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A Review

Connexins: synthesis, post-translational modifications and trafficking in health and disease

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Abstract: Connexins are tetraspan transmembrane proteins that form gap junctions and facilitate direct intercellular communication, a critical feature for the development, function and homeostasis of tissues and organs. In addition, a growing number of gap junction-independent functions are being ascribed to these proteins. The connexin gene family is under extensive regulation at the transcriptional and post-transcriptional level, and undergoes numerous modifications at the protein level, including phosphorylation, which ultimately affects their trafficking, stability and function. Here, we summarize these key regulatory events, with emphasis on how these affect their multi-functionality in health and disease.

Keywords: connexins; gap junctions; transcription; translation; post-translational modifications; trafficking.

1. Introduction

Since the cloning of the first connexins in the 1980's, steady progress towards elucidating their regulation and function as signalling hubs and mediators of direct intercellular communication has been made [1-3]. All connexins share a conserved four-transmembrane domain structure that assembles into hexameric pores known as connexons that can integrate into the cell membrane (Figure 1). Hundreds to thousands of these connexons typically dock with opposing connexons in an adjacent cell, creating intercellular channels forming a clustered gap junction plaque that permits direct flux of ions and small cytosolic signalling molecules between cells, commonly referred to as gap junctional intercellular communication (GJIC) (Figure 1). More recently, connexons have been shown to act as "hemichannels" to facilitate direct exchange of molecules between the cell cytosol and the extracellular milieu under specific conditions [4]. Additionally, numerous non-canonical channel-independent functions have been described, in particular for connexin 43 (Cx43), that are mediated through direct protein interactions and modulation of signalling pathways [5]. The complexity and isoform-specificity of the connexin gene family is reflected by their links to numerous human diseases, many of which are rare syndromes with unique genotype-phenotype associations [6,7]. This latter phenomenon is underscored by the observation that mutations in different connexins

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48 can cause the same disease, whereas varying mutations in one connexin gene can result in vastly
49 divergent diseases and phenotypes. Dysregulation of connexins is also increasingly linked to many
50 common, and often morbid, medical conditions such as stroke, heart attack and cancer, which have
51 been linked to the discovery of an expanding number of new functional attributes through both gap
52 junction-dependent and -independent mechanisms [2,3,6-8]. As such, exploring the clinical and
53 therapeutic potential of connexins as drug targets is pertinent and ongoing [9-11]. Towards this, a
54 deeper understanding of how these genes and proteins are regulated and function is essential. This
55 review aims to summarize and underscore important and unique mechanisms that regulate connexin
56 function in healthy and diseased states, which ultimately shed light on clinical observations and
57 future therapeutic opportunities.
58

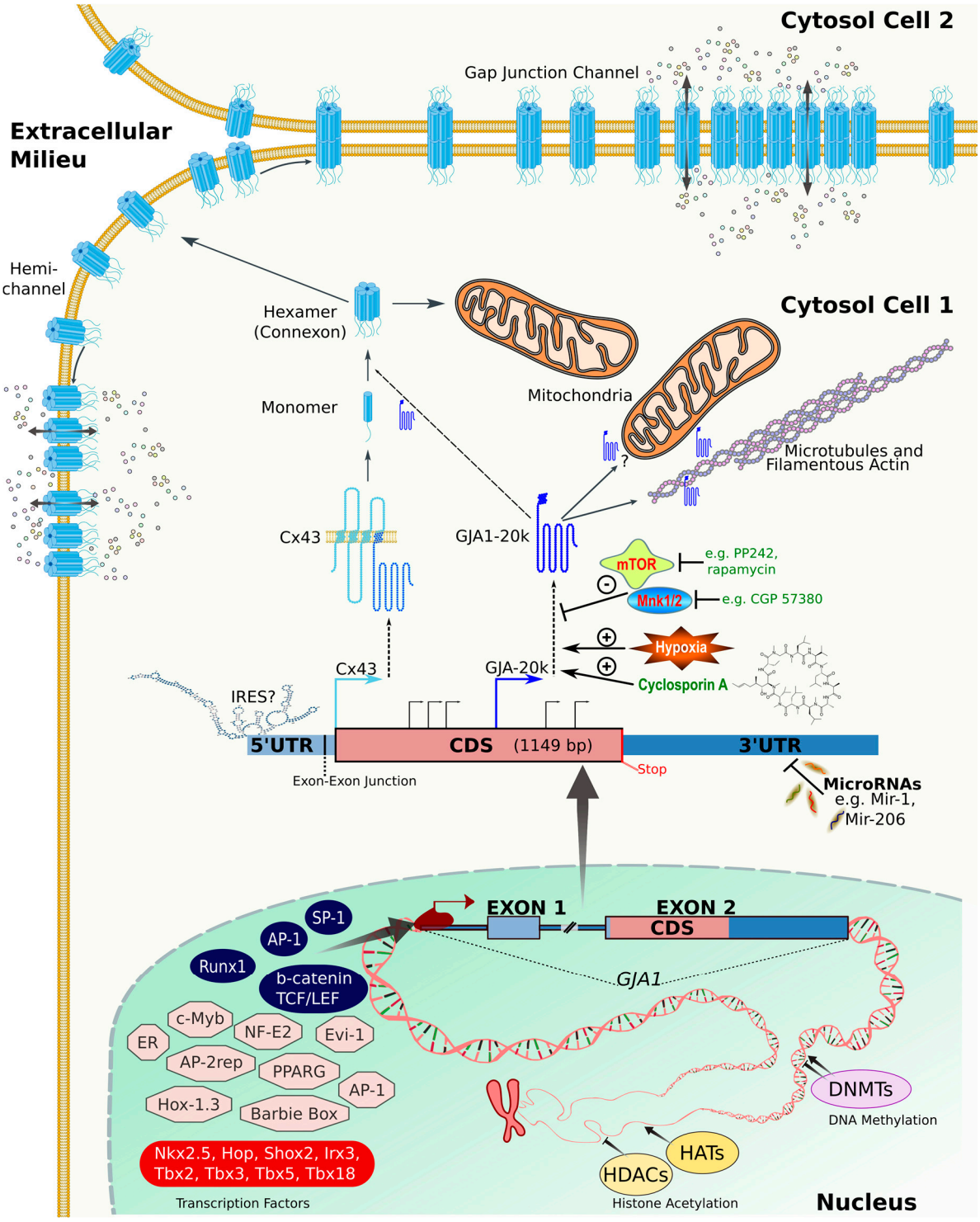

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Figure 1. Connexins forms hexameric connexons permeable to small molecules acting either as hemichannels or intercellular channels. The human *GJA1* gene, encoding for Cx43, contains two exons spanning a genomic region of 14168 bp. Exon 1 contains 256bp of the 5'UTR whereas Exon 2 encompasses 16 bp of the 5'UTR, the entire coding region (1149 bp), and the entire 3'UTR region (1748 bp). Transcription of mRNA (3169 bp) is under regulation by numerous transcription factors as indicated in figure an in main text. Notably, Sp-1 and AP-1 are key regulators of Cx43 mRNA expression (grouped in blue). Multiple tissue-specific promoters are active, which has been well described in the heart (grouped in red). Additional transcription factors (grouped in light red) are derived from promoter analysis using the online Lasagna-Search tool (using a very strict cut-off of $p<0.0001$ and Transfac transcription factor binding sites) [12]. Epigenetics regulate transcription including through promoter hypermethylation by DNA methyltransferase enzymes (DNMTs). Acetylation histone acetyltransferase enzymes (HATs) promote transcription, and the reverse reaction is mediated by histone deacetylases (HDACs). The transcript is also regulated by numerous microRNAs (see main text for details). In addition to full length Cx43 (43kDa), the same mRNA can produce multiple truncated forms via internal translation initiation (indicated by arrows, most notably the 20kDa form GJA1-20k). Truncated forms are also under translational regulation by a number of pathways such as mTOR and Mnk1/2, and can be induced by inhibitors of these pathways as well as by other specific drugs such as Cyclosporin A. GJA1-20k is also induced by pathological states such as hypoxia. The function of GJA1-20k may include interaction with mitochondria and regulation of the actin cytoskeleton as well as regulation of Cx43 oligomerization and trafficking to the membrane. See main text for further details related to the figure.

