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Connexin hemichannels and cochlear function

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Abstract

Connexins play vital roles in hearing, including promoting cochlear development and sustaining auditory function in the mature cochlea. Mutations in connexins expressed in the cochlear epithelium, Cx26 and Cx30, cause sensorineural deafness and in the case of Cx26, is one of the most common causes of non-syndromic, hereditary deafness. Connexins function as gap junction channels and as hemichannels, which mediate intercellular and transmembrane signaling, respectively. Both channel configurations can play important, but very different roles in the cochlea. The potential roles connexin hemichannels can play are discussed both in normal cochlear function and in promoting pathogenesis that can lead to hearing loss.

Keywords

hemichannels; connexins; deafness; pannexins; ATP

Introduction

Genetic studies have linked mutations in two connexin (Cx) genes, *GJB2* and *GJB6*, which encode Cx26 and Cx30, respectively, to inherited forms of autosomal, recessive, nonsyndromic sensorineural deafness [1, 2]. Uniquely among ion channels, Cxs can adopt two channel configurations that impact cellular functions in very different ways. One configuration is that of a gap junction (GJ) channel, formed by the docking of two hexameric hemichannels, which functions in direct intercellular signaling. The other configuration is that of an undocked hemichannel, which functions in transmembrane and paracrine signaling.

Mutations in *GJB2* are common and account for as much as 50% of severe-to-profound autosomal, recessive deafness across diverse ethnic populations [3]. These mutations are generally deletions, truncations and frameshifts that result in loss of GJ- and hemichannel-mediated functions. However, there are dominant mutations in *GJB2* linked to syndromes in which deafness is accompanied by infectious and neoplastic skin disorders [4, 5]. Syndromic deafness mutations are generally missense mutations and aberrant hemichannel function is a

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common feature. Since individuals with Cx26 loss-of-function mutations lack skin pathology, Cx26-linked deafness syndromes appear to be gain-of-function disorders. Moreover, the lack of GJ channel function in some syndromic mutants suggests that gain of function pertains to hemichannels.

The cochlear epithelia and expression Cx26 and Cx30

The cochlea has three fluid-filled compartments (Fig. 1), the scala tympani and the scala vestibuli, which contain perilymph similar in composition to plasma and the scala media, which contains endolymph that is high in K^+ and low in Na^+ and Ca^{2+} . The hair cells and the surrounding supporting cells of the sensory organ of Corti are largely bathed in perilymph except at their apical surfaces where they face endolymph. The endolymph-filled scala media also rests at a high positive potential, the endolymphatic potential (EP), which is generated by the specialized epithelia, the stria vascularis, of the lateral wall. The EP is critical for proper signal transduction in hair cells by creating a sufficient driving force for K^+ entry, depolarization and transmitter release onto the central processes of the spiral ganglion neurons, the primary afferents in the auditory pathway.

Immunostaining has shown widespread and overlapping expression of both Cx26 and Cx30 in supporting cells of the sensory epithelium and in the lateral wall; expression is absent in hair cells [6–8]. Although coupling is extensive, there are two independent coupled cellular networks, one comprised of the supporting cells of the organ of Corti and the cells that extend into the spiral ligament and the other comprised of fibrocytes, basal cells and intermediate cells in the lateral wall [9].

Cx26 and Cx30 have been shown to co-localize to the same GJ plaques suggesting that heteromeric channels form. Functional studies that have examined properties of GJ channels in cell lines expressing Cx26 and/or Cx30 as well as in cochlear supporting cells in rodents indicate that Cx26 and Cx30 can and do form heteromeric channels [10–12], although homomeric channels in the cochlea also likely occur. A recent study in human cochlea using super-resolution fluorescence microscopy showed that Cx26 and Cx30 reside in separate GJ plaques in the lateral wall of [13]. Thus, the degree of heteromerization and its function in human cochlea remains unknown.

Cx hemichannels and the cochlear sensory epithelium

Immunofluorescence staining using antibodies directed against the extracellular loops of Cx26 was reported in supporting cells in mouse organotypic cultures, notably in the apical membranes exposed to endolymph [14]. These hemichannels would be exposed to a low “extracellular” Ca^{2+} environment (~20 μ M), a condition that favors hemichannel opening. Functional studies have shown increased uptake of fluorescent dyes in several different supporting cell types isolated from guinea pig cochleae upon exposure to low extracellular Ca^{2+} [15]. Dye uptake was blocked by 18-AGA, a non-specific inhibitor of GJ channels, but not by inhibitors of P2x receptors.

