesicles, Cx43 binding partners that form macromolecular complexes [51, 92] may serve as a scaffold to transfer information, facilitate transport mechanisms or regulate signalling pathways.

Cx expression and GJ intercellular communication (GJIC) are regulated during the cell cycle [8]. Plaques of Cx43 have been detected in the plasma membrane during the S and G2 phases, and these cells show increased GJIC (as visualized by dye transfer) compared to cells in G0/G1 [94,95]. Additionally, decreased GJIC [96,97] associated with high levels of Cx43 phosphorylation [98,99], as well as the presence of large vesicular clusters of Cx43 in the cytoplasm [97,99], have also been observed during mitosis. The functional consequences and the ultimate fate of the cytoplasmic double membrane Cx43-vesicles during and after mitosis are not yet clear.

Cxs have also been identified on single membrane vesicles derived from various sources, referred to as exosomes [16,18]. In addition, Cxs have been included in the composition of membrane vesicles designed to mimic the protein content of exosomes to create a more effective delivery particle [100]. The presence of Cx43 on these membrane vesicles increases the efficacy of liposomes in delivering anti-inflammatory peptides into Cx43-expressing cells, suggesting that delivery is dependent on Cx43 expression in the recipient cells [16]. This and other evidence indicate that Cx43 participates in intercellular communication involving membrane vesicles.

5. Horizontal transfer of DNA fragments

Intriguingly, cytoplasmic DNA that originates in the genome but remains associated with the plasma membrane has been described [101]. These DNA fragments have become of interest for the study of innate immunity, particularly in the case of lupus erythaematosus [102]. Deep sequencing studies have demonstrated that these DNA fragments probably originate from the centromeric and pericentromeric regions of all chromosomes and are precisely removed [103]. These studies have also demonstrated that this DNA can be transcribed at the plasma membrane by RNA polymerase II converted at this site into RNA. The physiological roles of these DNA and RNA transcripts remain uncertain.

The BindN + and DP-Bind computational methods [43,104] predicted several DNA-binding sites in the Cx43 and Cx26 sequences located in the NTD, E2 and CTD that could directly participate in the anchoring of DNA to the membrane (Fig. 4 and Supp. Fig. 2). It would be interesting to investigate if Cxs, particularly Cx43 and Cx26, are involved in the modulation of signal transduction in the same cell or in the target cell.

6. RNA metabolism and gene expression

Others and we have previously found that proteins that interact with Cx43 participate in RNA-related processes [92,105] such as transcription, splicing, processing, transport and RNA chaperones by helping the RNA to form secondary or tertiary structures [92,105]. Proteins containing one or more RNA-binding domains have a variety of RNA binding preferences and functions and are involved in each step of RNA metabolism including splicing, processing, transport and localization. Besides, these structured RNAs can act as a signal for other RNA-binding proteins (RBPs) that mediate gene regulation. Thus, Cxs through their RNA-binding domains may be involved in RNA metabolism and a synchronized protein production by orchestrating protein-RNA complexes in the same cell or in the target cell. Additionally, several reports have suggested that Cx43 regulates gene transcription, but the mechanism has not yet been described. We cannot also disregard the possibility that Cxs may interact with and recruit extracellular DNA involved in innate immunity, the activation of inflammatory pathways, cancer or the spread of cell damage [106–111] through the DNA-binding motif located in extracellular loop EL2 (Fig. 4 and Supp. Fig. 2).

The potential activity of individual DNA-binding motifs (peptides released by physiological cleavage) [56,64] may be a powerful tool capable of binding specific DNA sites to control gene expression or chromatin structure [112,113]. Several peptides (in the Cx43 sequence)