2. Connexins: from gene to protein

2.1. Gene structure and splicing

Twenty-one human genes and twenty mouse genes encoding for connexin proteins have been identified of which nineteen are considered orthologous pairs [13,14]. The genes tend to have distinct chromosomal locations, although there are some regions of the genome containing clusters of connexin genes [13]. Most connexin genes share a common structure consisting of two exons separated by an intron of variable size. The majority of the 5'-UTR (untranslated region) is localized in exon 1, whereas the entire coding region and the 3'-UTR are found in exon 2. Some connexin genes contain more than two exons (for the 5'UTR of the transcript), such as; human *GJA5* (Cx40) [15], which contains three exons producing two distinct and tissue-specific transcripts, and *GJB6* (Cx30), described to contain six exons that allows for tissue-specific splicing [16]. Examples in mouse includes the genes *Gjb1* (Cx32) [17], *Gja1* (Cx43) [18] and *Gjc1* (Cx45) [19]. In a few cases, the coding region is also distributed over more than one exon [20-23]. A basal promoter (P1) is typically found within 300 bp upstream of the transcription initiation site of exon 1 [24]. However, splice isoforms have been reported due to alternate promoter usage, yielding different transcripts with the coding region being unaltered. As such, a deeper understanding of connexin gene structure, promoter usage and splicing pattern is required for a full understanding of their impact in connexin-related diseases. For example, the human *GJB1* gene encoding Cx32 is contains at least three exons: E1, E1B and the coding exon E2, and produces two different alternatively spliced transcripts by using two tissue-specific promoters (P1 and P2) [25]. It is thus pertinent to include this region in mutational screening of dominant X-linked Charcot-Marie-Tooth (CMTX1) disease, a type of neuropathy that can be caused by mutations in Cx32 leading to defects in Schwann cell function, at least in cases where no mutations are found in the Cx32 coding region. Indeed, recent studies have identified mutations affecting *GJB1* splicing [26] and even deletion of the *GJB1* P2 promoter [27], as underlying causes of CMTX1. Others have shown that splicing mutations in *GJC2* encoding Cx47 can cause a severe form of Pelizaeus-Merzbacher-like disease [28]. Another splice-site mutation, in *GJB2* encoding Cx26, has been suggested to cause a mild post-lingual onset form of hearing loss [29].

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In addition to these more well-described biological phenomena, a few connexin pseudogenes (genes thought to originate from decay of genes that stems from duplication through evolution) have been identified in the human genome. The *GJA1* pseudogene (*GJA1P*) is located on human chromosome 5 whereas the regular *GJA1* gene encoding for Cx43 is located on chromosome 6. Although most pseudogenes are thought to be non-functional, *GJA1P* appears to be transcribed, possibly even translated, and may regulate tumor growth [30,31]. Mutations in *GJA1P* has also been associated with nonsyndromic deafness [32]. Functionally, *GJA1P* may influence *GJA1* expression levels, by acting as a microRNA sponge [33]. In contrast, *GJA6P* seems to be a non-functional pseudogene, originated from the mouse *Gja6* connexin gene encoding Cx33 which has no human counterpart (Gene ID: 100126825). Another potential pseudogene has been inferred for *GJA4* (Gene ID: 100421028) encoding Cx37. The role of pseudogenes in disease is an emerging field, particularly among genes causing multiple different diseases or syndromic diseases, such as connexins.

2.2. Transcription factors and epigenetics

Connexins are expressed distinctively in almost all vertebrate cell types (excluding erythrocytes, mature sperm cells and differentiated skeletal muscle cells) [34]. Some connexins (notably Cx43) are expressed in numerous cell types, whereas others show a more restricted expression profile (e.g. Cx50 that is mainly expressed in the cells of the lens). This spatio-temporal expression pattern is in large part controlled by transcription factors and epigenetic mechanisms. Several transcription factors acting as regulators of basal (ubiquitous) or cell-specific gene activity, and their upstream signal transduction pathways, have now been implicated in the control of connexin expression (Figure 1). Notably, specificity protein 1 (Sp1), an important basal transcription factor that binds to GC box sequences in promoter regions, has been reported to favour transcriptional initiation of several connexin genes including Cx26 [35], Cx32 [36,37], Cx40 [15,38-42] and Cx43 [40,43-48]. Examples of other important regulators that control connexin gene expression include: (i) Activator protein 1 (AP1) transcription factor; composed of proteins belonging to the c-Fos, c-Jun, ATF and JDP families that promote positive regulation. AP-1 sites have mainly been described in Cx43 [43,49,50], whereas putative sites have been identified in the Cx45 promoter [51]. (ii) The Wnt pathway; activation of this pathway leads to the formation of nuclear β catenin/TCF complexes that acts as transcription factors binding to specific TCF/LEF motifs present in the promoter of human *GJA1* and mouse *Gja1* encoding Cx43 [52]. From a physiological and disease point of view this may also be relevant. For example, one study showed Wnt signalling could modulate Cx43-dependent GJIC in the heart, which ultimately may contribute to altered impulse propagation and arrhythmia in the myopathic heart [53]. The importance of GJIC in the heart is well documented and several cell-specific transcription factors have been shown to either activate or repress connexin gene expression in this setting (Figure 1, reviewed in [24,54]). These studies have revealed a role of: (i) Homeobox proteins, transcription factors with a unique DNA binding domain that target gene promoter sequences by self-complementarity, e.g., Nkx2.5, Hop, Shox2, Irx3 (ii) T-box proteins, transcription factors that possess a domain that recognizes a DNA binding element, e.g., Tbx2, Tbx3, Tbx5, Tbx18, and (iii) GATA proteins, important regulators of specific gene expression in different tissue, e.g., Gata4, in alteration of connexin gene expression [24,54].

Besides the well-described transcriptional regulation of the cardiac connexins, other cases of tissue-specific regulation have been reported (for an overview see [24]). Cx32 transcription has been found to be positively regulated by HNF-1 via Sp1 in liver cells [55], by Mist1 in secretory pancreatic acinar cells [56], and by the Sox10 in synergy with the early growth response-2 gene (Egr2) in Schwann cells [57]. This exemplifies how different transcription factors act in a tissue-dependent fashion. Complex transcriptional control thus allows for tissue-specific regulation of connexin-expression. It also facilitates rapid response to environmental changes, e.g., progesterone and oestrogen act as positive and negative regulators respectively of Cx43 transcription in the myometrium during pregnancy and labour [58]. Transcription factors are also important during pathological states, e.g., ischemia where multiple connexins are emerging as important injury response mediators. Their roles in complex

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disease such as cancer are also being unravelled. In breast cancer for example, Cx43 has been proposed to play a biphasic role acting both as a tumour promotor and a tumour suppressor depending on context such as cancer subtype and stage [3]. The aforementioned role of progesterone and oestrogen as regulators of Cx43 expression may be of importance in this setting. Recent evidence also suggests that the transcription factor FOXP3 directly binds to and inhibits RUNX1 in mammary epithelial cells, whereas in the absence of FOXP3 in breast tumours, RUNX1 downregulates Cx43 expression [59]. Understanding the role of transcription factors will provide further insight into loss and overexpression of connexins during tumour progression and other pathological states.

Connexin expression is also under significant epigenetic regulation (for recent extensive reviews see [24,60]). Two major epigenetic mechanisms have been described to regulate transcriptional control: DNA methylation and histone acetylation. Connexin gene inactivation due to hypermethylation of CpG islands in the promoter region has been described in various human carcinomas e.g. Cx26 in lung [61] and breast [62], Cx32 in a renal cell carcinoma cell line [63] and Cx43 in breast cancer [64]. In addition, a gradual decrease in Cx32 and Cx43 mRNA expression levels associates with promoter hypermethylation in Helicobacter pylori-associated gastric tumorigenesis [65]. Transcriptional silencing via promoter hypermethylation is mediated by the enzyme DNA methyltransferase (DNMT). The use of demethylating drugs (DNMTs inhibitors), such as 5-aza-2-deoxycytidine and 5-azacytidine, has been proposed as a potential therapeutic solution in cancer as an increase connexin expression and/or GJIC has been demonstrated in specific cases [63,66,67]. However, the correlation between hypermethylation and gene expression is not always direct and differs between connexin isoforms [67].

