Title

Recent insights into gap junction biogenesis in the cochlea

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Abstract

In the cochlea, connexin 26 (Cx26) and connexin 30 (Cx30) co-assemble into two types of homomeric and heteromeric gap junctions between adjacent non-sensory epithelial cells. These channels provide a mechanical coupling between connected cells, and their activity is critical to maintain cochlear homeostasis. Many of the mutations in *GJB2* or *GJB6*, which encode CX26 and CX30 in humans, impair the formation of membrane channels and cause autosomal syndromic and non-syndromic hearing loss. Thus, deciphering the connexin trafficking pathways *in situ* should represent a major step forward in understanding the pathogenic significance of many of these mutations. A growing body of evidence now suggests that Cx26/Cx30 heteromeric and Cx30 homomeric channels display distinct assembly mechanisms. Here we review the most recent advances that have been made towards unravelling the biogenesis and stability of these gap junctions in the cochlea.

1. Introduction

Hearing loss is the most common congenital sensory impairment. About 1–3 in 1000 children are affected at birth or during early childhood by severe deafness, which is defined as prelingual hearing loss, with at least half of all cases attributable to genetic causes.[1] Mutations in *GJB2* and *GJB6*, which encode connexins 26 and 30 (CX26 and CX30) involved in inner ear homeostasis, are found in patients with autosomal dominant or recessive non-syndromic hearing loss.[2-4] Besides these non-syndromic forms of deafness, *GJB2* and *GJB6* mutations also cause several types of skin disorders which are associated or not with hearing deficits.[5] In mammals, sounds are perceived through mechanosensory hair cells located within the sensory epithelium of the cochlea (i.e. the organ of Corti). Within the organ of Corti, sensory inner and outer hair cells, and non-sensory supporting cells are organized in a regular mosaic pattern that extends along the basal-to-apical axis of the cochlear duct. Cx26 and Cx30 gap junction proteins are believed to promote the rapid removal of K^+ away from the base of sensory hair cells, resulting in the recycling of this ion back to the endolymph to maintain cochlear homeostasis.[6,7] However, gap junctions may serve additional roles in the cochlea. There is evidence proving that intercellular fluxes of second messengers such as inositol phosphates and Ca^{2+} ions may regulate cochlear physiology. Indeed, an impaired transfer of the Ca^{2+}-mobilizing molecule inositol 1,4,5-trisphosphate has been suggested as a cause of recessive deafness due to a specific *GJB2* mutation.[8] In addition, gap junctions within the cochlear sensory epithelium of immature mice are permeable to fluorescent analogues of D-glucose,[9] pointing to a role for connexins in the transport of energy substrates.

In the cochlea, Cx26 and Cx30 co-assemble into two types of gap junctions, which form a syncytium extending from the spiral limbus to the cochlear spiral ligament. On the one hand, Cx30 mainly forms homomeric channels between adjacent Deiters' cells, i.e. the supporting cells which surround the outer sensory hair cells.[10-12] On the other hand, Cx30 co-assembles with Cx26 into heteromeric channels which connect the other supporting cell types (**Figure 1**).[12,13] Why Deiters' cells are preferentially connected via Cx30 homomeric channels still remains unclear. However, it is worth noting here that Cx30-mediated intercellular communication would be involved in epithelial repair following the loss of sensory hair cells.[10] In the organ of Corti, the outer hair cells are often more vulnerable than inner hair cells, and also more susceptible to die.[14] Lesions caused by loss of sensory hair cells are closed by the supporting cells that surround each one. In mice lacking Cx30, only one, rather than all, of the supporting cells that contacted an individual dying hair cell closes the lesion, indicating a dysregulation of cellular repair responses.[10] These observations suggest that

intercellular communication via Cx30 homomeric channels between Deiters' cells, in direct vicinity of the outer hair cells, could be needed to ensure epithelial repair in the case of sensory hair cell death.

Permeability studies using HeLa cells co-transfected with Cx26 and Cx30 revealed that heteromeric channels transfer both cations and anions, unlike Cx30 homomeric channels, which can transfer cationic tracers only.[15] Moreover, cationic dyes diffuse farther in cells co-expressing Cx26 and Cx30 than in cells expressing Cx26 or Cx30 alone.[15] In the same vein, Sun et al.[12] have shown that cochlear gap junction channels co-assembled from Cx26 and Cx30 show faster intercellular Ca^{2+} signalling than homomeric counterparts. Overall, these data provide evidence that Cx26/Cx30 heteromeric channels exhibit specific selectivity and biophysical properties. Little is known about the physiological control of gap junction states (« open » versus « closed » conformation) in the cochlea. However, there is evidence proving that gap junctional conductance between adjacent cochlear supporting cells is voltage-dependent[16,17] and can be regulated by intrinsic factors such as nitric oxide[18] or turgor pressure.[19] Moreover, Todt et al.[20] have shown that hydrogen peroxide inhibits gap junctional coupling in the cochlea. This observation could be related to pathophysiological conditions such as noise-induced hearing loss, aminoglycoside-related ototoxicity and presbycusis, which are known to be associated with production of free radicals.

