REVIEW ARTICLE

Life cycle of connexins in health and disease

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Evaluation of the human genome suggests that all members of the connexin family of gap-junction proteins have now been successfully identified. This large and diverse family of proteins facilitates a number of vital cellular functions coupled with their roles, which range from the intercellular propagation of electrical signals to the selective intercellular passage of small regulatory molecules. Importantly, the extent of gap-junctional intercellular communication is under the direct control of regulatory events associated with channel assembly and turnover, as the vast majority of connexins have remarkably short half-lives of only a few hours. Since most cell types express multiple members of the connexin family, compensatory mechanisms exist to salvage tissue function in cases when one connexin is mutated or lost. However, numerous studies of the last decade have revealed that mutations in connexin genes can also lead to severe and debilitating diseases. In many cases, single point mutations lead to dramatic effects on connexin trafficking, assembly and channel function. This review will assess the current understanding of wild-type and selected disease-linked mutant connexin transport through the secretory pathway, gap-junction assembly at the cell surface, internalization and degradation.

Key words: assembly, connexin, gap junction, transport.

INTRODUCTION

Gap junctions are classically defined as clusters of a few to hundreds of tightly packed intercellular channels that, in the simplest assessment, function to allow small molecules to be directly exchanged between adjoining cells [1] (Figure 1). The function of gap junctions in cell and tissue biology is of the utmost importance as GJIC (gap-junctional intercellular communication) exists in nearly every mammalian cell type [2,3]. This diverse and ubiquitous distribution of gap junctions is possible due to the fact that the connexin family consists of 20 members in the mouse and 21 members in humans (Table 1) [4,5]. While the channels assembled from connexin family members serve a common purpose of allowing the intercellular exchange of small metabolites, second messengers and electrical signals, the diversity of function is attributed to the subset of connexins that are expressed in any one cell type [6]. While not all channels are the same, they share the property of excluding molecules that exceed 1 kDa in size [1,7–9]. Importantly, smaller molecules that differ in size, shape and charge can also be included or excluded from passing through distinctly different gap-junction channel subtypes, resulting in a wide variety of molecular transjunctional selectivity (Figure 1) [10–12]. Collectively, secondary messengers and small metabolites are deemed to be the molecular constituents that are directly passed from one cell to another. Important transjunctional molecules include cAMP, InsP_3, adenosine, ADP and ATP, to name only a few [10–12]. The intermixing of connexin subunits within the same channel becomes even more important as we attempt to understand the mechanisms associated with connexin-linked autosomal recessive and dominant diseases.

DIVERSITY OF CONNEXIN EXPRESSION

Given the large number of connexins, it is not surprising to learn that their cellular and tissue distribution is overlapping, yet distinct. The scope of the present review prevents a detailed temporal and spatial assessment of connexin expression in all cell and tissue types, but, as one assesses this protein family, there are three fundamental principles that come to the forefront. First, many tissues and cell types express two or more members of the connexin family. For example, keratinocytes express at least Cx26 (connexin26), Cx30, Cx30.3, Cx31, Cx31.1 and Cx43 (Table 2) [13–17]. Likewise, cardiomyocytes express Cx40, Cx43 and Cx45 [8,18,19] and hepatocytes express Cx26 and Cx32 [20–23]. Collectively, co-expression of multiple connexin family members within the same cell type allows for possible compensatory mechanisms to overcome the loss or mutation of one connexin family member. This principle has been demonstrated effectively in connexin-gene-ablation studies, where the prevalence of disease or the incidence of abnormal development is far less than might be predicted if no compensatory mechanisms existed between co-expressed connexin family members [24,25]. In one example, Cx57 appears to be restricted to horizontal cells, yet Cx57-null mice exhibit no behavioural or obvious anatomical defects [26], suggesting the existence of a possible compensatory mechanism by an unknown connexin. In another case, humans suffering from loss-of-function mutations in Cx26 are deaf, but no accompanying liver diseases have been reported where Cx26 and Cx32 are co-expressed in hepatocytes [27–29]. A second principle to note from the connexin distribution patterns is that even though two or more connexins may be co-expressed in the same cell, the resulting channels formed cannot always compensate for the loss or mutation of a connexin family member [6]. This point is exemplified in the skin, since a subset of loss-of-function Cx26 mutations that result in human deafness also manifest as skin disease, even though the epidermis is rich, with multiple members of the connexin family [30,31]. The third notable observation, from the examination of connexin expression patterns in mammals, is that the most ubiquitously expressed connexin is Cx43. It

Abbreviations used: AT, N-terminus; CL, cytoplasmic loop; CT, C-terminus; Cx*x*, connexin*x* (the notation is used only for numbered connexins and not for the generic term); EL, extracellular loop; ER, endoplasmic reticulum; ERAD, ER-associated-degradation; ERGIC, ER–Golgi intermediate compartment; GFP, green fluorescent protein; GJIC, gap-junctional intercellular communication; MAGUK, membrane-associated guanylate kinase; NOV, nephroblastoma overexpressed gene; ODDD, oculodentodigital dysplasia; PKK, palmoplantar keratoderma; ZO, zonula occludens.

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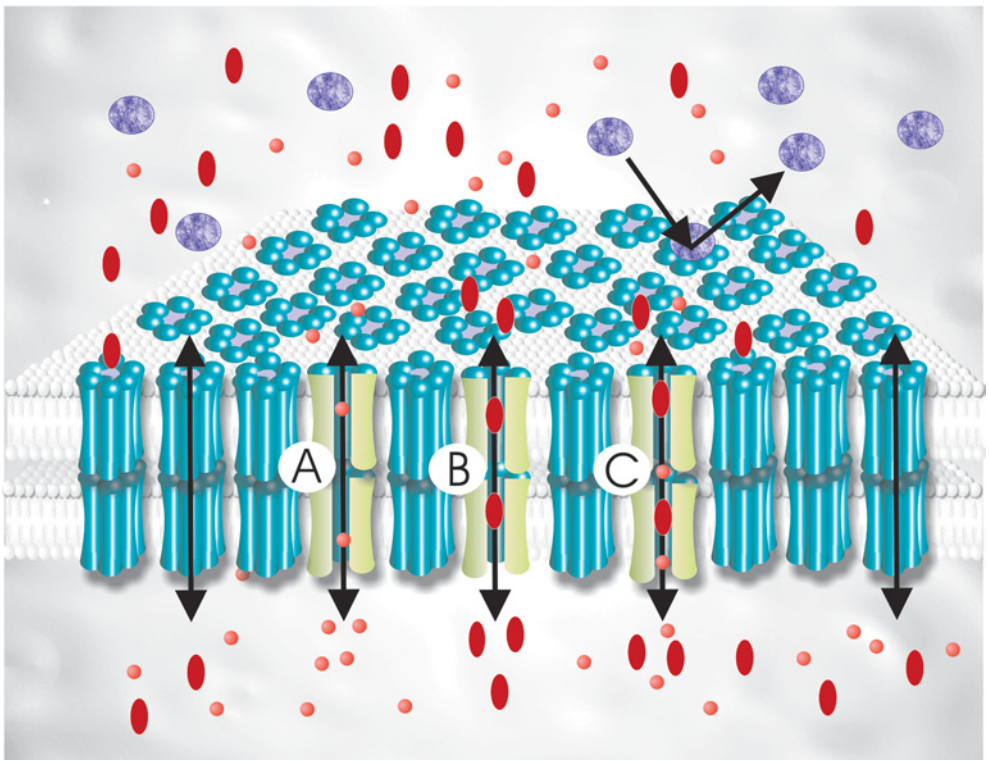

Figure 1 Schematic diagram illustrating the selective trans-junctional properties of gap junctions in intercellular communication

Gap-junction channels can be permeable to small molecules (**A**), small molecules with elongated shapes (**B**) or combinations of both molecular shapes (**C**). It is also important to note that the charge of the trans-junctional molecule also governs permeability characteristics. Gap junctions are typically not permeable to molecules exceeding 1 kDa (purple).