Histone acetylation and deacetylation causing chromatin decondensation and condensation respectively, constitutes another important mechanism of epigenetic regulation of connexin transcription [24,60]. While acetylation is catalysed by histone acetyltransferase (HAT) enzymes and promotes transcription, the reverse reaction is mediated by histone deacetylase (HDAC) enzymes. Histone acetylation also affects connexin expression, and inhibitors of HDAC enzymes (HDACi), such as trichostatin A, sodium butyrate, and 4-phenylbutarate, have been shown to enhance connexin and GJIC in a variety of cell populations including in cancer cells [68], in which therapeutic and preventive roles for specific HDACi has been proposed. Histone deacetylase inhibition has also been shown to reduce Cx43 expression and gap junction communication in cardiac cells [69], which has implications with regards to potential side-effects such as slow ventricular conduction or arrhythmias. Therefore, the action of HDACi seems to be connexin and cell type dependent. Curiously, Cx43 has been shown to influence histone acetylation of other genes; in a human pulmonary giant cell carcinoma cell line, the follistatin-like 1 (FSTL1) promoter was shown to be associated with acetylated histones H3 and H4 upon Cx43 transfection. Cx43 was proposed to act as a "histone deacetylase inhibitor" that modulates gene expression and inhibits tumour invasion [70].

The potential therapeutic role of epigenetic regulations has broad interest, in particular in complex diseases such as cancer as exemplified above, however the non-specific nature of this gene regulatory mode complicates more direct and specific therapeutic targeting. Moreover, research is needed to determine if connexins levels are mainly mediated via HDACi histone modification [68,71] or via non-histone protein modification of transcription factors or connexin protein modification such as phosphorylation [72-74].

2.3. RNA stability and microRNAs

Micro-RNAs (miRNAs) are short single-stranded non-coding RNAs that can regulate expression at a post-transcriptional level by base pairing to mRNA sequences (usually located at the 3'UTR region), reducing protein expression levels via mRNA degradation, translational inhibition or transient mRNA sequestration. Numerous microRNAs have been predicted to downregulate the expression of different connexin genes (for recent reviews see [60,75]). Cx43 is by far the best studied connexin,

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and a number of functional microRNAs targeting this gene have been identified including miR-1, miR-23a, miR-186, miR-200a, miR-206 and miR-381 in human breast cancer [76], miR-20a in human prostate cancer [77], miR-221/222 in glioblastoma multiforme [78] and miR-206, miR-1 and miR-133 in cardiac myocytes and during skeletal myoblast differentiation [79-81].

Regulation of connexin expression by miRNAs has been described to be active in various disease states for example in cancer by affecting hallmarks such as proliferation and invasion [77,78]. In therapeutic settings options include targeting miRNAs that regulate connexins in order to reverse the malignant phenotype. This has been shown in several studies including in human glioblastoma cells where inhibition of miR-221/22 activity with antisense oligonucleotides lead to the upregulation of Cx43 and restoration of GJIC [78].

As mentioned above, miR-1 acts in cardiac muscle and downregulates Cx43 expression. This has been related to several cardio-pathologies in humans including the regulation of cardiac arrhythmogenic potential [81]. In contrast, loss of miR-1, and thus increased Cx43 expression, has been linked to myotonic dystrophy [82]. Interestingly, a severe congenital heart defect, tetralogy of Fallot, is associated with downregulation of miR-1 and miR-206, thought to lead to an increase on Cx43 protein levels [83]. MiR-1 downregulation of Cx43 in the bladder musculature has been also appointed to have a role in overactive bladder syndrome [84].

Connexins are also implicated in joint and bone disease [85]. Cx43 has an important role in osteoblast growth and differentiation, and various miRNAs (including miR-23a [86] and miR144-3p [87]) have been shown to target Cx43 in this setting. Cx43 can also influence the expression of miRNAs themselves, notably miR-21 in osteocytes, a pathway linked to osteocyte apoptosis and osteoclast formation/recruitment [88]. Moreover, direct transfer of miRNAs — through gap junctions — has been described, and is thought to play a role in bone development [89] as well as in aspects of tumour growth and tumour dormancy [3].

In addition to miRNAs, connexin transcript stability can be regulated by RNA-binding proteins (RBPs), such as human antigen R (HuR) that stabilizes the Cx43 mRNA by binding adenylate/uridine-rich elements (AREs) in the 3′UTR [90]. Other examples include S1516-binding protein elements which may regulate Cx43 expression, particularly in Ras-transformed cancers [91]. For further insight into the epigenetic regulation of connexins, including by miRNAs, we refer to other more exhaustive recent reviews [60,75].

2.4. Translational Regulation

2.4.1 IRES

Due to the key role of connexins in sustaining many cellular functions and tissue physiology, it has been suggested that connexin expression needs to be maintained at all times, even under conditions where the classical cap-dependent mRNA translation pathway is suppressed, such as during mitosis, apoptosis, differentiation, senescence or cell stress [92,93]. Several internal ribosome entry site (IRESs) elements have been reported in the mRNA of connexins, notably in Cx43 [94] (Figure 1), Cx32 [95] and Cx26 [96]. An IRES is a nucleotide sequence usually located within the 5′UTR of the mRNA, which in contrast with the canonical translation mechanism, allows for cap-independent translation initiation, a process regulated by specific RBPs also known as IRES trans-acting factors (ITAFs) [97,98]. However, numerous other translation initiation mechanisms are thought to exist [99], and whether true IRES-mediated translation occurs in the aforementioned connexins and other family members, is subject to caution and additional specific molecular assays are warranted [100]. Additional work is also needed towards elucidating their functional relevance. One study suggests that IRES-mediated translation of Cx26 and Cx43 occur in density-inhibited cancer cells (where cap-dependent translation is reduced), thus leading to the induction of GJIC and potentially reduced

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tumor growth [96]. Some data also points towards an important role of IRES-translation of connexins in human physiology. Notably, a specific mutation in the 5'UTR Cx32 IRES sequence has been linked to neurodegenerative Charcot–Marie–Tooth disease [95].

2.4.2 Alternative translation of truncated connexin forms

Most IRES sequences are located in the 5'UTR, yet a few examples exist (notably Notch2 [101]) where an IRES sequence is located within the coding region allowing translation of truncated protein forms. A similar mechanism has been proposed for Cx55.5 in Zebrafish, in which an 11-kDa truncated C-terminal form is produced and localizes to the nucleus of outer retina cells [102,103].

In mammalian cells, the presence of truncated forms of Cx43 is often observed in immunoblots. In particular, a 20 kiloDalton form (named GJA1-20k) is highly prevalent in cultured cells, which was described to arise from the Cx43 coding sequence and correspond to the C-terminal tail [104]. More recently, Smyth and Shaw described that GJA1-20k and several other less prevalent truncated forms can occur in normal tissue, and is due to internal translation initiation events [105]. Multiple groups confirmed this observation and further delineated key regulatory pathways such as the mTOR [105,106] and the MAPK-Mnk1/2 [106] signalling cascades, as well as important physiological conditions such as hypoxia [107] (Figure 1). Although an internal IRES element has been suggested [107], evidence suggests a highly unusual cap-dependent mechanism is critical for the efficient synthesis of these truncated forms [75,106].

The C-terminus of Cx43 has been extensively studied and is implicated in the regulation of a variety of biological events such as cell migration and proliferation, neuronal differentiation and cytoskeletal changes (for a recent review see [5]). However, functional roles for specific internally truncated forms of Cx43 are currently being elucidated. Thus far, GJA1-20k has been shown to act as a potential chaperone for Cx43 [105,108] that facilitates microtubule-based mitochondrial transport and mitochondrial network integrity [109] (For details, see section 4.3). Additionally, loss of GJA1-20k (but not full length Cx43) has been reported in early-stage human breast cancers, and its re-expression in cell lines regulated by p53 activation via miR-125b [110]. Roles for these truncated forms of connexins in complex genetic disease is of future interest considering recent advancements in the potential for pharmacologic modulation of internal translation[75].

3. Post-translational regulation of connexins

Post-translational modification of connexin proteins regulates many important aspects of their life-cycle including synthesis, trafficking, channel gating and protein-protein interactions. While highly conserved, variations occur throughout the connexin family in protein sequence, size of intracellular N-/C-terminus and loop regions. Connexin extracellular loop regions contain disulfide bridges that form between cysteines to maintain membrane topology and facilitate docking with opposing connexons allowing the formation of gap junctions [111]. Unlike many other membrane-bound proteins, connexins are not glycosylated, with membrane trafficking and protein folding being regulated through alternative pathways (for details, see section 4). The relatively unstructured nature of intracellular connexin domains makes for an ideal environment for post-translational modification to induce conformational changes that regulate protein-protein interactions. The majority of connexins contain multiple consensus sites for modifications through phosphorylation, *S*-nitrosylation, SUMOylation and others. There have been several recent and comprehensive reviews on connexin post-translational modifications [5,112-114]. Instead of recapitulating these articles we will highlight some of the main aspects of post-translational modifications of connexins and discuss their relevance in human disease.