Various roles for hemichannels in the cochlea have been proposed, mainly linked to release of signaling molecules such as ATP and IP3 (summarized in Fig. 1A). Mechanical

stimulation was suggested to induce hemichannel opening linking ATP release to acoustic stimulation-induced basilar membrane vibrations. ATP can dampen the electromotility of outer hair cells through activation of P2 receptors providing a negative feedback mechanism that can regulate hearing sensitivity and protect against cell damage at high sound intensities [16]. Propagation of intercellular Ca^{2+} waves through the cochlear epithelium also was attributed to ATP release through hemichannels [17]. Focally applied ATP in mouse cochlear organotypic cultures was shown to activate purinergic receptors in neighboring cells, initiating Ca^{2+} release and propagated Ca^{2+} waves. Ca^{2+} responses failed to propagate in cultures from mice lacking Cx26, but continued to propagate in cultures from mice lacking P2x7 receptors or pannexin-1 (Panx1) channels. Although the role Ca^{2+} waves play is unclear, Ca^{2+} signaling may affect connexin expression thereby affecting cochlear homeostasis [18]. In addition to ATP, hemichannels were reported to release IP3 [19]. Immunofluorescent labeling showed staining of IP3 receptors in the sensory epithelium both intracellularly and at the cell surface. Release of IP3 increased upon exposure to Ca^{2+} -free solutions and was blocked by several Cx channel inhibitors, but not with the P2x receptor antagonist, PPADS. Because extracellular IP3 can be stable due to a lack of degradative enzymes, it could provide an additional mechanism by which Ca^{2+} signals can be initiated and spread over distances through regenerative release of IP3. Finally, ATP release through hemichannels was suggested to activate inward K^+ currents that promote K^+ entry into supporting cells, thereby potentially functioning to reduce extracellular accumulation of K^+ near hair cells [20].

Cx hemichannels and the lateral wall

The stria vascularis with the lateral wall is responsible for generating the EP and is comprised of a multi-cell layer consisting of fibrocytes, basal cells and intermediate cells and a monolayer of marginal cells whose apical ends face the scala media; an intrastrial space separates the two cell layers (Fig. 1B). Cxs are expressed in fibrocytes, basal cells and intermediate cells and the abundant GJs between these cells establishes an electrical syncytium. Cx expression is lacking in marginal cells. Na^+/K^+ ATPases and $\text{Na}^+/\text{K}^+/\text{Cl}^-$ (NCKK) cotransporters in fibrocytes mediate uptake of K^+ from the perilymph, which diffuses to the basal and intermediate cells via GJs and exits into the intrastrial space via Kir4.1 channels [21]. Tight junctions electrically isolate the intrastrial space from the perilymph, endolymph and capillary blood. The intrastrial space has been shown to have low K^+ concentration and to reside at a high positive potential [22]. K^+ in the intrastrial space is taken up by Na^+/K^+ -ATPases and NCKKs into the marginal cells, which moves into the endolymph via KCNQ1/KCNE1 channels.

An important factor in the net movement of K^+ from perilymph to endolymph is the unusually depolarized membrane potentials of the fibrocytes, basal cells and intermediate cells, which are in the range of 0 mV to +4 mV. This potential contributes to a favorable electrochemical gradient that moves K^+ out of the intermediate cells into the intrastrial space. The origin of this depolarized membrane potential remains unknown, but it is feasible that Cx hemichannels serve this role, although this possibility has not been directly tested. Conditional deletion of Cx26 using different Cre lines showed either little or modest reductions in EP that did not correlate with the degree of hearing loss [23]. Cx30 null mice

showed a substantial reduction in EP [24], but reduced Cx26 expression was also evident in these mice and deafness could be prevented when Cx26 expression was maintained [25, 26]. Thus, maintenance of either Cx26 or Cx30 expression appears to largely preserve the EP. A reduced EP with reductions in expression of both Cxs could result in insufficient numbers of hemichannels to maintain depolarization of the syncytial cells and reduced coupling that could produce deficient metabolic communication and/or signaling. Cx30 null mice also showed possible damage to endothelial cells of blood vessels in the stria vascularis, which would disrupt the electrical isolation of intrastrial space and lead to a collapse of the EP separate from GJ or hemichannel function [27].