Table 1 Mouse and homologous human connexin family members

Mouse connexins	Human connexins
Cx23	Cx23
	Cx25
Cx26	Cx26
Cx29	Cx30.2
Cx30	Cx30
Cx30.2	Cx31.9
Cx30.3	Cx30.3
Cx31	Cx31
Cx31.1	Cx31.1
Cx32	Cx32
Cx33	
Cx36	Cx36
Cx37	Cx37
Cx39	Cx40.1
Cx40	Cx40
Cx43	Cx43
Cx45	Cx45
Cx46	Cx46
Cx47	Cx47
Cx50	Cx50
	Cx59
Cx57	Cx62

Table 2 Representative tissues and cell types where mouse connexin family members are found

Mouse connexins	Representative tissue/organ	Representative cell type
Cx23	–	–
Cx26	Liver, skin	Hepatocytes, keratinocytes
Cx29	Brain	Oligodendrocytes
Cx30	Skin	Keratinocytes
Cx30.2	Testis	Smooth-muscle cells
Cx30.3	Skin	Keratinocytes
Cx31	Skin	Keratinocytes
Cx31.1	Skin	Keratinocytes
Cx32	Liver, nervous	Hepatocytes, Schwann cells
Cx33	Testes	Sertoli cells
Cx36	Retina, nervous	Neurons
Cx37	Blood vessels	Endothelial cells
Cx39	Developing muscle	Myocytes
Cx40	Heart, skin	Cardiamyocytes, keratinocytes
Cx43	Heart, skin	Cardiomyocytes, keratinocytes
Cx45	Heart, skin	Cardiomyocytes, keratinocytes
Cx46	Lens	Lens fibre cells
Cx47	Nervous	Oligodendrocytes
Cx50	Lens	Lens fibre cells
Cx57	Retina	Horizontal cells

is now known that Cx43 is endogenously expressed in at least 35 distinct tissues encompassing over 35 cell types that include cardiomyocytes, keratinocytes, astrocytes, endothelial cells and smooth-muscle cells among many others (Table 3). Not surprisingly, over 2100 papers have focused on examining Cx43 biosynthesis, post-translational modifications, trafficking, assembly, turnover and channel function.

MOLECULAR ARCHITECTURE OF GAP JUNCTIONS

Zimmer et al. [32] used a combination of sequence analysis, limited molecular proteolysis and site-directed antibodies to

Table 3 Tissue and cell distribution of Cx43

Tissue	Cell type	Selected references
Cardiac	Cardiomyocytes	[213,214]
	Smooth-muscle cells	[215–218]
Oral cavity	Keratinocytes	[219]
Dental pulp	Odontoblasts	[220]
	Pulp cells	[220,221]
	Periodontal fibroblasts	[222,223]
Salivary glands	Myoepithelial cells	[224–226]
Oesophagus	Epithelial cells	[227,228]
Stomach	Epithelial cells	[227]
Gastroduodenal	Muscle cells	[229]
Small intestine	Muscle cells	[227]
	Cells of Cajal	[230–232]
Colon	Muscle cells	[233]
Pancreas	Endocrine β-cells	[234]
Pituitary gland	Cells of the anterior and posterior pituitary	[234,235]
Parathyroid gland	–	[234]
Thyroid gland	Thyroid epithelial cells	[176,234,236]
Adrenal gland	–	[234]
Skin	Keratinocytes	[16,237]
	Dermal fibroblasts	[238]
Muscle (myogenesis)	Myoblasts	[239,240]
Brain	Astrocytes	[241,242]
	Leptomeningeal cells	[241]
	Ependymal cells	[241]
Testis	Sertoli cells	[243,244]
	Leydig cells	[243,244]
Ovary	Granulosa cells	[245,246]
Uterus	Myometrial cells	[245]
Oviduct	Epithelial cells	[247]
	Smooth-muscle cells	[247]
Mammary gland	Epithelial cells	[248,249]
Lung	Alveolar epithelial cells	[250,251]
Trachea	Smooth-muscle cells	[117]
Bone	Osteoblasts	[252,253]
	Osteoclasts	[253,254]
	Osteocytes	[253,255]
Cartilage	Chondrocytes	[256]
Kidney	Vascular cells, glomerular cells	[257,258]
Bladder	Smooth-muscle cells	[217,259]
	Suburothelial interstitial cells	[260]
Retina	Retinal glial cells	[261]
Thymus	Thymic epithelial cells	[262]
Bone marrow	Stromal cells	[263,264]
Lymph node	Follicular dendritic cells	[264,265]
Spleen	Follicular dendritic cells	[264,265]
Tonsil	Tonsilar epithelial cells	[264,265]

propose a topological model for connexins that has remained essentially unchanged over the years [32]. Connexins are polytopic integral membrane proteins where the polypeptide backbone threads through the membrane four times, yielding two extracellular loops (EL-1 and EL-2), a cytoplasmic loop (CL) with both the N-terminus (AT) and the C-terminus (CT) exposed to the cytoplasm (Figure 2). This basic topology was confirmed for Cx43, Cx32 and Cx26 by many investigators [23,33–37]. To date, no exceptions to this connexin architecture have been discovered, although the topology of every member of the connexin family has not been specifically examined. Sequence conservation amongst the connexin family members is most evident within the four transmembrane domains, the two EL domains and the AT. Reciprocally, the highest degree of diversity among connexins is in the sequence and size of the CL region and both the size and post-translational modified status of the CT domain. The EL domains are connected by intramolecular disulphide bonds, as all connexins have three conserved cysteine residues in each EL [38–41]. Connexins oligomerize into hexamers commonly referred to as either 'connexons' or 'hemichannels' (Figure 2). Two adjacent connexons from apposing plasma membranes dock to form a complete gap-junction channel [42]. Individual gap-junction channels are arranged in hexagonal arrays that are often referred to as 'gap-junction plaques' [42] (Figure 2). The packing of connexin subunits within a channel yields an aqueous channel with a diameter of approx. 2 nm [43].

As a first assessment, it would appear that only 21 gap-junction channel subtypes would be possible in humans if connexins could only oligomerize in a homomeric fashion and dock with connexons of the same type. However, the possible channel subtypes increases exponentially when two compatible connexins are co-expressed in the same cell and are capable of assembling both homomeric and heteromeric channels (Figure 2). Heteromeric Cx26/Cx32 connexons have been shown to exist in the liver [44], Cx46/Cx50 connexons in the lens [45,46] and Cx26/Cx30 connexons in the cochlea [47]. Likewise, a substantial body of evidence suggests that Cx43 and Cx45 form heteromeric channels (Figure 2) [8,48–50], which are predicted to exist in the myocardium. Under these conditions each cell could contribute up to 14 variations, yielding 196 possible complete channel subtypes (Figure 2). However, the complexity of theoretically possible channel subtypes explodes when one considers the fact that many cell types express three or more connexins. The epidermis has been reported to differentially express at least seven connexins that are precisely regulated by the state of keratinocyte differentiation [51,52]. To add to the level of possible channel complexity, it is now well established that both heteromeric [44] and heterotypic [46,53–57] gap-junction channels exist in addition to their homomeric and homotypic counterparts. However, channel diversity is tempered by the fact that not all co-expressed connexins can form heteromeric connexons or heterotypic channels [45]. For example Cx26 has been convincingly demonstrated to be capable of co-oligomerizing with Cx32 (i.e. hepatocytes) [44], but this same connexin is unable to co-oligomerize with Cx43 [58]. The importance and sophistication of connexin arrangement in the epidermis may allow for the establishment of specific gap-junction compartments in the different strata. In wound healing, Cx26 and Cx30 are up-regulated at the wound edge, whereas Cx31 and Cx43 are reciprocally down-regulated, pointing to unique functions of channel subtypes [15]. Thus the overall complexity of GJIC is governed in a large part by the connexin constituents of the channels.

CONNEXIN BIOSYNTHESIS

Remarkably, connexin proteins have a short half-life of only a few hours. This short lifespan of a connexin has been well-documented in cultured cells as well as in the three-dimensional milieu presented by native tissue environments [59–62]. Thus, for reasons that remain elusive, connexins are pre-programmed to be continually biosynthesized and degraded. It is probable that connexins retain a short half-life to respond to physiological requirements to either up- or down-regulate the extent of gap-junction coupling. This response mechanism is best exemplified in the myometrium, where it is proposed that steroid hormones promote the dramatic 5-fold increase in total gap junctions just prior to labour onset [63–67]. Conversely, gap junctions are rapidly cleared following labour re-establishing a steady-state level of GJIC in the uterus [68]. While other physiological changes may not demand such a dramatic change in gap-junction status as this, the general ability of cells to govern their overall level of GJIC by altering the expression levels of connexins coupled