Current knowledge suggests that gap junction biogenesis usually occurs in a kind of "two-step mechanism", which requires successively microtubules and actin cytoskeletal components. First, hexameric connexons assembled in the *trans*-Golgi network are trafficked along microtubules to non-junctional plasma membrane.[21,22] Secondly, hemichannels associate with cortical actin through actin-binding proteins zonula occludens which regulate delivery of connexins from the periphery to pre-existing gap junction plaque (GJP).[23,24] This peripheral membrane region containing non-junctional hemichannels and surrounding the GJP is called "perinexus".[25] Besides mutations that affect the channel function itself, many of the disease-causing mutations in *GJB2* or *GJB6* impair the trafficking and assembly of Cx26 and Cx30, what prevents the formation of gap junctions.[5,26,27] A growing body of evidence now suggests that Cx26/Cx30 heteromeric and Cx30 homomeric channels display distinct assembly mechanisms. Here we review the most recent advances that have been made towards understanding the biogenesis and stability of these gap junctions in the cochlea. These findings could represent a step toward unravelling the pathogenic significance of many of these mutations.

2.1. Biogenesis and stability of Cx26/Cx30 heteromeric channels between adjacent inner sulcus cells

With the exception of Deiters' cells, Cx26 and Cx30 mostly co-assemble into large gap junction plaques which connect adjacent non-sensory supporting cells.[12,13] Amongst these cells are the inner sulcus cells, which are located medially to the inner hair cell layer (**Figure 1**). It is worth noting here that Cx26 and Cx30 are not functionally equivalent. *GJB2* and *GJB6*, which encode CX26 and CX30 respectively, are two contiguous genes that are found within 50kb in the same DFNB1 deafness locus on chromosome 13.[2] Therefore, *GJB2* and *GJB6* are co-regulated and display extensive overlapping expressions in the cochlea.[28] Although there is robust evidence for the direct involvement of Cx26 in cochlear functions, the contribution of Cx30 is unclear since deletion of Cx30 strongly downregulates Cx26 in both humans and mice.[28-31] Moreover, several studies report that mutations in *GJB2* have trans-dominant negative effects on Cx30.[32-36] Loss of Cx26 leads to drastic reduction in Cx30 GJP area and is associated with excessive endocytosis in inner suclus cells.[37] Together, these findings led to consider Cx26 as the key organizer of the Cx26/Cx30 gap junction macromolecular complex.

Whereas gap junctions usually assemble into lipid rafts domains,[38] we observed that Cx26/Cx30 GJPs are fully devoid of lipid rafts and actin filaments. This observation is consistent with the low affinity of Cx26 for cholesterol, which is abundantly present in lipid raft fractions.[39,40] In contrast, both actin filaments and lipid rafts are enriched at tricellular junctions, i.e. at the crossroad between three adjacent GJPs.[41,42] Since lipid rafts are frequently the site of cell surface delivery of membrane proteins,[43] we hypothesized that lipid raft-associated tricellular junctions could promote cell surface delivery of Cx26/Cx30 oligomers. Indeed, tricellular junctions are usually enriched in a variety of adhesion molecules,[44,45] among which are cadherins.[46,47] These proteins are known to regulate microtubule dynamics and to promote microtubule anchoring at the cell-cell border.[48-50] The combined activity of cadherins and microtubules would be consistent with findings showing that Cx26/Cx30 GJP formation in the cochlea strongly relies on an intact microtubule network.[41] We observed that N-cadherin associates with lipid rafts at tricellular junction sites. In addition, the cadherin-associated submembrane microtubule network, presumably involved in delivering Cx26/Cx30 oligomers to the cell surface, was exclusively present in the immediate vicinity of N-cadherin-containing tricellular junctions. Using an *in situ* proximity ligation assay, we detected the presence of Cx26/Cx30 oligomers within lipid raft-enriched tricellular junctions. These findings were consistent with previous *in vitro* data showing the

presence of non-junctional Cx26 within lipid rafts, whereas Cx26-containing channels were excluded from lipid raft fractions. On the basis of these results, the authors suggested that lipid rafts may be involved in trafficking non-junctional membrane Cx26 to non-lipid raft GJPs.[51]