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298 *3.1 Phosphorylation*

299 Phosphorylation is a key regulator of connexin proteins, hemichannels and gap junction channels
300 [115-117]. The addition of phosphate groups to specific amino acids including serine (Ser, S),
301 threonine (Thr, T) or tyrosine (Tyr, Y) leads to changes in charge, hydrophobicity and potentially
302 alterations in protein structure resulting from formation of hydrogen bond networks [118]. These can
303 alter the way the connexin protein interacts with itself e.g. channel regulation or with other proteins
304 e.g. trafficking and protein-protein interactions.

305 Phosphorylation has been reported in a large number of connexins, e.g. Cx31 [119], Cx32 [120-122],
306 Cx37, Cx40 and Cx45 [123,124], Cx43 [125,126], Cx46 and Cx50 [127-129] and Cx47 [130]. The majority
307 of phosphorylation events are reported within connexin C-terminus, with the exception of Cx26
308 which is not phosphorylated in its short 11a.a. C-terminus [131,132]. However, mass spectrometry
309 has demonstrated multiple potential Cx26 phosphorylation sites in the N-terminus, which are
310 differentially regulated by hydroxylation, and further putative sites in the cytoplasmic loop, although
311 the functions of these Cx26 phosphorylation sites are unknown [133,134]. There are some reports of
312 intracellular loop phosphorylation, e.g. Cx56 [135] and Cx35 [136], although this does not appear to
313 be the case for Cx43 or other connexins [5,137,138]. There are no reports of N-terminus
314 phosphorylation in other connexins, although Cx43-Ser5 is a potential candidate site [139]. The C-
315 terminus of connexins are intrinsically disordered protein (IDP) regions with a high Ser/Thr/Tyr
316 content as described for Cx32, Cx40, Cx43, Cx45 and Cx50, [140-144]. Stable alpha helical regions have
317 been identified by nuclear magnetic resonance (NMR) and circular dichroism (CD) in the C-terminus
318 of Cx43 [141,145] and other connexins e.g. Cx37, Cx45 and Cx50 [146-149]. However, stable alpha-
319 helices are not a common a feature of the connexin C-terminus. For instance, Cx40 only forms
320 dynamic alpha-helices between Cys267-Gly285 [150]. Several lines of evidence such as electrophoretic
321 shifts on SDS-page gels, and NMR analysis suggest that phosphorylation by enzymes such as MAPK
322 and PKC, result in differential transient, increases connexin C-terminal alpha helical content
323 [123,144,151-153].

324 The significance of the formation of alpha helical domains is the potential for higher order secondary
325 structures that regulate channel gating and protein partner binding. In Cx43, it has been
326 demonstrated that the C-terminus interacts with the intercellular loop to regulate channel functions
327 in a "ball-and-chain" type mechanism [154,155], although other factors relating to phosphorylation,
328 e.g. charge and hydrophobicity, may also influence channel gating. Multisite phosphorylation of
329 proteins is known to alter protein half-life, docking and intracellular localization which may also
330 influence gap junction signalling [143,144,156]. Connexin 43, the most widely studied of the connexin
331 family, has 30 putative phosphorylation sites which have been extensively demonstrated to be post-
332 translationally modified leading to alterations in gap junction signalling. For detailed reviews of these
333 phosphorylation sites and their effects on channel regulation see [5,138,139,157-159]. The effects of
334 post-translational modifications on connexins are also shown in Table 1.

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346 **Table 1: Connexin post-translational modifications and functional effects**

Connexin/Residue	PTM	GJIC	Expression	Refs.
Cx26[m.s./a]:				
M1/ K15/ K102/ K103/ K105/ K108/ K112/ K116	Acetylation	ND	ND	[133,134]
N14/ N113/ N170/ N176	Hydroxylation	ND	ND	
E42/ E47/ E114	carboxylation	ND	ND	
K61/ R75/ K221/ K223	Methylation	ND	ND	
T123/ T177/ S183/ T186/ (Y233 or Y235 or Y240)	Phosphorylation	ND	ND	
Cx31(m):				
263[b]	CK1	No change	No change	[119]
266[b]	CK1	No change	No change	[119]
Cx32:				
S229	PKC	Increase/ Decrease	Increase/Decrease	[160]
S233	PKA/PKC	Increase/ Decrease	Increase/Decrease	[115,133,160,161]
S240	ND	ND	ND	[133]
Y7/ Y243	EGFR tyrosine kinase	ND	ND	[162]
Cx35/Cx36:				
S110	PKA/ PKG	No change	Decrease	[136,163-165]
S276/293	PKA/ PKG	No change	Decrease	[136,163-166]
S289	PKG (NO mediated)	ND	Decrease	[165]
Cx37:				
S275/ S285/ S302/ S319 / S321/ S325/ S329	PKC	Increased	Decrease	[123]
Cx43:				
S5[m.s.]	ND	ND	ND	[139]
K144	SUMO	Increase	Increase	[167]
K237	SUMO	Increase	Increase	[167]
S244[m.s.]	CAMKII	ND	ND	[168]
Y247[c]	Src	Decrease	Decrease[c]	[115,141,169-173]
S255 [m.s.]	CAMKII	ND	ND	[168]
	P34cdc2	Decrease	Decrease	[174,175]
	MAPK	No change/ Decrease	No change	[115,143,176,177]
S257 [m.s.]	PKG/ CAMKII	ND	ND	[168]
S262[d]	P34cdc2	Decrease	Decrease	[174,175]
	MAPK	Decrease	Decrease/ no change	[115,143,176,178,179]
	PKCε[a]	Decrease	Decrease	[176-178]
Y265[c]	Src	Decrease	Decrease[c]	[115,141,169-173]
C271	Nitrosylation	Increase	No change	[180]
S279[e]	MAPK	Decrease	Decrease/ no change	[143,169,176]
	CDK5		Decrease	[181]
S282[e]	MAPK	Decrease	Decrease/ no change	[143,169,176] [181]

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	CDK5	Decrease	Decrease	
S296 m.s.	CAMKII	ND	No change	[168,182]
S297 m.s.	CAMKII/ PKCε	ND	No change	[168,182]
S306 m.s.	CAMKII	Decrease	Decrease associated with De-Phosph.	[168,182,183]
S314 m.s.	CAMKII	ND	ND	[168]
S325 m.s.	CAMKII	ND	ND	[168]
	CK1	Increase	Increase	[184]
S328 m.s.	CAMKII	ND	ND	[168]
	CK1	Increase	Increase	[184]
S330 m.s.	CAMKII	ND	ND	[168]
	CK1	Increase	Increase	[184]
S364 m.s.	CAMKII	ND	ND	[168]
	PKA	Increase	Increase	[115,185-187]
S365 m.s.	CAMKII	ND	ND	[168]
	PKA	Increase	Increase	
	PKC	Decrease	Decrease	[115,188-190]
S368[f]	PKCα	Increase/	Increase	[188-192]
	PKCε	Preserved/ Decrease[g]	Decrease	[115,189-195]
S369 m.s.	CAMKII	ND	ND	
	PKA	Increase	No change	[168]
	PKC	Increase	Increase	[5,115,188-190]
S372 m.s.	CAMKII	ND	ND	[168]
	PKC	Decrease	Decrease	[5,143,188,189,196,197]
S373[g, m.s.]	Akt	Increase	Increase[e]	[196,197]
	CAMKII	ND	ND	[168]
	PKC	Decrease	Decrease	
	PKA	Increase	Increase	[115,143,188-190]
Cx45:				
S326/ Y337/ S381/ S382/ S384/ S385/ S387/ S393 m.s.	CAMKII	ND	ND	[124]
S326/ S382/ S384/ S387/ S393 m.s.	CK1	ND	ND	[124]
Cx46 (Cx56 Chick homologue):				
S118	PKCε	ND	Decrease	[135,198]
Cx50:				
S363	CK1	Increase	Increase	[115,189]

Table Notes:

a. mass spec identified a number of potential phosphorylation sites in Cx26 but did not test functions, although mutations at many of these sites are associated with disease pathology [134].
b. direct phosphorylation not shown, S263 and S266 on Cx31 contain consensus sequence for Ck1 which when deleted alters functions.
c. SRC may not alter function of formed gap junctions.
d. Currently debated as to whether Cx43-S262 is a CDK1/CDC2/ PKC/ MAPK site, several lines of evidence indicate that this is most likely an ERK regulated site [143].
e. Functions of S279/S282 typically shown by single phosphorylation antibodies or multiple site directed mutagenesis including both residues. decrease GJIC as a result of reduced open probability.
f. phosphorylation of S368 by phorbyl esters e.g. TPA are associated with PKCε phosphorylation and reduced communication. In ischemia, treatment by peptides e.g. rotagaptide increase 368 phosphorylation by PKCα leading to increases in GJIC.
g. While initial phosphorylation at S373 is associated with a temporal increase in GJ size, it is thought to be the start in the process that leads to internalization.