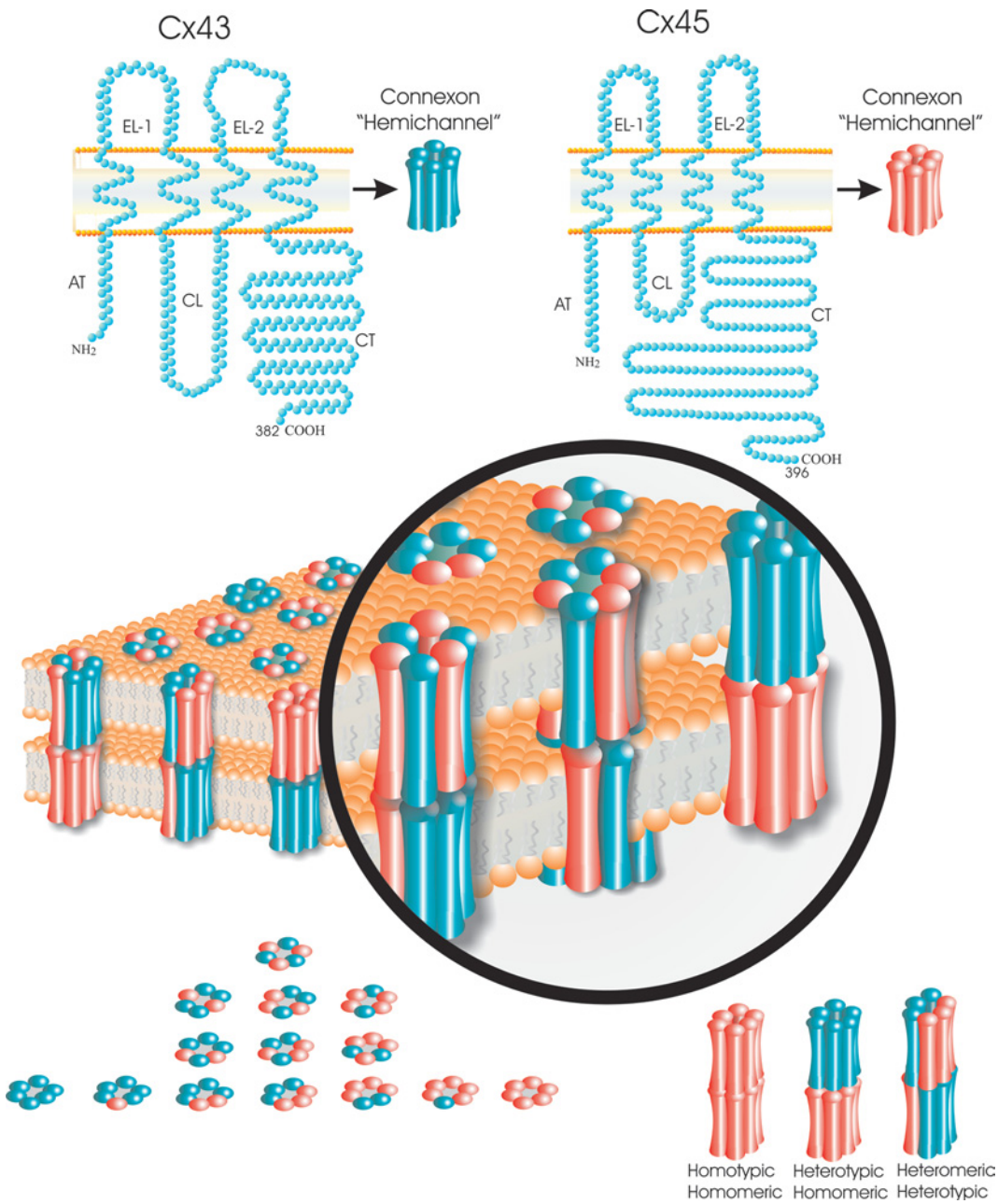

Figure 2 Assembly of connexins into gap junctions

Cx43 and Cx45, as examples of connexin family members, typically thread through the membrane four times, with the AT, CT and CL exposed to the cytoplasm. Connexin arrangement in the membrane also yields two extracellular loops designated EL-1 and EL-2. Six connexins oligomerize into a connexon or hemichannel that docks in homotypic, heterotypic and combined heterotypic/heteromeric arrangements. In total, as many as 14 different connexon arrangements can form when two members of the connexin family intermix.

to assembly and degradation events allows for an exquisite level of regulation that extends beyond the rapid channel opening and closure events associated with channel gating [9,39].

In keeping with classical integral membrane proteins, connexins are thought to co-translationally thread into the ER (endoplasmic reticulum) via the translocon and encoded start and stop transfer sequences. One possible exception to this rule is Cx26, which has been shown to be capable of being both post- and co-translationally imported to the ER membranes [69] and even to be directly imported into the plasma membrane [70]. However, there remains no direct evidence that Cx26 is post-translationally imported into any membrane compartment *in vivo*. Although connexins are not glycosylated, it is reasonable to speculate that they are appraised by one or more molecular chaperones as they reach their final folded and stable state. The fact that cysteine residues form disulphide bonds between the extracellular loops (domains initially exposed to the lumen of the ER) [40] suggests that connexins are under the surveillance of at least protein disulphide-isomerases. Furthermore, it is predicted that other molecular chaperones are engaged in assisting the stable folding of connexins while they exist as transient residents of the ER.

Connexin oligomerization has been reported to occur during their resident time within the ER [71–77]. This finding, although

somewhat predictable, appears not to be the case for at least Cx43 and Cx46, since both of these connexins have been demonstrated to be present in monomeric form in the Golgi apparatus and appear to oligomerize in the *trans*-Golgi network [78,79]. Recently, questions have been raised as to whether early connexin oligomerization is a product of overexpression or of cell-free systems where the concentration of connexin subunits is artificially elevated with native oligomerization of connexins occurring after their exit from the ER. It has been shown that high expression levels of Cx32 can drive the formation of intracellular gap-junction-like structures in BHK (baby-hamster kidney) cells [80], raising concerns that aberrant and premature oligomerization and gap-junction assembly can be induced. It is possible that oligomerization of connexin subunits is a progressive event where subunits begin to associate in the ER and the ER–Golgi intermediate compartment (ERGIC), with stable oligomers being established in the late-Golgi compartments that include the *trans*-Golgi network. Further studies are required to resolve the apparent discrepancies in the compartment or compartments where complete connexin oligomerization is achieved. Regardless of where connexon oligomerization is completed, the resulting hemichannels are predicted to be gated closed to protect and maintain the integrity of the lumens of these intracellular compartments.

CONNEXIN TRANSPORT TO THE CELL SURFACE

Upon exiting the ER, properly folded connexins are expected to pass through the ERGIC prior to entering the *cis*-Golgi network (Figure 3). Several studies have revealed that connexins pass through the Golgi apparatus [78,79,81–86]. However, other studies have reported that Cx26 can reach the cell surface via a Golgi-independent pathway [87–91]. In a recent study we directly compared the secretory pathway followed by both Cx26 and Cx43 in the same cells and found that both connexins followed similar secretory pathways with differential dependences on intact microtubules [85]. The extent to which connexins are substrates for post-translational modifications in the secretory pathway during transport to the cell surface is not well understood. Some evidence suggests that Cx43 is transiently phosphorylated early in the secretory pathway [81,92], yet the vast majority of Cx43 phosphorylation is thought to occur when it reaches the plasma membrane [93,94]. This review will not summarize the phosphorylation status of connexins, as several excellent reviews exist on this topic [93,94]. Interestingly, even though connexins are integral membrane proteins, one recent report convincingly showed that Cx32 is post-translationally prenylated [95]. Since prenylation is normally used to anchor proteins to lipid bilayers it is not clear what role this modification plays in anchoring Cx32, as it would seem to be an unnecessary post-translational modification.

Studies in live cells, using fluorescent-protein-tagged connexins, revealed that connexins, on exiting the *trans*-Golgi network, appear to enter a variety of transport intermediates of different sizes and shapes that are used for delivery of the connexin cargo to the cell surface [83,85,96]. These pleiomorphic vesicles and tubular extensions can be seen emanating from the *trans*-Golgi network [85]. Several lines of evidence also suggest that connexin transport is mediated in part by microtubules [83,97–99]. Although microtubules facilitate the delivery of connexin transport carriers to the cell surface, they appear not to be essential, but rather act to improve the efficiency of the delivery process. Upon delivery of connexons to the cell surface it is expected that they would continue to be gated closed to prevent uncontrolled exchange of small molecules to the extracellular environment. However, a new paradigm is emerging where connexons or hemichannels may transiently gate open for regulated exchange between the cytosol and extracellular environment [100–102]. The most studied of these exchanges is ATP release from the cytosol in response to elevations in Ca^{2+}, as has been documented in retinal pigment epithelial cells [103].