Cx hemichannels and the developing cochlea

Prior to the onset of hearing, inner hair cells in rodent cochleae release glutamate onto the processes of spiral ganglion neurons suggesting the presence of spontaneous activity, which can support neuronal survival and guide wiring of the auditory pathways [28]. Bursts of spontaneous spiking in spiral ganglion neurons were attributed to ATP released from supporting cells that, in turn, depolarized inner hair cells to cause glutamate release. Moreover, spontaneous ATP release could be enhanced by low extracellular Ca^{2+} and blocked by Cx channel inhibitors octanol and carbenoxolone. ATP release elicited synchronized activities of neighboring inner hair cells suggesting those encoding similar frequencies could be synchronized, helping refine the tonotopic segregation of neuronal projections in auditory pathways.

An alternative view as to the origin of spontaneous activity suggests that currents through the transduction channels in the apical membranes of hair cells, enhanced by the low- Ca^{2+} environment of the endolymph, can provide a sufficient depolarizing influence [29]. It has also been suggested that ATP concentrations produced by release from supporting cells may be insufficient to generate a depolarizing response [18]. In fact, endogenous levels of ATP around inner hair cells were shown to have a hyperpolarizing effect through the activation of SK type K^+ channels that may be linked to P2x3 receptors, which respond to nanomolar concentrations of ATP [30]. P2x3 receptors show an apical-to-basal gradient of expression in the cochlea [31], which could explain tonotopic differences in inner hair cell activity observed along the cochlea [30]. Thus, ATP release from hemichannels in supporting cells may not be necessary for spontaneous activity in hair cells, but could still play an important role in modulating electrical activity, thereby contributing to the refining of the auditory neural circuits in the brainstem.

Conditional deletion of Cx26 was also shown to have effects on cochlear development and to impair postnatal development of the organ of Corti (reviewed in [32]). Whether hemichannels specifically contribute to early cochlear development is unknown.

Enter Pannexins

Pannexins function as membrane channels with a similar capacity as Cx hemichannels to mediate the transmembrane flux of larger molecules such as ATP [33]. Although they bear no sequence similarity to Cxs, pannexins and Cxs adopt similar membrane topologies and

are inhibited by many of the same pharmacological agents suggesting they share common structural motifs. Thus, attributing dye uptake and release of ATP to Cx hemichannels based pharmacological inhibition can be problematic [34]. This issue now affects interpretation of previous data in the cochlea with pannexins, particularly Panx1, which is expressed in some supporting cells in the sensory epithelium and in the lateral wall [35].

Using a Panx1 cKO mouse and a foxg1-Cre mouse line to delete Panx1 [36], ATP release was shown to be substantially reduced in tissue extracted from the lateral wall. Cx26 cKO mice generated with pax2-Cre as well as Cx30 KO mice showed no changes in ATP release. However, previous examination of Cx26 cKO mice generated using pax-2 Cre showed no statistical difference in Cx26 protein expression in the stria vascularis [37]. The lack of an effect on ATP release in mice null for Cx30, which also show reduced levels of Cx26 [25], argues in favor of Panx1 as the principle source of ATP release in the lateral wall.

Additional considerations for assessing contributions of Panx1 channels and Cx hemichannels in cochlea include the reported increases in ATP release and dye uptake in cochlear supporting cells with reduced extracellular Ca^{2+} [16]. Open probability of Cx hemichannels, but not Panx1 channels, increases in low extracellular Ca^{2+} [38]. However, Panx1 can exhibit Ca^{2+} sensitivity indirectly through its activation by P2x7 receptors, but is inconsistent with the lack of an effect of P2x channel blockers in ATP-mediated Ca^{2+} signaling. Also, Ca^{2+} responses in supporting cells failed to propagate in cochlear organotypic cultures from mice lacking Cx26 expression, but continued to propagate in cultures from mice globally null for Panx1 or P2x7 receptors [17]. Of note, Cx hemichannels can be activated at physiological Ca^{2+} levels in the 1–2 mM range [39]. Thus, various conditions may activate Cx hemichannels, separate from reductions in extracellular Ca^{2+} .