Abbreviations: ND, not demonstrated; (m), mouse; m.s., mass spectrometry based identification approach.

Phosphorylation is a key regulator of physiological states in tissues and changes in the phosphorylation status has been observed in several disease states. Within the vasculature,

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heterocellular endothelial cell-smooth muscle cell contacts, called the myoendothelial junctions (MEJs), express Cx37, Cx40 and Cx43 allowing for the direct exchange of intercellular signalling ions and molecules such as Ca^{2+} and IP_3 [199,200]. At the MEJ, Cx43 and Cx37 are regulated by post-translational modifications including phosphorylation and *S*-nitrosylation (for details, see section 3.2). In vitro and ex vivo data demonstrate that gap junctions at MEJs allow for the movement of Ca^{2+} and IP_3 between endothelial and vascular smooth muscle cells, which is in part regulated via Cx43-Ser368 [201].

In the healthy heart, Cx43 is primarily localized to the intercalated disc region of cardiomyocytes. Opening of Cx43-containing channels and signal conduction is facilitated by phosphorylation at residues including Ser365, 325, 328, 330 [202-204]. Phosphorylation acts as a molecular switch, regulating gap junction opening. In ventricular arrhythmias following myocardial infarction, raised intracellular [Ca^{2+}] leads to de-phosphorylation of Cx43-Ser365, which acts as the gatekeeper to phosphorylation of Cx43-Ser368. This resulting increase in Cx43-Ser368 reduces GJIC and promotes a redistribution of Cx43 to lateral regions of the cardiac myocytes, disrupting signalling in the heart [204-206].

Formation of large cardiac gap junction plaques at the intercalated disc is modulated through Cx43 interactions with ZO-1 [207-210]. In turn, this protein-protein interaction is regulated by PKC phosphorylation of Cx43 at Ser368 which inhibits ZO-1 mediated disassembly of gap junctions [211]. In ischemic heart disease Cx43 is lost at the intercalated disc, but Cx43-Ser368 phosphorylation can act to indirectly stabilize the protein [192]. Multiple studies have investigated the effects of targeting the C-terminus of Cx43 in ischemia/ reperfusion injuries, reducing infarct size and other diseases [212,213]. A peptide that mimics the terminal region of the Cx43 known as ACT1 can disrupt Cx43/ZO-1 interaction [210,214]. This peptide promotes phosphorylation of Cx43-Ser368 via upregulation of PKC-ε activity, inhibits Cx43-ZO-1 binding and improves cardiac function following ischemic insult in mice [211]. Similar results have been found for other connexin mimetic peptides targeting the Cx43 C-terminus e.g. AAP10 and Rotagaptide (ZP123), causing increases in Cx43-Ser368 phosphorylation through PKC-α (reported to stabilize protein expression and increase GJIC) associated with improved cardiac functions in experimental animal models and early tests demonstrating no adverse effects in humans [215-220]. However, it should be noted that a similar peptide, Danegaptide (a stabilized form of Rotagaptide), failed to change clinical outcomes in ischemic reperfusion injuries in human Phase II testing (NCT01977755, completed 2016) [221].

In vascular disease, phosphorylation-mediated connexin-protein interactions and GJIC have been found to regulate disease state. Oxidized phospholipids found within atherosclerotic plaques increase MAPK and PKC phosphorylation of Cx43 and are associated with increased inflammation and cellular proliferation [125,222-225]. In response to release of growth factors in disease, Cx43 is phosphorylated at MAPK residues (Cx43-Ser255, -Ser262, -Ser279, -Ser282) promoting direct interactions with the cyclin E/CDK2 complex and enhancing smooth muscle cell proliferation [126]. Conversely PKC phosphorylation of Cx37 alters GJIC which is linked with growth suppressive effects e.g. reducing vasculogenesis and angiogenesis [226-231]. Mutation of all seven Cx37 Ser>Ala essentially closes Cx37 GJ and hemichannels and inhibits both proliferation and cell death, whereas mutation of only 3 (Cx37-Ser275, -Ser302 and -Ser328) partially inhibits channel opening and decreases cellular death in rat insulinoma cells [123].

Phosphorylation also plays an important role in altered localisation and function of connexins in cancer [3]. Several oncogenes and proto-oncogenes robustly inhibits GJIC including HRAS [232], c-Src [233] and v-Src [173]. Curiously, the tyrosine-protein kinase c-Src has a reciprocal relationship with Cx43 that regulates its activity, where Cx43 is shown to bind with phosphatases (e.g. PTEN and Csk) reducing c-Src activity [234]. Conversely, Src phosphorylation of tyrosine residues on Cx43 (Cx43-Tyr243/-Tyr265) mediates interactions with endosomal machinery, leading to internalization of Cx43 and reduced expression [113,235]. Numerous tumour promoters such as phorbol esters also

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rapidly inhibit Cx43-mediated GJIC [236-238], through PKC- and ERK-mediated phosphorylation events [177,239]. Conversely, loss of phosphorylation can also negatively affect GJIC. One recent study showed that the levels of total Cx43 protein and Cx43 phosphorylated at Ser368 and Ser279/282 were high in normal tissue but low to absent in malignant pancreatic tissue [74]. Altered Cx43-phosphorylation can be indicative of prognosis in some tumours such as gliomas [240]. Phosphorylation of other connexins can also affect GJIC and the cancer phenotype, notably PKC-mediated phosphorylation of Cx37 [123]. Targeting dysregulated phosphorylation events of connexins in cancer may be one therapeutic angle towards restoring connexin function or GJIC. Indeed the chemotherapeutic drug gefitinib has been suggested to upregulate GJIC by inhibiting Src and PKC-modulated Cx43 phosphorylation [241]. Conversely, resistance to cisplatin-based chemotherapy has been suggested to be due to Src-induced Cx43 phosphorylation and loss of GJIC [242].

During wound healing, phosphorylation may also play a role in coordinating GJIC and connexin redistribution [243-245]. Initial responses to wounding include a generalized loss in Cx43 which may be modulated by increases in cAMP. In wound models, 8-Bromo-cAMP treated embryonic stem cells promote enhanced wound repair associated with reduced membrane bound Cx43, disruption in Cx43-ZO-1 interactions and reduced GJIC [246]. However, the mechanisms regulating this are unclear since cAMP associated kinases have been previously described to increase PKA mediated Cx43 synthesis, phosphorylation (Cx43-Ser364), GJ assembly and GJIC in other model systems [185,247]. Phosphorylation is extremely dynamic within the wound and appears to be coordinated with the stage of repair. Initial increases in Cx43-Ser373 driven by AKT can be seen between 1-30 minutes, disrupting interactions with ZO-1, initially stabilizing Cx43 at the membrane, but is followed by rapid internalization of Cx43 [196]. Following wounding, transient increases (24-72 hours) in PKC mediated Cx43-Ser368 phosphorylation in regions proximal to the injured sited are associated with a loss of GJIC [248,249]. These data and others suggest that a combination of phosphorylation events sequentially regulate connexin signalling during wound repair [250].

In disease states such as diabetes, non-healing wounds lead to complications including ulcerations in skin tissues. In streptozotocin-induced diabetic mice, Cx43 dynamics are different from normal skin tissues with increased expression of dermal Cx43 associated with reduction in keratinocyte migration [251]. Similar observations have been made in human diabetic ulcers, with Cx43 found to remain at elevated levels as compared to normal skin wounds [252]. In vitro and ex vivo evidence suggests that peptides aimed at disrupting gap junction and hemichannel communication, e.g. Gap27 can increase wound healing associated with increased Cx43-Ser368 phosphorylation [249]. Recent studies have also shown that increases in Cx43-Ser368 phosphorylation following topical application of the ACT1 peptide is associated with clinically significant improvements in scar reduction and wound closure rates [253].