GAP-JUNCTION ASSEMBLY AT THE CELL SURFACE

Once inserted into the plasma membranes, connexons appear to freely diffuse within the lipid bilayer [85] and, under the guidance of specific N- and E-cadherin-based adhesion events [104–106], dock with connexons from adjacent cells to form gap-junction channels (Figure 3). Recent studies have suggested that N-cadherin and Cx43 may in fact co-assemble, suggesting direct cross-talk between adherens junctions and gap junctions [106]. Interestingly, some evidence using GFP (green fluorescent protein)-tagged Cx43 has been generated that suggests that, once apposing connexons have docked, there may be a delay in the functional coupling until channels coalesce into plaques, as defined by their characteristic punctate structures [107]. However, more recent studies using disease-linked connexin mutants has indicated that coupling status is not always directly correlated with the clear optical-microscopic identification of gap-junction plaques [108], suggesting that either small gap junctions or even individual gap-junction channels could acquire a functionally active state in the absence of a channel-clustering event.

The clustering of individual gap-junction channels from non-junctional membranes appears to be a continually active and dynamic process. A key study, where a novel tetracysteine epitope was tagged to Cx43 together with the use of two optically distinct ligands covalently bound to the tetracysteine epitope, was used in a pulse–chase experiment to show that gap junctions formed from the outer margins of the plaques, whereas aged channels were localized to the centre of the plaques [109]. This surprising finding, namely that old and new channels could be distinctly separated within the gap-junction plaque, was confirmed later using GFP-tagged Cx43 and FRAP (fluorescent recovery after photobleaching) [83]. One interpretation of these findings is that the older channels in the middle of a plaque are destined for internalization and degradation (Figure 3). This hypothesis is supported by the fact that fragments of tetracysteine-tagged Cx43 gap-junction plaques, when viewed under the electron microscope, appear to bud from inner elements of the gap-junction plaque [109].

GAP-JUNCTION INTERNALIZATION

The internalization of gap junctions, connexons and/or connexins has been a subject of considerable focus in recent years. Many years ago, large double-membrane vesicular structures termed ‘annular junctions’ were identified by electron microscopy, and it was proposed that these structures were the products of one cell internalizing either the entire gap junction or a fragment of it [110–115] (Figure 3). With the generation of connexin-specific antibodies, which could be used to immunolabel annular junctions, it became apparent that these double-membrane structures indeed consisted in large part of connexin proteins [116–119]. In 2001, anti-Cx43 antibody microinjection studies, together with live imaging of GFP-tagged Cx43, revealed that the origin of annular junctions was in fact from pre-existing gap-junction plaques at cell–cell interfaces [120]. These findings were further validated by a number of studies using fluorescent-protein- or epitope-tagged connexins [109,121]. Since the term ‘annular junction’ is descriptive in nature and does not implicitly refer to these structures originating from gap junctions, we propose here

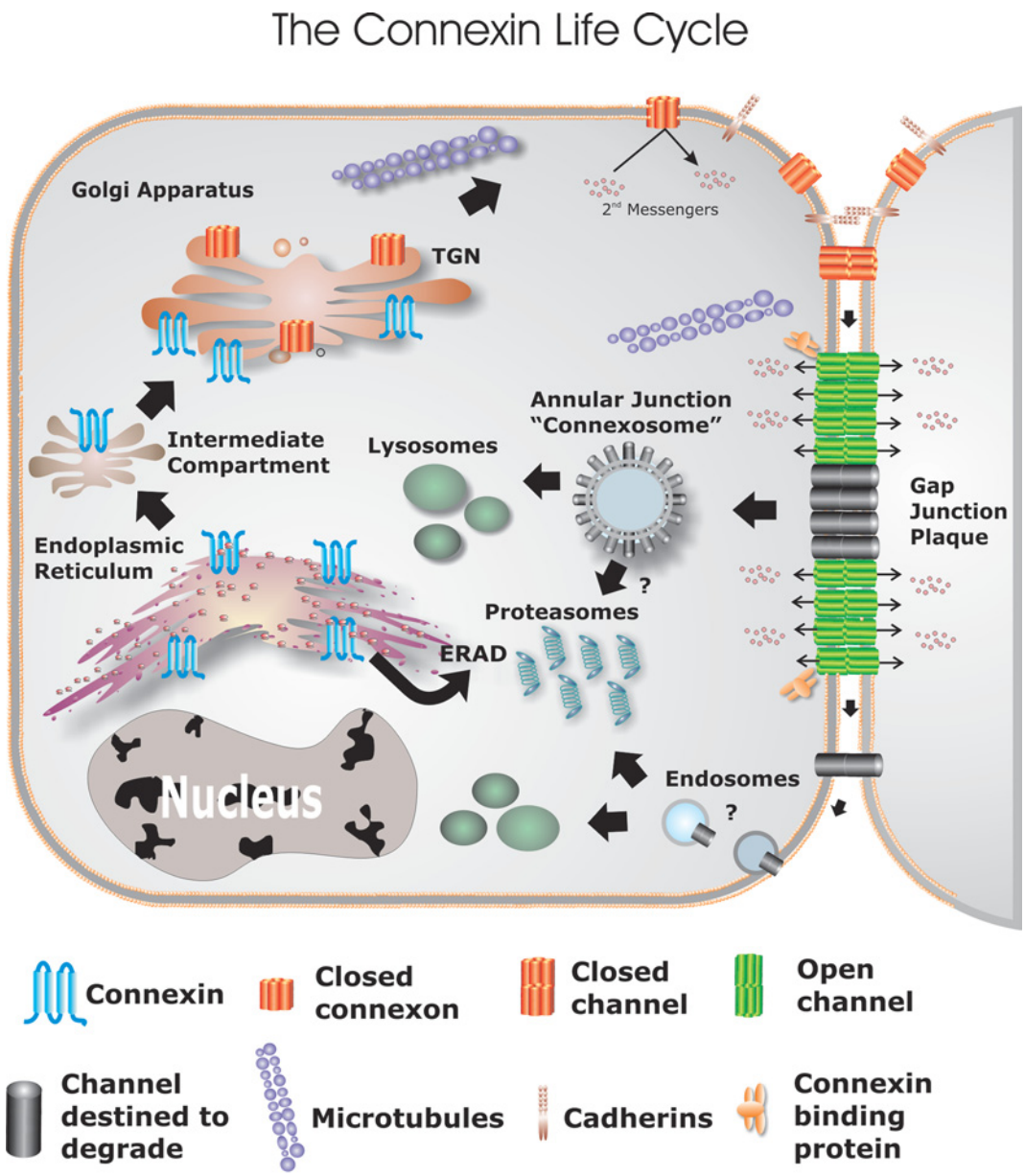

Figure 3 Life cycle of a connexin

Connexins typically co-translationally insert into the ER. If properly folded, it is expected that connexins are spared from ERAD, whereas in other cases they may be targeted for ERAD. For at least some members of the connexin family, complete oligomerization is delayed until the connexin passes through the intermediate compartment and reaches the distal elements of the Golgi apparatus, namely the TGN (*trans*-Golgi network). Pleiomorphic vesicles and transport intermediates are thought to deliver closed connexons to the cell surface, a process that is facilitated by microtubules. Connexons may function as hemichannels and exchange small molecules with the extracellular environment or laterally diffuse in a closed state to sites of cell–cell apposition and dock with connexons from an opposing cell. In conjunction with cadherin-based cell adhesion, gap-junction channels cluster into plaques, open and exchange secondary messengers. New gap-junction channels are recruited to the margins of gap-junction plaques and older channels are found in the centre of the plaques. Several connexin-binding proteins have been identified, and it is likely that one or more of these binding proteins regulate plaque formation and stability, possibly by acting as scaffolds to cytoskeletal elements. Gap-junction plaques and fragments of gap-junction plaques are internalized into one of two adjacent cells as a double-membrane structure commonly referred to as an annular junction, but renamed in the present review as connexosomes. Other pathways for connexin internalization may exist where connexons disassemble and enter the cell by classical endocytic pathways. Internalized gap junctions are targeted for degradation in lysosomes, although some evidence suggests a role in proteasomal degradation.

to term these structures 'connexosomes'. The rationale for the use of this nomenclature is based on several principles.

(1) The internalization of a double-membrane structure into one of two apposing cells is novel, and is clearly distinct from the majority of other proposed mechanisms for the turnover of junctional complexes [122–124]. However, one study suggests that two apposing membranes containing claudin-positive tight junctions may in fact internalize into one of the two adjacent cells [125].