A final point on pannexins in the cochlea, conditional deletion of Panx1 using mice from KOMP (UC Davis, CA) and foxg1-Cre or pax2-Cre mouse line to drive deletion was reported to cause moderate to severe hearing loss [40]. However, another study found no hearing deficits in Panx1 null mice, generated by Genentech (San Francisco, CA) and a ubiquitously active Cre deleter mouse to achieve widespread deletion of Panx1 [41]. Global deletion of Panx1 did not alter expression of Cx26 or Cx30. Thus, unlike Cx26, in which all mouse models agree that Cx26 is essential for hearing, the importance of Panx1 in hearing requires further examination.

Syndromic deafness mutations and hemichannels

There is a growing list of missense mutations in Cx26 associated with syndromic deafness in which aberrant hemichannel function is a common feature [5, 42]. The most studied Cx-linked deafness syndrome is keratitis-ichthyosis-deafness (KID). Mutations at 8 positions in Cx26 have been identified [42] and most of them cluster in the N-terminal domain (NT) and at the border of the first transmembrane (TM1) and first extracellular loop (E1) domains (Fig. 2A), which contribute to the aqueous pore and to channel gating and regulation by voltage, Ca^{2+} and pH [43]. When superimposed on the crystal structure of Cx26 [44], KID mutations localize to the pore and to interfaces between subunits (Figs. 2B).

Several KID mutations have been shown to weaken Cx26 hemichannel inhibition by extracellular Ca^{2+} , suggesting a mechanism for cellular dysfunction caused by excessive hemichannel opening. However, the G45E mutation, which is associated with a fatal form of KID syndrome [45], maintains robust Ca^{2+} inhibition [46]. Biophysical studies demonstrated that G45 is a pore-lining residue and that the G45E mutation increases hemichannel permeability to Ca^{2+} [46]. This gain-of-function provides a compelling explanation for the serious phenotype G45E patients' experience, namely excessive Ca^{2+} entry leading to cell dysfunction and/or death triggered by Ca^{2+} -dependent signaling cascades.

Using substituted cysteine accessibility, several KID mutations, like G45E, have been shown to occur at pore-lining residues [46–48], suggesting that altered hemichannel permeability may be a common mechanism of disease pathogenesis. Effects on permeability would depend on the nature of the mutation, which together with other aberrant hemichannel properties could result in heterogeneous phenotypes among patients carrying different KID mutations.

Aberrant Cx26 hemichannel function has not been explicitly tested in the cochlea. Although the lack of skin disorders with loss of Cx26 implicates a gain of function in syndromic deafness, both loss and gain of Cx26 function could be detrimental to hearing. Loss of Cx26 function is a leading cause of nonsyndromic deafness, but it is unknown whether loss of hemichannel function is a contributing factor. In syndromic cases, gain of hemichannel function is a possible contributor to hearing loss, but some mutants function as GJ channels, which also may behave aberrantly and contribute to disease. Mutants that do not function as homomeric GJ channels may function when co-expressed with WT Cx26 and/or with Cx30. Aberrant heteromization has been described in the S17F KID syndrome mutant, which leads to gain of function via heteromeric hemichannels that normally do not form [49].

Conclusions

Several roles for Cx hemichannels in the cochlea have been proposed, mainly linked to ATP release from supporting cells. The more recent reports of Panx1 expression in supporting cells requires re-analyses of the origins of ATP release. Reports of the effects of deleting Panx1 in mice, both with respect to ATP release and hearing loss, are not in agreement and may be due to differences in the mouse lines and the mechanisms used to generate Panx1 and Cx26 KO animals. In disease, a convincing argument can be made for aberrantly-functioning Cx26 hemichannels as contributors to hearing loss, whether or not hemichannels have a significant role to play physiologically. Unequivocal experimental evidence for aberrant hemichannels in hearing loss, however, is lacking and awaits the development of specific tools to distinguish contributions of hemichannels, GJs and pannexin channels in cochlear physiology and pathology.