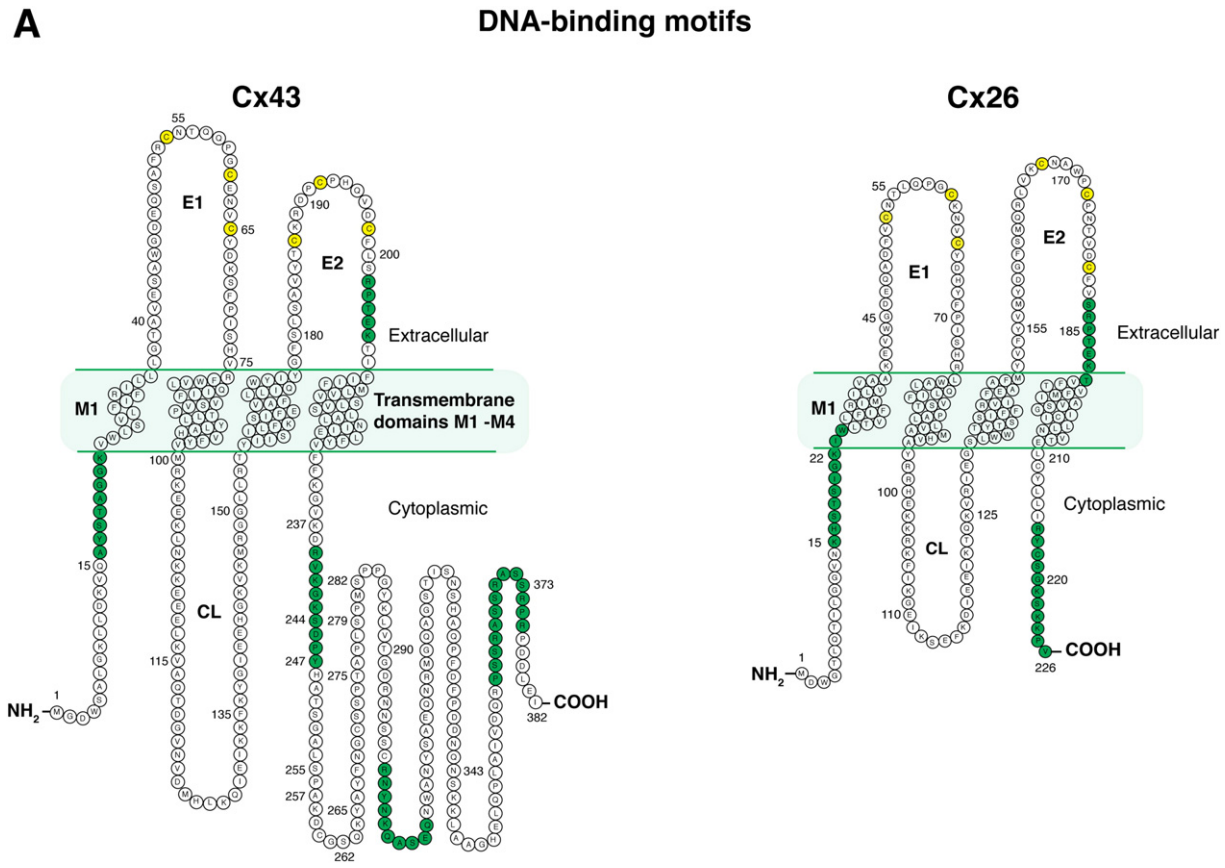

Fig. 4. Predicted DNA-binding motifs in the Cx43 and Cx26 sequences. The topological diagrams show the amino acid sequence of Cx43 (A) and Cx26 (B) with the predicted DNA-binding residues (highlighted in green) using the BindN+ (specificity: 85%) and DP-Bind (probability >0.5) computational tools (see Suppl. Fig. 2).

have already proposed the profile of connexins as therapeutic targets for cardiac reperfusion injury in heart attack patients, tissue regeneration and wound healing or narcolepsy [114]. Further studies will clarify the role played by Cxs and their peptides in the regulation of gene expression, chromatin structure and RNA metabolism.

7. Concluding remarks

Here, we identify several potential RNA- and DNA-binding motifs in the sequences of Cx43 and Cx26. Whether these putative motifs have any function *in vivo* remains unknown at this stage. However, the location of the motifs, together with the known function of connexins, makes this an intriguing possibility for future research. A full understanding of how these putative binding motifs might be involved in connexin and microvesicles functions will require detailed studies that include functional and structural characterization. We expect that future analyses will expand on the diverse functions of connexins and identify which specific RNA or DNA molecules bind to regulate cellular signalling and the differential roles of connexins, especially during tumourigenesis and metastasis. Furthermore, Cx-positive vesicles have valuable potential as a therapeutic resource for drug delivery vehicles, improving molecular transport across the plasma membrane barrier.

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Author contributions

MV-E performed the analysis and prepared the Figs. AV-V, MR-C-M and AV-S provided technical support. EF provided helpful suggestions. AV-S, JLM and EV provided critical input. MDM made the original observation and wrote the paper. All authors discussed the data and commented on the manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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