(2) Immunogold and immunofluorescence labelling studies in conjunction with ultrastructural characteristics would strongly suggest connexosomes are connexin-enriched [116,120]. Although connexosomes have not yet been subcellularly fractionated or biochemically assessed, immunogold labelling revealed that

connexins are found throughout the spherical structure, with the possible exception of small areas which may represent locations where the connexosome separated from the plasma membrane.

(3) In keeping with recent developments, where novel endosomal compartments are assigned distinctive names (i.e. signalosomes [126]), the uniqueness of connexosomes, including their novel origin from the cell surface, would provide suitable rationale for the assignment of a distinct name.

(4) Given that connexosomes are large and involve a double-membrane mechanism of internalization, these structures cannot be assigned as classical endosomes or phagosomes, and thus need to be considered as a specialized intracellular compartment. It is important to note that connexin-enriched connexosomes likely include many other molecules, since several connexin-binding proteins have been identified (see the section 'Connexin binding-partners' below). In addition, connexosomes are predicted to contain molecules necessary for directing their internalization and intracellular fate.

While annular junctions (connexosomes) have been identified in a variety of cell types, it is important to note that these structures are difficult to detect in some cell types (e.g. hepatocytes). This raises the very real possibility that connexins may also be internalized via more classical endosomal mechanisms. In some cases gap junctions have been shown to disassemble at the cell surface into small aggregates [127], perhaps in preparation for internalization. In a recent study examining epidermal-growth-factor-induced loss of gap junctions from the cell surface, the process was inhibited by hyper-osmotic sucrose treatment, suggesting the possibility of a clathrin-mediated internalization pathway for Cx43 [128]. In support of this pathway, other studies have identified connexins in close proximity to clathrin-coated pits [116] or clathrin co-localization with Cx43 [129]. Interestingly, Cx43 has been shown to bind to caveolin-1 [130], suggesting that Cx43 may be targeted to lipid rafts and internalized in a caveolae-dependent pathway. The existence of one or more pathways, in addition to connexosomes, for connexin and gap-junction internalization is particularly relevant if connexins have any capacity to recycle back to the cell surface. It would be difficult to envisage a mechanism where connexosomes are disassembled inside the cell such that connexin subunits or connexons could be recycled. However, if a recycling pathway does exist for connexins, it would most likely involve classically understood endosomes or recycling endosomes. In support of such a notion, a recent study has suggested that, under conditions of cytosolic stress, non-junctional cell-surface Cx43 internalizes and recycles back to the cell surface to facilitate gap-junction formation [131]. Clearly, additional studies are required to determine all the pathways responsible for connexin internalization and to assess the possibility that connexin recycling exists under both normal and/or abnormal conditions.

CONNEXIN DEGRADATION

Initially the degradation of connexins and gap junctions was proposed to follow the well-understood pathway for integral plasma-membrane proteins, terminating in their delivery to lysosomes [114,116,132,133] (Figure 3). This paradigm was challenged in the mid-1990s, when compelling evidence was reported that the degradation of Cx43 was delayed in the presence of inhibitors of proteasomes or in cells that lack the E1-ubiquitin-activating enzyme [61]. In support of connexin degradation in proteasomes, Cx43 has now been demonstrated to be a suitable substrate for ubiquitin [61,128,134]. New evidence suggests that Cx43 is subject to mono-ubiquitination, which probably serves as an internalization signal, as opposed to poly-ubiquitination, which typically targets molecules to proteasomes [134]. Nevertheless, a substantial body of evidence, primarily from the use of proteasomal inhibitors, suggests that proteasomes play either direct or indirect roles in regulating connexin degradation [61,135,136]. However, drug-based studies, together with localization studies, convincingly argue for degradation of connexins in lysosomes [128,134,137–140]. This apparent discrepancy may be resolved in part by the observation that a subpopulation of Cx43 and Cx32 can apparently be reverse-translocated from the ER into the cytosol in proteasomal-inhibitor-treated cells, a process that is inhibited by cytosolic stress [141]. Consequently, it is possible to model a scenario where proteasomes are responsible for ER-associated-degradation (ERAD), whereas lysosomes solely degrade connexins that cycle through the plasma membrane. In support of such a model, non-junctional cell-surface Cx43 has been reported to be re-routed from being targeted to lysosomes under conditions of cytosolic stress [131]. Overall it is possible that polyubiquitinated connexins are targeted for ERAD and mono-ubiquitin connexins are tagged for internalization, with their final fate being degradation in lysosomes. Such a model would be in keeping with the findings of most of the previous studies, but it remains to be determined whether this model of connexin degradation can be further substantiated and whether all connexins are subject to the same fate.

CONNEXIN-BINDING PARTNERS

Since several connexins are post-translationally phosphorylated, protein kinases, and probably phosphatases too, must interact with members of the connexin family at least transiently. The kinases known to be involved include v- and c-src kinase, protein kinase C, MAPK (mitogen-activated protein kinase), cdc2 kinase (also known as cyclin-dependent kinase), casein kinase 1 and protein kinase A (Figure 4). Excellent recent reviews are currently available that assess the roles and function of connexin phosphorylation, so this topic will not be discussed further here [93,142–144]. A second classification of connexin-binding proteins began to emerge when proteins not involved in phosphorylation/dephosphorylation events were identified. The first of these molecules shown to bind connexins was ZO-1 (zonula occludens 1) [145,146], which was previously considered as a tight-junction-associated protein on the basis of its proposed role in tight-junction assembly [147]. It is now understood that ZO-1 binds to Cx31.9, Cx32, Cx36, Cx43, Cx46 and Cx50 in addition to Cx43 [148–155]. A second member of the MAGUK (membrane-associated guanylate kinase) family, ZO-2, has also been demonstrated to bind to Cx43 [152,156]. While it has been known for some time that these MAGUK family members bind to connexins, their functional roles are only beginning to be understood. Singh et al. [156] examined ZO-1 and ZO-2 binding to Cx43 at different stages of the cell cycle and found that ZO-1 bound preferentially with Cx43 in cells that were quiescent, suggesting that ZO-1 interaction with Cx43 may contribute to the stability of gap junctions. A similar role for the MAGUK family members has been proposed for localizing and stabilizing occludin in tight junctions [157]. Another study, using a Cx43-binding dominant-negative fragment of ZO-1, revealed that inhibition of ZO-1/Cx43 binding decreased GJIC and promoted a lipid raft localization of Cx43 in osteoblast-derived cells, suggesting a role for ZO-1 in regulating gap-junction assembly [158]. Consistent with the regulation of gap-junction formation, blocking ZO-1 binding to Cx43 has been reported to increase the size of gap junctions in HeLa cells [159], a position supported by ZO-1/Cx43