Because of the relatively short half-life of connexins (usually 1–5h), the GJP is in a dynamic state, constantly remodeled through both recruitment of newly synthesized hemichannels to the outer periphery of the GJP and endocytosis of older components from the center of the plaque.[21,52] In agreement with the latter model, we could suggest that, owing to low affinity of Cx26 for cholesterol abundantly present in lipid rafts,[39,40] Cx26/Cx30 hemichannels rapidly diffuse laterally out of lipid raft-enriched tricellular junctions and accrue along the outer periphery of the pre-existing GJP, where each hemichannel docks with another from the neighbouring cell to form a new heteromeric channel. The inhibition of N-cadherin-based homophilic interactions induced a significant reduction in the summed Cx26/Cx30 GJP length per inner sulcus cell and a concomitant accumulation of Cx26/Cx30 oligomers within the cytoplasm.[42] These data support that cadherin-based tricellular adherens junctions promote the microtubule-mediated trafficking of Cx26/Cx30 oligomers to the cell surface and further assembly into GJPs (**Figure 2**). That said, we cannot rule out the possibility that Cx26/Cx30 hemichannels could also be delivered at other sites of the plasma membrane.

Very few proteins have been shown to interact with Cx26/Cx30 channels in the cochlea. Recently, the transmembrane protein TMEM43 was found to associate with Cx26 and Cx30 gap junction proteins in cochlear non-sensory supporting cells.[53] The authors identified a nonsense *TMEM43* variant (p.Arg372Ter) in two Asian families, which leads to disruption of connexin-linked function and autosomal dominant auditory spectrum disorder. *In vivo*, this variant does not affect the biogenesis or stability of Cx26/Cx30 GJPs, but it disrupts prehearing supporting cell conductance, mediated primarily by gap junctions.[53]

2.2. Biogenesis and stability of Cx30 homomeric channels between adjacent Deiters' cells

Unlike most cochlear non-sensory supporting cell types, Deiters' cells are mainly connected via Cx30 homomeric channels.[10-12] Once they have been delivered to non-junctional plasma membrane, connexin hemichannels usually associate with cortical actin filaments and/or with actin-binding proteins which regulate delivery of connexins from the periphery to pre-existing GJPs. In this sense, we observed that non-junctional Cx30 hemichannels mostly interact with ß-actin isoforms in the perinexus of the GJP. An *in vitro* organotypic model revealed that

Cx30 hemichannels strongly rely on the actin network for gap junction biogenesis and stability (**Figure 3**).[54]

Having explored the role of cytoskeletal components, we identified the transmembrane protein ephrin-B2, which belongs to the Eph/ephrin family, as a potential candidate for regulating Cx30 gap junction biogenesis and stability. In humans, *EFNB2* haploinsufficiency has been shown to cause a syndromic neurodevelopmental disorder including a progressive sensorineural hearing loss beginning in the first decades.[55] Eph receptors and their membrane-bound ligands are both divided into two A and B classes on the basis of sequence homology and binding affinity. There is now evidence proving that the Eph/ephrin system plays a leading role in the development and function of the cochlea in mammals.[56,57] Among a large number of physiological and pathological processes, Eph and ephrin proteins have been shown to control gap junction communication.[58,59] An *Efnb2*$^{+/H2BGFP}$ gene reporter mouse model, in which a nuclear *H2BGFP* gene, has been knocked into the *Efnb2* locus, revealed that ephrin-B2 is weakly expressed in sensory hair cells, but is broadly expressed in non-sensory supporting cell types.[60] This expression profile, distinguishing sensory and non-sensory supporting cells, made this protein a candidate for regulating cochlear gap junctions.

Our recent observations suggest that ephrin-B2 promotes the assembly of Cx30 GJPs into caveolae membrane domains between adjacent non-sensory Deiters' cells. Most connexins interact with components of caveolae membrane domains, such as caveolin proteins, which in turn regulate gap junction communication.[61,62] An *in situ* proximity ligation assay revealed that ephrin-B2 preferentially interacts with Cx30 in the periphery of the GJPs, i.e. where newly synthesized connexin hemichannels accrue to the GJP. Moreover, we observed that heterozygous mice encoding an *Efnb2* null allele display excessive clathrin-mediated internalization of Cx30 GJPs in early postnatal stages. Finally, an *in vitro* organotypic assay revealed that ectopic activation of ephrin-B2 reverse signalling promotes the internalization of Cx30 GJPs. These data argue in favor of a cell-autonomous, Eph receptor-independent role of ephrin-B2 in the assembly and sequestration of Cx30 GJPs into caveolae membrane domains (**Figure 3**).[63] Recently, Tajima et al.[64] have shown that degradation of GJPs is a key event in the progressive development of age-related hearing loss in mice. Thus, an early GJP disassembly and endocytosis could certainly play a role in the pathogenic process leading to progressive sensorineural hearing loss due to *Efnb2*/*EFNB2* haploinsufficiency.