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Highlights

- Connexin hemichannels composed of Cx26 and/or Cx30 have been proposed to play multiple important roles in developing and mature cochlea
- ATP release by hemichannels may play a prominent role in cochlear function, but controversy has emerged between hemichannels and Panx1 channels as principal contributors to ATP release
- Aberrant Cx26 hemichannel function likely contributes to cochlear pathogenesis in syndromic sensorineural deafness

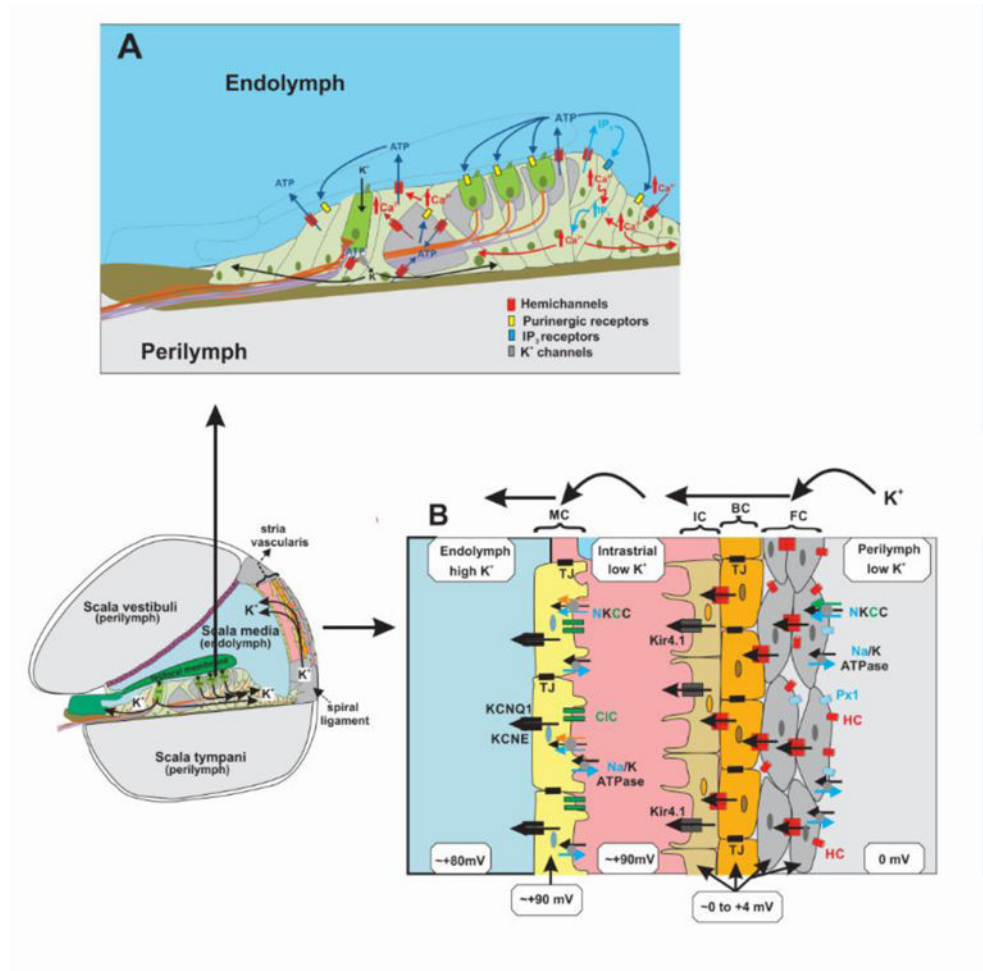


Figure 1. Illustrations of the cochlear ducts and epithelial cell networks depicting proposed functions of hemichannels. A) Expanded view of the sensory epithelium. Hemichannels in supporting cells can release ATP to modulate hair cell electromotility, activate K⁺ channels, reduce K⁺ accumulation and initiate Ca²⁺ waves. Ca²⁺ signaling could also involve hemichannel-mediated IP₃ release and direct Ca²⁺ entry. B) Expanded view of the stria vascularis in the lateral wall. Hemichannels can depolarize the multi-cell layer consisting of fibrocytes (FC), basal cells (BC) and intermediate cells (IC). These cells are separated from the marginal cells (MC) by an electrically-insulated intrastrial space. Shown are the key pumps, transporters, ion channels, the potentials and K⁺ concentrations of the cellular and extracellular compartments that move K⁺ from perilymph to endolymph; Na/K ATPases, N/K/Cl (NKCC) co-transporters, Cl⁻ channels (CIC), Kir4.1 and KCNQ1/KCNE K⁺ channels, Cx hemichannels (HC) and gap junctions (GJs), Panx1 channels (Px1) and tight junctions (TJs).