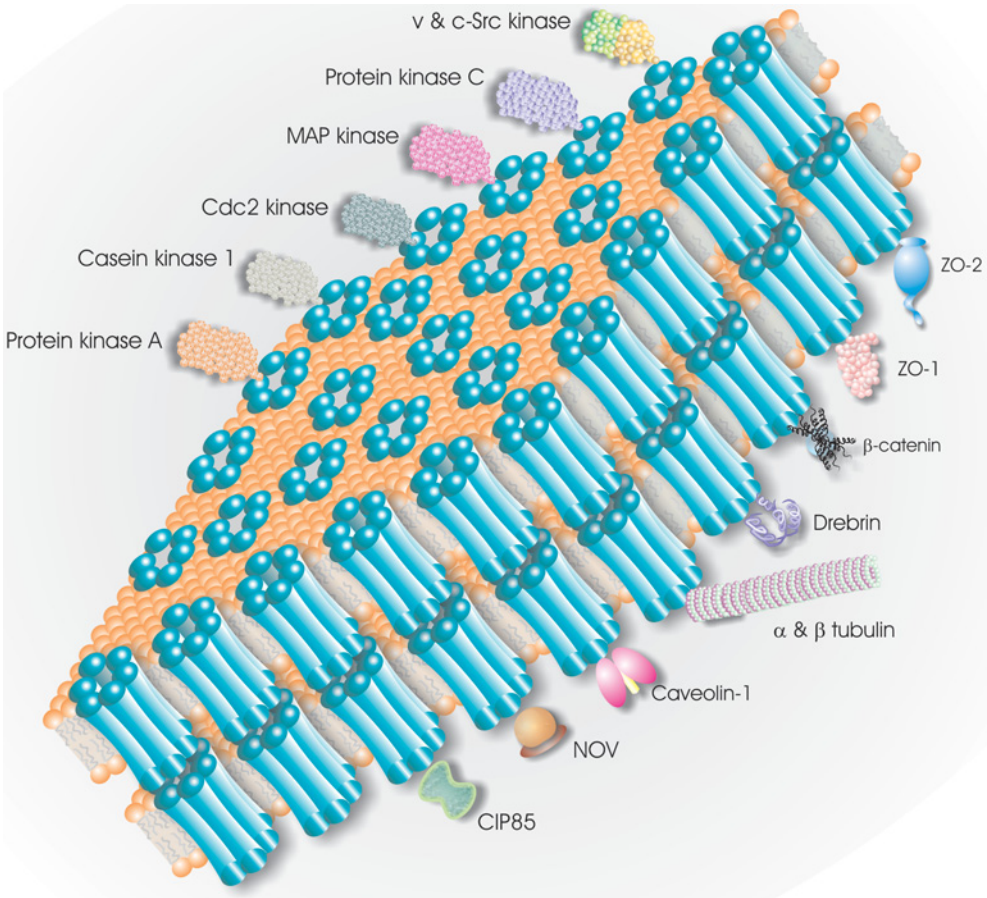

Figure 4 Cx43-binding proteins

Protein kinases known to phosphorylate Cx43 are shown along the top of a diagrammatically represented gap-junction plaque. A number of scaffolding proteins and proteins of unknown function that have been shown to bind directly or indirectly to Cx43 are shown along the bottom of the gap-junction plaque. It is important to note that it is not necessarily expected that all proteins shown here bind to Cx43 while it is a resident of the gap-junction plaque. MAP kinase, mitogen-activated protein kinase; CIP85, Cx43-interacting protein of 85 kDa.

co-localization studies in cardiomyocytes [160]. These same authors provided additional evidence that ZO-1 controlled gap-junction channel accretion at the margins of gap junctions and turnover thus regulating gap-junction size [161]. Collectively, these studies suggest that ZO-1 binding to Cx43 acts to regulate gap-junction size and stability.

In addition to connexins binding to MAGUK family members, evidence now suggests that β-catenin can be co-immunoprecipitated and co-localizes with Cx43 [162]. This interaction, whether direct or indirect, opens up the possibility that β-catenin plays a role in cell signalling and regulation of GJIC with cross-talk activity with the Wnt signalling pathway. Likewise, another potential signalling molecule that was found to bind to the CT of Cx43 is NOV/CCN3 [nephroblastoma overexpressed gene/CTGF (connective-tissue growth factor), Cyr61/Cef10 and nephroblastoma overexpressed gene] [163,164], suggesting a role for Cx43–NOV interactions in growth suppression. Mechanistically this protein–protein interaction is surprising, given that the CT domain of Cx43 would not normally be expected to encounter a soluble secreted factor like NOV. Further studies are necessary to uncover the mechanism governing this interaction.

A series of other protein–connexin interactions have been discovered that may play a role in regulating the life cycle of connexins. It is now well established that both α- and β-tubulin bind to the C-tail of Cx43 [149] and microtubules bind to a motif encoded close to the fourth transmembrane domain [165]. This finding is in keeping with previous and recent studies that support a role for microtubules in regulating connexin transport intermediate trafficking as part of the events leading to gap-junction assembly and removal [83,85,97,99,166,167]. However, it has been alternatively proposed that gap junctions may serve as microtubule-anchoring locations, thus regulating cellular activity associated with microtubule function [165]. In any event, gap junctions can continue to function in intercellular communication in the absence of intact microtubules, suggesting a non-essential, but a synergistic, role for Cx43–microtubule interactions.

The actin-binding protein drebrin has also been shown to bind to the CT of Cx43 and this suggests that Cx43 is possibly bridged to microfilaments. Silencing of drebrin resulted in reduced GJIC, accompanied by the internalization of Cx43, suggesting a role for drebrin in maintaining gap junctions at the cell surface [168]. Interestingly, cAMP-induced clustering of Cx43 gap-junction channels was abolished when microfilaments were disrupted further, suggesting a role for actin microfilaments in gap-junction maintenance [169]. In accordance with connexin-binding proteins regulating the stability of gap junctions, a novel connexin-interacting protein, CIP85 (Cx43-interacting protein of 85 kDa), was shown to bind to two proline-rich motifs of Cx43 and may play a role in regulating the turnover of Cx43-containing gap junctions [170]. Likewise, Cx43 has also been found to bind

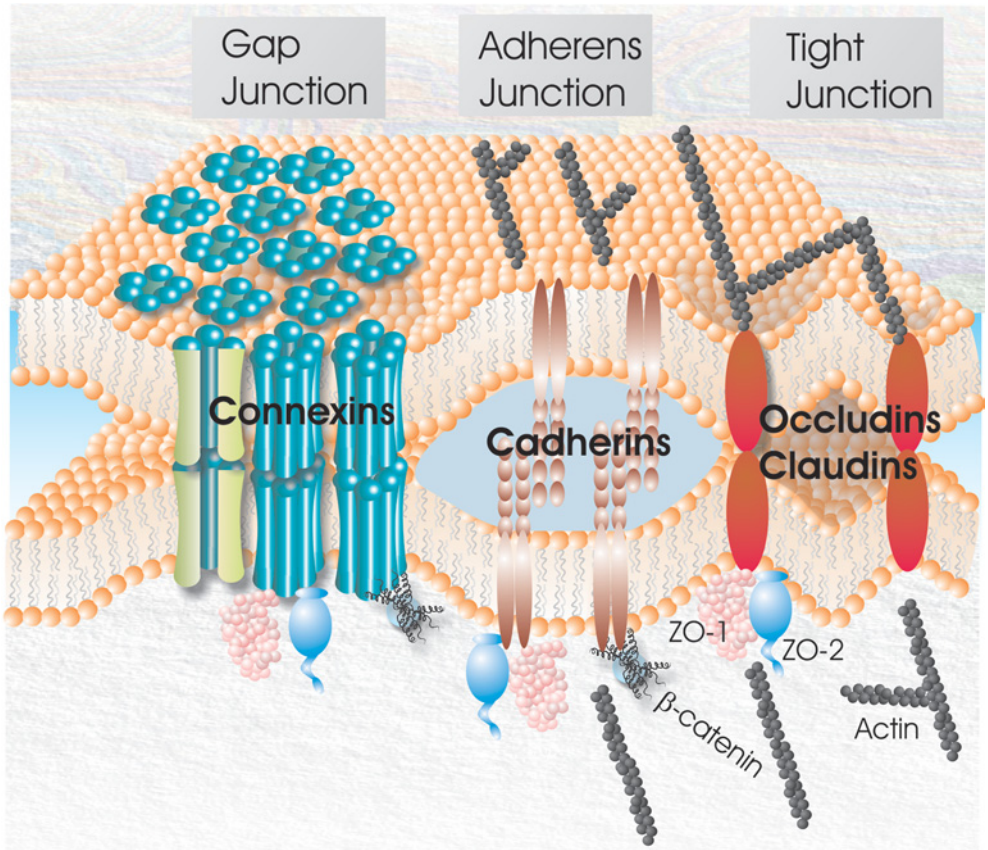

Figure 5 Junctional complexes arranged in a nexus

Gap junctions composed of connexins, adherens junctions consisting of cadherins and tight junctions made up of occludins and claudins are often closely arranged in epithelial cells and share common binding proteins that scaffold to actin and microfilaments. Binding-protein-mediated cross-talk allows these three junctional complexes to act as a nexus and be governed by some common regulatory events.

to caveolin-1 [130], generating further evidence that caveolae may play a role in internalization of Cx43. To date only an E3-ubiquitin ligase, called OCP2 (organ of Corti protein 2), has been reported to bind to Cx26 [171]. Given the evidence that connexins are substrates for ubiquitination, the implication for this interaction could be traced to either gap-junction internalization or degradation.

It is without doubt that more connexin-binding proteins will be discovered in the coming years. While this is a necessary first step, the difficulty comes in assigning clear and definitive functions to these interactions. To date, the vast majority of Cx43-binding proteins can be assigned to putative functions in regulating some part of the life cycle of connexins but attention needs to be given to the possibility that one or more of these interactions could regulate the gating properties of the channels.