3.2 S-Nitrosylation

S-nitrosylation occurs through covalent binding of nitric oxide (NO) to reactive cysteine(s) and can result in structural alterations of proteins leading to functional changes [254]. Protein *S*-nitrosylation is highly dependent on the cysteine oxidation state and surrounding amino acids, meaning that not all cysteines in a protein can be *S*-nitrosylated. While there are cysteine residues on the extracellular loops of all connexins, these have not been demonstrated to be *S*-nitrosylation targets [255]. Within the C-terminus of Cx43 there are three cysteines (Cx43-Cys260, -Cys271 and -Cys298) but only Cx43-Cys271 has been demonstrated to be *S*-nitrosylated, leading to an increase in GJ permeability in endothelial cells and at the MEJ [180]. Direct *S*-nitrosylation of other connexins has not been demonstrated, although there are multiple lines of evidence, demonstrating that NO activation leads to regulation of gap junction and hemichannel signalling [256]. Within the vasculature, NO plays an important role in vasodilation. Figueroa et al. found that vascular connexins channels formed by Cx37, Cx40 and Cx43 are activated by and directly permeable to NO, and have suggested that this is

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an alternative method to NO transfer across plasma membranes [257]. Cx37, is enriched at the MEJ of resistance arteries and is reported to be important in the regulation of NO mediated Ca^{2+} regulation via reducing Cx37-mediated gap junctional coupling between endothelial cells and smooth muscle cells [258]. However, unlike Cx43, the effects of NO on Cx37 gap junction channels are thought to be indirect, with no known cysteine modification occurring. Rather the phosphorylated tyrosine residue (Cx37-Tyr332) is protected from de-phosphorylation by SHP-2 phosphatase, which is inhibited in the presence of NO, reducing MEJ transfer of Ca^{2+} signalling through Cx37 GJ [259]. Thus S-Nitrosylation appears to have diverse effects depending on GJ composition particularly at the MEJ [259].

3.3 Other post-translational modifications: SUMOylation, ubiquitination and acetylation

A number of post-translational modifications are associated with regulation of connexin protein turnover e.g. ubiquitination, SUMOylation and acetylation. Small ubiquitin-like modifier proteins e.g. (SUMO-1/-2/-3) interact with lysine residues on proteins altering protein targeting and turnover [260]. So far there is only evidence for direct Cx43 SUMOylation at lysines (Cx43-Lys144, -Lys237) within its intracellular loop and C-terminus [167]. Overexpression of all three SUMO proteins in HeLa cells increases Cx43 expression, promotes gap junction formation and increases signalling. However, the exact mechanism by which SUMOylation regulates protein expression is not known. The amino acids sequences surrounding Cx43-Lys144 and Cx43-Lys237 are not common motifs associated with SUMOylation, although the same motifs of a conserved Lys144 followed by upstream large hydrophobic amino acid (valine) are found in at least six other connexins suggesting a common regulatory pathway [167].

Once at the plasma membrane, the majority of connexins are rapidly turned over with half-lives estimated between 1.5-5 hours for Cx43 and Cx26 and up to 24 hours for other isoforms such as Cx46 [119,261-264]. While connexins use a multitude of pathways for internalisation and degradation, the process typically involves formation of an endosome (termed connexosome [265]), where older gap junctions are internalised to be targeted to the lysosome for degradation, although there is also evidence for endosomal recycling back to the membrane [210,266,267]. Endosomal formation is driven by multiple proteins in complex including interactions with ZO-1, tubulin and others. In the case of Cx43 this interaction (with ZO-1) is regulated via Cx43-Ser373 and Cx43-Ser368 phosphorylation [196,210,268,269]. Mono-ubiquitinylation typically acts as a signal for internalisation of proteins via endosomes to lysosomes leading to degradation [270,271]. Multiple covalently linked ubiquitin molecules bind lysine residues within the target protein, which are then recognised by receptors and targeted for degradation by the 26S proteasome [272-274] and by autophagy [275-277]. Recent evidence has demonstrated a complementary role for Cx43 in regulating autophagy, in that Cx43 at the plasma membrane interacts with several pre-autophagosomal proteins including Atg16, but not other autophagosome proteins such as LC3, [278]. When the cells are under stress, such as nutrient depletion, Cx43 becomes ubiquitinylated and internalized causing recruitment of other factors (Atg5, Atg12 and LC3) to form fully functional autophagosomes. While regulated autophagy can have a protective effect in stressed cells, there is also evidence linking aberrant autophagy and Cx43 degradation from intercalated discs to heart failure [279], suggesting the potential for a novel pharmacologic approach to treat cardiac failure.

Proteasomal-ubiquitin pathways have been proposed to indirectly regulate Cx43 through interaction with the ZO-1 protein, thus disrupting part of the process that is critical for Cx43 membrane organisation [210,280]. Multiple studies suggest that other connexin proteins, e.g. Cx50, Cx43 and Cx31.1, are regulated by ubiquitination [281]. Several studies show that ubiquitin regulates internalisation of Cx43 via clathrin mediated endocytosis, by both YXXO tyrosine-dependent sorting signal and tyrosine-independent, EP15-dependent pathways [282,283]. However, the route through which ubiquitin regulates the connexins has not been fully delineated, with studies in Cx43 demonstrating that the C-terminal lysines are dispensable for protein turnover [284]. Despite this, there is increasing evidence that Cx43 is modified in response to ubiquitin, and corresponding ligases

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are controlled in part by phosphorylation events e.g. MAPK and PKC phosphorylation [285,286]. A number of ubiquitin binding proteins e.g. EPS15, p62, Hrs and TSG101 are recruited to Cx43 to facilitate its internalisation and sorting to the lysosome [287,288]. In addition, TSG101 has been found to interact with Cx30.2, Cx31, Cx36, and Cx45 [288]. While classic lysine based motifs may not be responsible for direct ubiquitin binding, more recent studies have shown that proline rich regions of the Cx43 C-terminus (xPPxY) bind to ubiquitin ligase. A number of ubiquitin ligases have also been associated with direct binding, internalization and degradation of Cx43 e.g. Trim21 [289], WWP1 [290] , SMURF2 [291] and NEDD4 [285-287,292]. NEDD4 also has been directly associated with loss of Cx43 at the plasma membrane in experimental models [285].

The process of degradation may be further regulated by connexin N-terminal acetylation which can act to regulate protein stability in the membrane. In mouse cardiac myocytes N-terminal acetylation through binding of P300/CBP associated factor with Cx43 leads to a loss of Cx43 at the intercalated disc, a lateral reorganisation of the protein, reduced gap junction formation in cardiac myocytes and internalisation in NIH-3T3 fibroblasts [72]. These patterns of dysorganisation of Cx43 are similar to those seen in mouse models of Duchenne cardiomyopathies where NO and oxidative stress lead to an imbalance in acetylation/ deacetylation and alterations in cardiac conduction. Similarly in dogs, cardiac pacing leads to increased Cx43 acetylation suggesting that this mechanisms is important in regulating signalling in physiology and pathology of the cardiac system [72,293,294].

4. Connexin Trafficking

Formation of gap junctions by connexins is regulated by the delivery of newly synthesized channels to the plasma membrane balanced by the removal of channels via endocytosis [261,295,296]. As mentioned above, since connexin turnover is generally quite rapid and influenced by post-translational modifications, the dynamic regulation of connexins by secretion and turnover provides a means to control gap junction formation, composition and thus, GJIC.

4.1 Control of oligomerization

Secretion of newly synthesized connexins from the endoplasmic reticulum (ER) through the Golgi apparatus is coordinately regulated with oligomerization into hexameric hemichannels [297]. Based on structural homology, connexins can be separated into two distinct oligomerization groups. GJB1-GJB7 (so called beta connexins, including Cx26 and Cx32) follow a more traditional pathway, where full oligomerization into hexamers is required prior to transport from the ER to the cis Golgi apparatus [298-300]. By contrast other connexins are stabilized by a connexin-specific quality control apparatus as monomers that are subsequently transported to the trans Golgi network (TGN) where they then have the capacity to oligomerize [299,301]. The best studied connexin known to oligomerize in the TGN is Cx43, although there is also experimental evidence for Cx40 and Cx46 oligomerization late in the secretory pathway as well [302,303]. By homology, it is likely that most non-beta connexins will also follow the late oligomerization pathway that has been demonstrated for Cx43 [297].