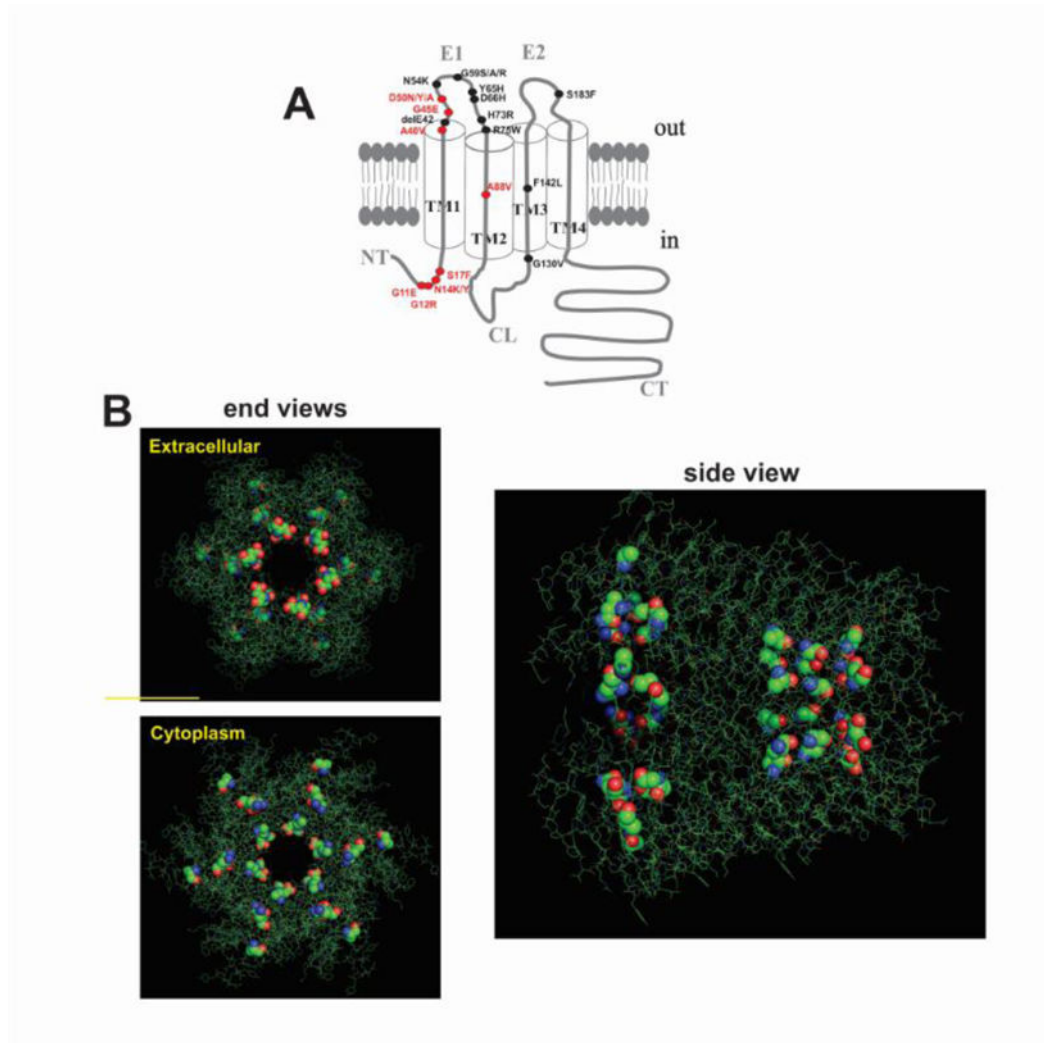


Figure 2. Cx26 syndromic deafness mutants. A) Topology of a Cx subunit consisting of four transmembrane domains (TM1-TM4), two extracellular loops (E1, E2), a cytoplasmic loop (CL) and amino (NT) and carboxy (CT) termini. Denoted are the 18 residues with mutations causing syndromic deafness; those associated with KID syndrome are indicated in red. B) Representation of six subunits (in green) around a central aqueous pore from the atomic structure of Cx26 (Protein Data Bank accession no. 2ZW3) published by [44]. The structure is displayed using the PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC. KID mutant residues are shown as spherical renditions. Shown are end views from the extracellular and cytoplasmic sides and a side view. The KID mutations often occur at residues facing the pore and at subunit interfaces.