CROSS-TALK AMONGST JUNCTIONAL COMPLEXES

Reports of earlier studies frequently described gap junctions as a nexus [172–174]. Although this terminology fell out of favour for a number of years, the discovery of connexin-binding proteins that have properties to potentially interconnect junctional complexes has resulted in the re-emergence of this term [175]. Gap-, tight- and adherens-junctional complexes often share close spatial proximity to each other in epithelial cells (Figure 5). Cx43 gap junctions have been reported to interdigitate with tight junctions near the beginning of the apical domain in polarized thyroid cells [176]. The fact that adherens junctions are thought to mediate gap-junction assembly [104] and, reciprocally, gap junctions mediate the assembly of adherens junctions [105,176], suggests that these two junctional complexes are closely associated. Substantial evidence has now been obtained that suggests that both ZO-1 and ZO-2 can bind to components in all three junctional complexes, further suggesting that there is significant interplay between gap, tight and adherens junctions [150,175,177,178]. Interestingly, it has recently been shown, in ZO-1-null epithelial cells, that ZO-2 may have compensated for the loss of ZO-1, as tight and adherens junctions that assembled appeared indistinguishable from those in wild-type cells [179]. The impact of ZO-1 elimination from these same epithelial cells on gap junctions has not been examined, but earlier competitive studies would predict that gap-junction size and stability would be affected [158,161]. Collectively ZO-1 and ZO-2 act as scaffolding proteins for actin and microfilaments. Such trijunctional links to microfilaments would in turn mandate that cross-talk regulation and interplay that growing evidence now supports. Additional molecules such as β-catenin and drebrin may also serve to facilitate junctional cross-talk. Interestingly, family members of the tight-junction proteins occludins and claudins have been shown to co-localize or co-immunoprecipate with Cx32, further suggesting that tight and gap junctions are intimately arranged in the cell [155,180]. Since these are integral tight-junction proteins, one could speculate that the interactions with Cx32 are indirect and may be a consequence of the junctional nexus. Overall, in order to fully understand the properties and

mechanisms that regulate gap junctions, it will be important to consider the cross-talk regulatory pathways that involve both tight and adherens junctions.

HUMAN DISEASES LINKED TO CONNEXIN MUTATIONS

At present, no fewer than eight distinct human diseases have been definitively linked to germline mutations in connexin family members. These diseases range from the rather common non-syndromic sensorineural deafness [181] to the exceptionally rare oculodentodigital dysplasia (ODDD) [182]. The first discovered connexin-linked human disease was chromosome-X-linked Charcot–Marie–Tooth disease [183] which is clinically manifested by progressive peripheral axon demyelination and limb weakness [184]. Intriguingly, over 270 mutations in Cx32 have been linked to Charcot–Marie–Tooth disease, the majority of which are point mutations that result in aberrant Cx32 trafficking, misassembly of gap-junction channels or abnormal gating properties [185,186]. Because of the broad scope of human diseases associated with connexin mutations, this present review will focus on Cx26 mutations that lead to deafness and, in some cases, a variety of skin diseases, and Cx43 mutations linked to ODDD.

CX26 MUTATIONS IN DEAFNESS AND SKIN DISEASE

It has now been shown that mutations in four connexins (Cx26, Cx30, Cx30.3 and Cx31) give rise to sensorineural hearing loss and hyperproliferative skin disorders [187]. These skin disorders include Vohwinkel's syndrome, keratitis–ichthyosis–deafness (KID), hystrix-like-ichthyosis-deafness (HID), Bart–Pumphrey syndrome and palmoplantar keratodermas (PKKs). Detailed clinical features of these diseases associated with abnormal keratinization and hypertrophy of the corneum, particularly in the palmar and plantar surfaces, are described elsewhere [188–190]. In the cochlea, Cx26 is predicted to play a vital role in potassium recycling [191]. In support of an essential role of Cx26 in the organ of Corti, cochlea-specific Cx26 gene ablation and loss-of-function Cx26 R75W ($Arg^{75} \rightarrow Trp$) transgenic gene knock-in studies resulted in mice with degenerate hair cells and deafness [192,193].

Intriguingly, a mutation in Cx26 may give rise to deafness and skin disease, or just deafness, suggesting that there is a complex interrelationship between functional changes in connexin genotype and the phenotype outcome. Now upwards of 100 missense, nonsense, frame-shift, insertion and deletion mutations in Cx26 have been found to be linked to deafness alone, with over a dozen additional mutations being linked to both deafness and skin disorders [187,188]. Figure 6 highlights over 50 of these mutations, the vast majority of which are missense mutations. Interestingly, the mutations that cause both disease states are located within the first third of the Cx26 molecule (Figure 6) and are typically autosomal dominant, as opposed to the far more common autosomal recessive mutations associated with non-syndromic sensorineural hearing loss [189]. It is tempting to speculate that the first third of the Cx26 molecule plays a critical role in how Cx26 mutants may co-oligomerize with its wild-type counterpart or other members of the connexin family co-expressed in the epidermis.

In order to begin to understand mechanistically how a single Cx26 mutation that causes a loss of molecular function can result in a skin phenotype in addition to deafness, it is necessary to determine how Cx26 mutants may co-exist, and possibly co-oligomerize, with other members of the connexin family. A commonly studied Cx26 missense mutation associated with deafness and PKK is R75W. This mutation results in the formation of non-functional gap-junction-like structures at the cell surface and is dominant over co-expressed wild-type Cx26 [194–197]. This mutant was found to have a trans-dominant inhibitory effect on co-expressed Cx43 [195], which is particularly interesting, since wild-type Cx43 and Cx26 do not appear to co-oligomerize [58]. A second well-studied Cx26 mutant is D66H ($Asp^{66} \rightarrow His$) [198,199], which also results in an inability to form functional gap-junction channels [195,196]. This mutant protein failed to reach, or be stabilized at, the cell surface, and was typically found localized to the *trans*-Golgi network [86]. As with the R75W mutant, the D66H mutant protein inhibited its Cx26 wild-type counterpart as well as endogenous Cx43 [86]. The G59A mutant protein also exhibited properties of being both dominant and trans-dominant with a robust inhibition of wild-type Cx26 and partial trans-dominant inhibitory activity on both Cx32 and Cx43 [86]. Collectively, these examples highlight the fact that there are at least two classifications of Cx26 mutant proteins: ones that reach the cell surface and do not function (i.e. R75W and G59A) and mutant proteins that exhibit a steady-state residence within an intracellular organelle (i.e. D66H). Nevertheless, both classifications of mutant protein can traffic to, or beyond, the intracellular locations where connexin oligomerization is expected to occur and both classifications of mutant proteins have successfully passed the 'quality-control' mechanisms associated with the ER that might target them for ERAD.

It is tempting to speculate that some Cx26 mutants cause both deafness and skin disease because of a mutation-specific increase in the ability of the mutant to interact (i.e. co-oligomerize) with one or more connexin family membranes co-expressed in the epidermis. Such an unfavourable interaction could invoke a loss of, or reduction in, overall GJIC. Although this is a compelling argument, caution must be used in reaching this conclusion. First, all of the studies performed to date have failed to mimic the human disease condition, as it is expected that the mutants are grossly overexpressed, resulting in mutant numbers far exceeding the 1:1 mutant-to-wild-type Cx26 ratio expected *in vivo* when examining autosomal dominant mutations. Secondly, it is possible that mutants and wild-type connexins compete for limited machinery needed to assemble and deliver connexins through the secretory pathway. Essentially, connexin-mutant-overexpressing cells could become inundated with mutant protein, resulting in a number of non-specific effects that could result in a decrease in GJIC. A third restriction may be the saturating level of suitable cell-surface space available for gap-junction formation at sites of cell–cell contact. In some of our studies we have observed that GJIC reaches a maximum and the additional expression of connexin family members fails to increase total GJIC (D. W. Laird, unpublished work). Consequently, although direct interaction between connexin mutants and wild-type connexins remains a viable mechanistic explanation, additional dose-controlled studies in biologically relevant models are necessary to decipher the mechanism involved.