Several lines of evidence suggest that the transition from monomeric to hexameric Cx43 requires a conformational change, largely centered on the third transmembrane domain (TM3) where it is stabilized in a monomeric conformation by motifs containing charged amino acids on both ends of the TM domain (Figure 2) [298,303]. At the cytoplasmic interface of the Cx43 TM3 domain is an LR motif containing a highly charged arginine residue and at the extracellular interface is a glutamine-containing motif with a QYFLYGF consensus sequence. The extracellular loop domain of Cx43 also interacts with a chaperone protein, ERp29, that is required to stabilize monomeric Cx43 [298].

By contrast, beta connexins lack charged residues adjacent to the TM3 domain. They instead have a di-tryptophan (WW) motif that is less stringently localized to the membrane/cytosol interface and they lack the ability to interact with ERp29. Thus, beta connexins are not stable as monomers and

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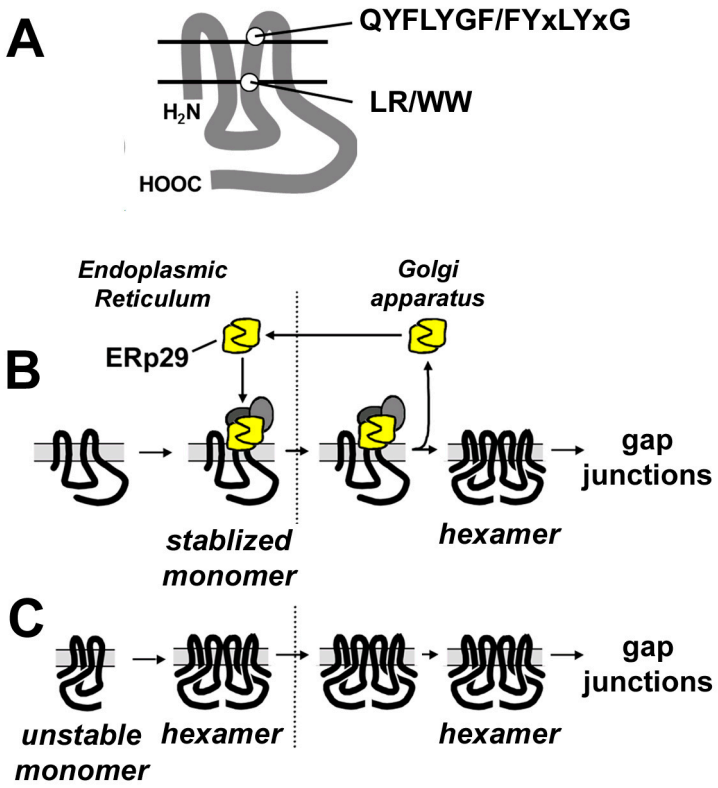
559 instead oligomerize in the ER (Figure 2) [298-300]. Since motifs associated with the TM3 domain also
560 have been implicated in regulating connexin hetero-oligomerization [297,304], this implicates a role
561 for spatial separation of connexin oligomerization in regulating the extent and stoichiometry of
562 heteromeric channel formation.



563

564 **Figure 2.** Differential connexin oligomerization. A. Line diagram showing two key connexin motifs
565 adjacent to the third transmembrane domain. Connexins (such as Cx43) which oligomerize in the
566 Golgi apparatus (B) have a cytosolic LR and extracellular QYFLYGF motif that interacts with ERp29
567 (yellow) and other putative chaperones (grey ovals) that stabilize monomeric connexins until they
568 reach compartments where they oligomerize into hexameric hemichannels. By contrast connexins
569 (such as Cx32) that have a WW and a FYxLYxG motif cannot interact with ERp29 are inserted into the
570 ER membrane as unstable monomers and so they immediately oligomerize (C).

571

572 *4.2 Connexin quality control*

573 The differences in quality control for Cx26 and Cx43 were directly observed for native connexins in
574 human airway epithelial cells derived from a cystic fibrosis (CF) patient expressing the CF
575 transmembrane conductance regulator (CFTR) protein harbouring the Fdel508 mutation [305]. In
576 these cells, Cx43 trafficking and function is impaired, yet Cx26 transport and assembly into gap
577 junction channels is normal. Interestingly, CFTR also interacts with ERp29 [306] and Cx43-mediated
578 GJIC by Fdel508-CFTR expressing cells is restored by treatment with 4-phenylbutyrate, a drug that
579 upregulates ERp29 expression [305,306]. In addition, 4-phenylbutyrate has been shown to upregulate
580 GJIC in several other contexts [307-312], further underscoring a role for ERp29 and other 4-
581 phenylbutyrate sensitive factors in connexin quality control.

582

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Aberrant accumulation of connexins in the ER clearly decreases the pool of connexins available to produce gap junction channels at the cell surface. However, ER accumulation of connexins has also been found to induce an unfolded protein response (UPR) that, in turn, has the capacity to impair cell function and lead to human disease. UPR induced by mutant connexins has been directly demonstrated for Cx50 mutations associated with cataract [313-315] and Cx31 mutations that cause the skin disease erythrokeratoderma variabilis (EKV) [316] or hearing impairment [317]. The association of UPR with human diseases related to misfolded connexins suggests the possibility that treatments alleviating ER stress, such as 4 phenylbutyrate, may have therapeutic value by promoting proper protein folding and trafficking as well as increasing GJIC. Also, as mentioned above, the ability of 4-phenylbutyrate to enhance GJIC also may contribute to its potential as an anti-cancer therapeutic, and may be related to increased ERp29 activity [318].

4.3 Connexin cytoplasmic domains and the cytoskeleton

In addition to motifs adjacent to the TM3 domain, there are several lines of evidence in support of connexin C-terminal domains in regulating connexin trafficking. As described above, in addition to containing several motifs that can be post-translationally modified, the semi-structured nature of the C-terminus [148,150,319] enables it to be conformationally labile and to interact with several different classes of cytosolic scaffold proteins and the cytoskeleton that can influence connexin targeting (reviewed in [5] for Cx43). For instance, several truncated connexins lack the ability to be efficiently trafficked to the plasma membrane or be endocytosed [320,321]. The connexin C-terminal domains also have the capacity to homo- and hetero-dimerize [148,150,154,322] as well as interact with other connexin domains, including the cytoplasmic loop [150,323,324] that can influence connexin targeting, oligomerization and function.

Interestingly, it was determined that there is reciprocal regulation of Cx43 and Cx46 in the lens, where conditions such as activation of PKC caused an increase in Cx46 transcription and expression that was associated with a concomitant decrease in Cx43, via ubiquitination and proteosomal degradation [325]. In fact, transfecting cells with Cx46 was sufficient to induce Cx43 degradation and this effect required the C-terminus of Cx46, since a Cx46 tail truncation mutant had no effect on Cx43 expression. Increased Cx50 also had no effect on Cx43. However, transfecting cells with a soluble Cx46 tail construct had the ability to decrease Cx43 expression. Since the decrease in Cx43 was induced by an intracellular pool of Cx46, this raises the possibility that crosstalk between Cx46 and Cx43 may be related to differential oligomerization [302]. However, this remains to be determined.

As another instance where the C-terminus plays a key role in regulating Cx43 trafficking, it has been shown that amino N-terminal truncated forms of Cx43 are also expressed by cells, through alternative internal translation via one of six different AUG initiation sites (see section 2.4.2) [326]. The most prominent of these is GJA1-20k, which consists of a portion of the TM4 domain as well as the entire C-terminus [105] (Figure 1). GJA1-20k expression promotes formation of Cx43 gap junction channels resulting in an increase in intercellular communication [105,108]. As discussed in section 2.4.2, alternative translation of Cx43, including production of GJA1-20k, is inhibited by mTOR [105,106] and Mnk1/2 kinases [106], suggesting that metabolic stress regulates gap junctional coupling through mTOR and Mnk1/2 mediated pathways as a means to protect cells both by enabling scarce metabolites to be distributed via intercellular communication as well as limiting damage by restricting generation of reactive oxygen species [327].

How GJA1-20k regulates channel formation by Cx43 is still under investigation. One intriguing possibility is that GJA1-20k acts as a chaperone protein that promotes Cx43 oligomerization, as was recently demonstrated to regulate the decrease in gap junction formation and function that can occur in the epithelial to mesenchyme transition [328] (Figure 1).