Cx43 MUTATIONS ASSOCIATED WITH ODDD

Previously, the pleiotropic developmental disorder ODDD was found to be linked to autosomal dominant mutations in the gene encoding Cx43 [182]. Patients suffering from this disorder exhibit syndactyly, craniofacial abnormalities, brittle nails, hair abnormalities, conductive hearing loss, lens defects, cornea defects, abnormalities of the teeth and occasional neurological and heart symptoms [182,200]. To date, 28 mutations in Cx43 have been identified, but only one frameshift mutation within the CT tail domain of Cx43 was found which would result in the loss of

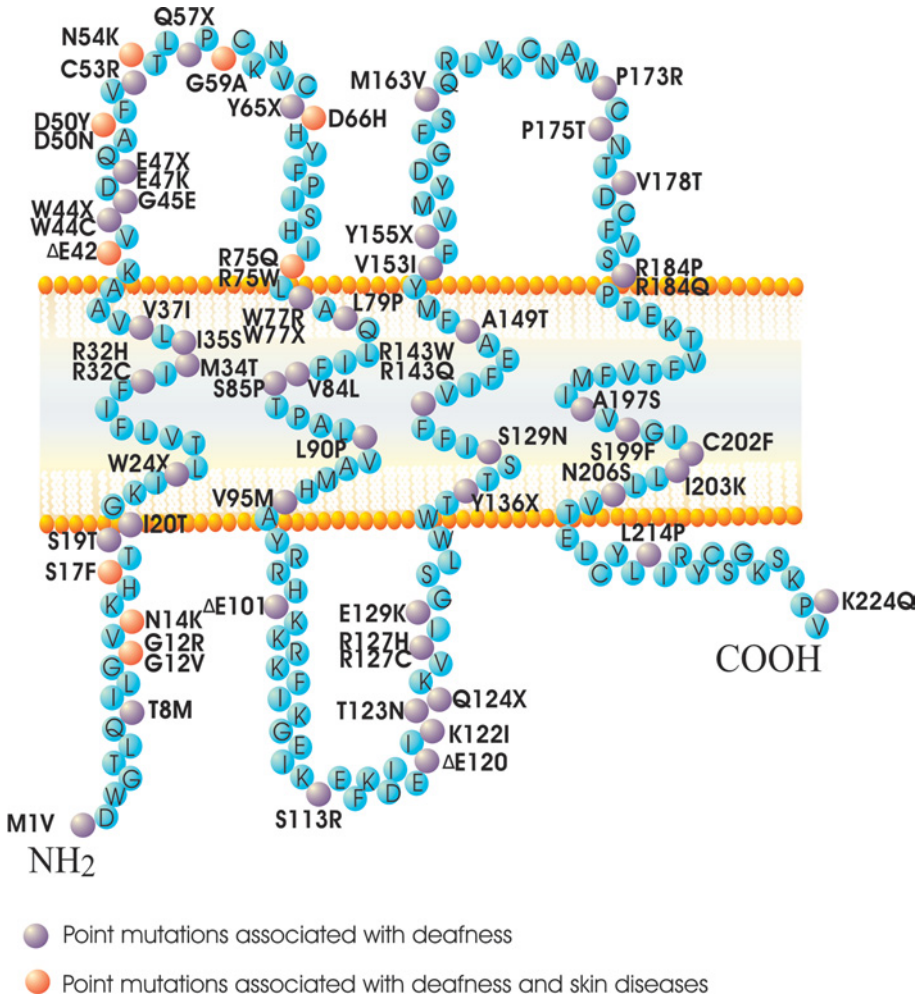

Figure 6 Deafness- and skin-disease-linked Cx26 mutations

Schematic diagram of Cx26 depicting a number of mutations associated with deafness (purple balls) and mutations associated with both deafness and skin diseases (orange balls).

many Cx43 phosphorylation sites and protein-binding domains (Figure 7) [200–207]. Since Cx43 is the most universal connexin found in the human body, it is somewhat remarkable that patients carrying this autosomal dominant mutation in the Cx43 gene are not considerably more ill than they appear to be. Although this disease is rare, it appears that most patients live long lives in relatively good health.

To begin to assess how Cx43 is linked to this pleiomorphic disease, a few studies have now examined the functional outcome of disease-linked missense Cx43 mutations. Collectively, in over-expressing cell models, all mutations examined thus far, including Y17S, G21R, A40V, F52dup (a codon duplication in the first extracellular loop), L90V, I130T, K134E, G138R and R202H, exhibited loss-of-function properties [84,208,209]. Although the majority of these mutant proteins trafficked to the cell surface and assembled into gap-junction-like plaques, F52dup and R202H mutant proteins have been reported to have a diminished capacity to reach the plasma membrane and cluster into plaque-like structures [209]. Since wild-type Cx43 would be expected to co-oligomerize with the co-expressed mutant, it was not surprising to find that several mutants could inhibit the function of co-expressed wild-type Cx43 in cultured cell lines [84,209]. However, at least in the case of the studies using G21R and G138R mutations, the mutant protein was predicted to be in 4–5-fold excess of wild-type Cx43 [84]. However, Shibayama et al. [209] showed that, for some mutants, it was possible to partially rescue their functional status by the presence of wild-type Cx43. Similar to the Cx26 mutants described above, there appears to be two classes of Cx43 mutants, namely mutants that reach the cell surface and assemble into plaque-like structures and mutants that appear to have substantial defects in their ability to efficiently traffic through the secretory pathway.

In order to more fully address the role of Cx43 in ODDD, it was deemed necessary to obtain or generate an animal model of this human disease. Fortuitously, a dominant *N*-ethyl-*N*-nitrosourea mutagenesis approach resulted in the generation of a mouse that exhibited a phenotype comparable with clinically described human ODDD [210]. Careful genetic analysis of this mouse model of ODDD revealed a G60S mutation in the EL-1 domain next to a P59H mutation recently described in humans [211]. The added advantage of examining the role of Cx43 in this model extends not only to physiological relevance, but also to the fact that both mutant and wild-type Cx43 would be expected to be transcribed at a 1:1 ratio, as expected in humans suffering from ODDD. Interestingly, these studies revealed the GJIC status in granulosa cells obtained from ovaries of these mice to be

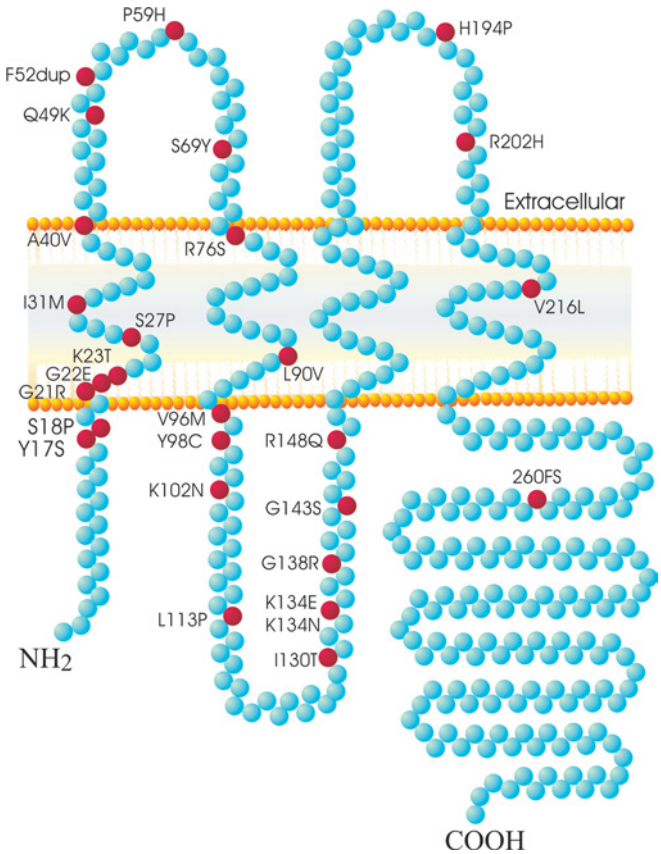

Figure 7 ODDD-linked Cx43 mutations

Schematic diagram of Cx43 depicting the locations of 28 mutations (red balls) linked to ODDD.

approx. 10–20 % normal coupling, suggesting that the mutant was dominantly inhibiting the function of co-expressed wild-type Cx43 [210]. Furthermore, the reduced coupling status in these animals correlated with the loss of total Cx43 protein, further suggesting that the mutant was destabilizing wild-type Cx43 [210]. In the event that these findings translate to humans, it is remarkable that patients with ODDD survive, given the drastic decrease in total Cx43-based GJIC. This finding would argue for extensive compensatory mechanisms being provided by other members of the connexin family, since we know that in mice where Cx43 is ablated, it is a lethal condition [212]. Further studies and additional animal models where mice harbouring true human ODDD-linked mutations will provide further insight into the disease status of patients with ODDD and the role compensatory mechanisms play.

CONCLUDING REMARKS

It is without doubt that the discovery of connexin-linked human diseases has raised the profile of gap-junction biology in the past decade. It is also remarkable that the large family of connexins serve so many essential physiological functions while compensatory mechanisms appear to rescue the mutation-based loss-of-function of some connexin members to minimize the overall damage to human health. The mechanism by which connexin mutants are related to human disease appears to be linked to the essential role of that particular connexin in a given tissue and also the impact of the mutant on other members of the connexin family. Clearly the establishment of additional animal models of human connexin-linked diseases will allow for the mechanisms involved to be dissected in tissue-relevant conditions. There will undeniably be exciting revelations of the function of connexins in health and disease as gap-junction biology expands in the coming years.

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