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Another likely role for GJA1-20k relates to cytoskeletal control of Cx43 trafficking, since it has been shown that the C-terminus of Cx43 and therefore, GJA-20k as well, interacts with both microtubules and filamentous actin [329-331]. Microtubules and actin perform complementary functions in regulating connexin trafficking, where microtubules help facilitate rapid transport of Cx43-containing vesicles to sites of junction formation [330], whereas actin has a more subtle role in regulating connexin trafficking, since quantitative live cell imaging shows that transport of Cx43-containing vesicles temporarily pauses when they interact with actin filaments, perhaps as a means to enhance sorting or to remodel vesicle composition [329]. Also, transfecting HeLa cells with GJA1-20k nucleates the formation of actin filaments [108], suggesting a role for GJA1-20k in altering the itinerary of Cx43 trafficking in the cell. Reverse regulation is also suggested by studies where gap junction inhibitors resulted in misalignment of actin filaments across the monolayer and reduced calcium signalling in rat astrocytes [332]. Furthermore, treatment of astrocytes with an actin polymerization inhibitor cytochalasin D or anti-actin antibodies reduced GJIC, as visualized by a reduction in the spread of microinjected neurobiotin between cells [333].

4.4 Regulation of gap junction plaque morphology

Actin has also been implicated in regulating gap junction plaque morphology. Double knockout of the actin capping protein tropomodulin 1 and intermediate filament protein CP49 in lens fiber cells led to a significant decrease in Cx46 plaque volume and increase in plaque number, affecting gap junction coupling and function in the lens tissue [334]. Regulation of plaque size by actin is likely to be coordinated by interactions involving the C-terminus of connexins and zonula occludens 1 (ZO-1). For example, EGFP-tagged Cx43 incapable of interacting with ZO-1 produces plaques that are not size regulated [335]. By contrast, the perimeter of gap junction plaques (the perinexus) is ringed by Cx43/ZO-1 complexes whereas the centre of plaques is largely devoid of ZO-1 [336]. Inhibiting Cx43/ZO-1 interactions causes an increase in gap junction plaque size. Consistent with this possibility, Cx43 phosphorylation inhibits ZO-1 binding and facilitates connexin channel endocytosis [337]. Additional roles for ZO-1, connexin phosphorylation and ubiquitinylation in regulating connexin endocytosis and degradation are described in sections 3.2 and 3.3, above.

Although the precise mechanism whereby ZO-1 limits plaque formation is still under investigation, it seems plausible that it may be analogous to the role of ZO-1 in regulating tight junctions, where claudin/ZO-1/actin interactions have a junction stabilizing influence on the apical junctional complex, whereas in the absence of ZO-1, there is increased access of myosin that increases tight junction dynamics and tension [338,339]. Consistent with this possibility, myosin VI has also been found to have a specific role in increasing gap junction plaque size, analogous to treatments inhibiting Cx43/ZO-1 interactions [340].

Whether regulation of plaque assembly strictly follows the perinexus model has recently been challenged by observations of Cx36 plaque formation [341]. Pulse chase experiments with Cx36 indicated addition of Cx36 to both the ends and the middle of pre-existing gap junction plaques, with diffusion of Cx36 throughout the plaque. When the experiments were repeated with Cx43, there appeared to be less diffusion of newly added Cx43 in pre-existing plaques [341]. Targeted delivery of connexins has only recently been observed. Through interactions with plus-end binding protein EB1 and the dynein/dynactin complex, microtubule plus-ends are tethered to adherens junctions at the plasma membrane, leading to the targeted deposition of connexin hemichannels and gap junction plaque formation [330]. These two models begin to bring to light the vast complexity of connexin trafficking and gap junction formation, suggesting a network of cytoskeleton and protein-binding partners tailored to specific connexins that was previously unrealized.

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A less understood but intriguing role for the cytoskeleton in gap junction biology is the creation of unique junctional sub-regions involved in gap junction dynamics. Using an EGFP-tagged Cx32 construct, particularly dynamic regions at the edges of gap junction plaques were observed as invaginated tubular structures, where plaque fragments pinched off into the cytoplasm [342]. These tubulovesicular extensions of gap junction plaques were recently observed with Cx36 and termed filadendrites [343]. Filadendrites at the edges of gap junction plaques appeared to be the same thickness as the plaque, suggesting that the filadendrites were a continuation of the gap junction plaques themselves. Filadendrites were also observed in interior regions of the gap junction plaques, but appeared to be much thinner than the gap junction plaques. From pulse-chase labelling of Cx36, it was observed that filadendrites exhibited some of the same dynamic properties as the earlier observed Cx32 invaginations, constantly pinching off and fusing with the gap junction plaque. Labelling of actin filaments showed co-localization with Cx36 filadendrites, suggesting that the actin cytoskeleton could be one of the drivers behind the formation of these dynamic structures. Treatment with the actin polymerization inhibitor Latrunculin A or actin depolymerization inducer Cytochalasin D reduced the presence of filadendrites, indicating that the driving force behind the dynamic gap junction plaques requires actin polymerization [343].

Similar structures have been noted at other junctions. Primary human keratinocytes treated with pemphigus vulgaris (PV) IgG containing antibodies targeted to the adherens junction protein desmoglein 3 (Dsg3) exhibited reorganized Dsg3 at the membrane into projections perpendicular to the membrane plane. These projections, termed linear arrays, are similar to the filadendrites in that they are sites of disassembly of junction components and active endocytosis at the junctions. Linear arrays also co-localized with actin filaments oriented perpendicular to the plasma membrane, similar to those observed in filadendrites. Furthermore, linear arrays were associated with decreased cell adhesion, suggesting a functional effect of these junctional sub-regions [344] [345]. A comparable structure formed by tight junction proteins, termed tight junction spikes, have been observed to correlate with treatments that enhance junction disassembly and paracellular leak, including oxidative stress induced by chronic alcohol exposure, transforming growth factor (TGF)-beta1 treatment, and inhibition of NF-kappaB [346-348]. In alveolar epithelial cells, actin filaments co-localized with the tight junction protein claudin-18 in tight junction spikes. Spikes were also found to be sites of budding and fusion of vesicles carrying tight junction proteins, both indicators of active tight junction remodelling. Treatment of lung alveolar epithelial cells with GM-CSF reduced actin filament co-localization with claudin-18 containing tight junction spikes, whereas keratinocyte growth factor treatment inhibited spike formation and instead promoted formation of cortical actin as opposed to actin stress fibres [348,349]. Taken together, these findings indicate that these similar junctional sub-regions observed universally across several different classes of intercellular junctions, including gap junctions, could represent a common mechanism of junction protein turnover, where the junctions partition themselves into unique filamentous structures. Whether these structures serve to restrict turnover of junction proteins to specific subdomains or whether they nucleate the formation of signalling complexes that recruit specialized subsets of cytosolic binding partners remains to be determined.

5. Conclusions and Future Perspectives

In order to fully understand the complex role of connexins in health and disease, it is essential to elucidate their regulation at all steps, from gene transcription, protein synthesis, post-translational modifications and trafficking, to their regulation at the cell membrane. This review is intended to highlight some of the progress made in these areas, giving examples of how this knowledge is pertinent for future therapeutic application. Going forward, understanding how modulation of connexins occurs at any of these stages will require additional work and insight, which over time may lead to more fruitful and safer strategies to alleviate patient suffering. For example, the

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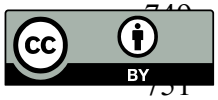
Danegaptide trials that were based on strong pre-clinical data suggested that alterations to the trafficking and increased Cx43 signalling in the heart would have a profound effect in reducing ischemic reperfusion injury and reduce cardiac tissue damage. However, Phase II clinical trials in humans failed to show an effect, highlighting the complex nature of targeting gap junctions as a treatment modality and also in deciphering differences in how connexins are regulated in model systems as opposed to human disease. Additional caution is also needed for therapeutic approaches in cancer, where it is now clear that connexins have distinct roles that both promote and inhibit cell growth and metastasis.

Despite substantial progress, it is important to acknowledge the complexity of gap junctions that serve as a conduit that enables cells to share thousands of different signalling molecules. Additionally, the complex connexin protein interactome underscores the non-junctional functions of connexins, including their ability to act as a signalling platform by acting as a site that promotes formation of multiprotein complexes. In particular, it is critical to identify connexin-specific functions which are unique and thus targetable. This is best approached by considering how connexins are influenced and regulated by multiple mechanisms, ranging from the level of the gene to post-translational modification to the specifically localized multi-protein complex.

Acknowledgments: TA acknowledges support from Instituto de Salud Carlos III grants PI13/00763, PI16/00772 and CPII16/00042, co-financed by the European Regional Development Fund (ERDF). Supported by NIH R01-AA025854 and R01-HL137112 (MK) and F31-HL139109 (KSL)

Conflicts of Interest: The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.



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