3. Conclusion

Over the past decades, several studies have been carried out to decipher the trafficking pathways of Cx26 and Cx30. However, a large majority of them reported data obtained in a variety of cell lines, using transfection of wild-type or fusion proteins in many cases.[65-68] Unsurprisingly, the findings presented here differ somewhat from these observations. It must be admitted that the behavior of fusion proteins *in vitro* could be very different to the one of equivalent wild-type proteins in native tissues. In this sense, Stout et al.[67] have shown that not only connexin type, but also fluorescent protein fusion tag determine structural stability of GJPs.

Our recent observations suggest that gap junction biogenesis in the cochlear sensory epithelium differs according to whether connexons are composed of Cx26 and Cx30, or composed of Cx30 only. Although important advances have been made, several questions remain about the regulation of gap junction dynamics in the cochlea. In this context, further studies will be needed to address connexin life cycle in normal and pathological conditions *in situ*.

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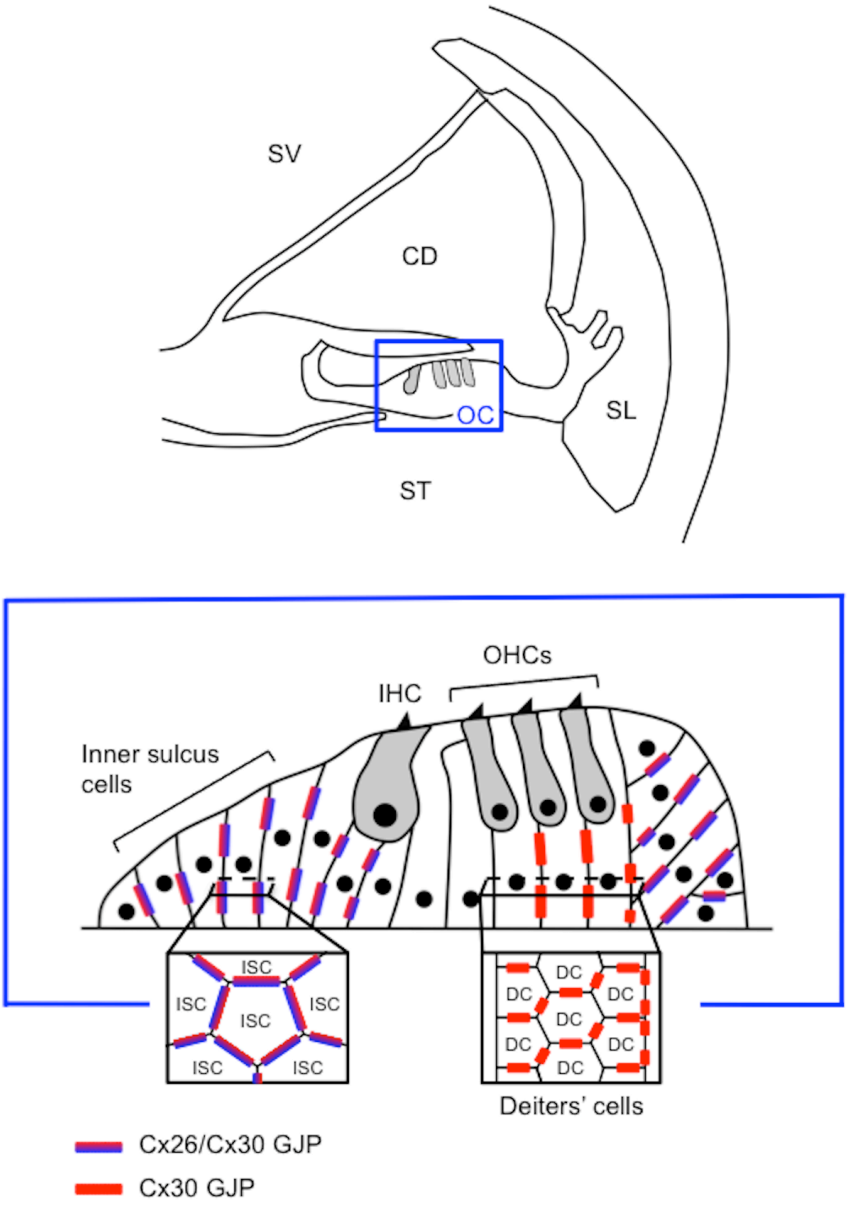

Figure 1. Schematic distribution of gap junction channels in the sensory epithelium of the cochlea. Upper panel: schematic representation of a cross-section through a single cochlear turn of a newborn mouse cochlea. The blue square delineates the organ of Corti. Lower panel: schematic representation of gap junction channel distribution between adjacent non-sensory epithelial cells within and adjacent to the organ of Corti. CD = cochlear duct; DC = Deiters' cell; GJP = gap junction plaque; IHC = inner hair cell; ISC = inner sulcus cell; OC = organ of Corti; OHCs = outer hair cells; SL = spiral ligament; ST = scala tympani; SV = scala vestibuli.

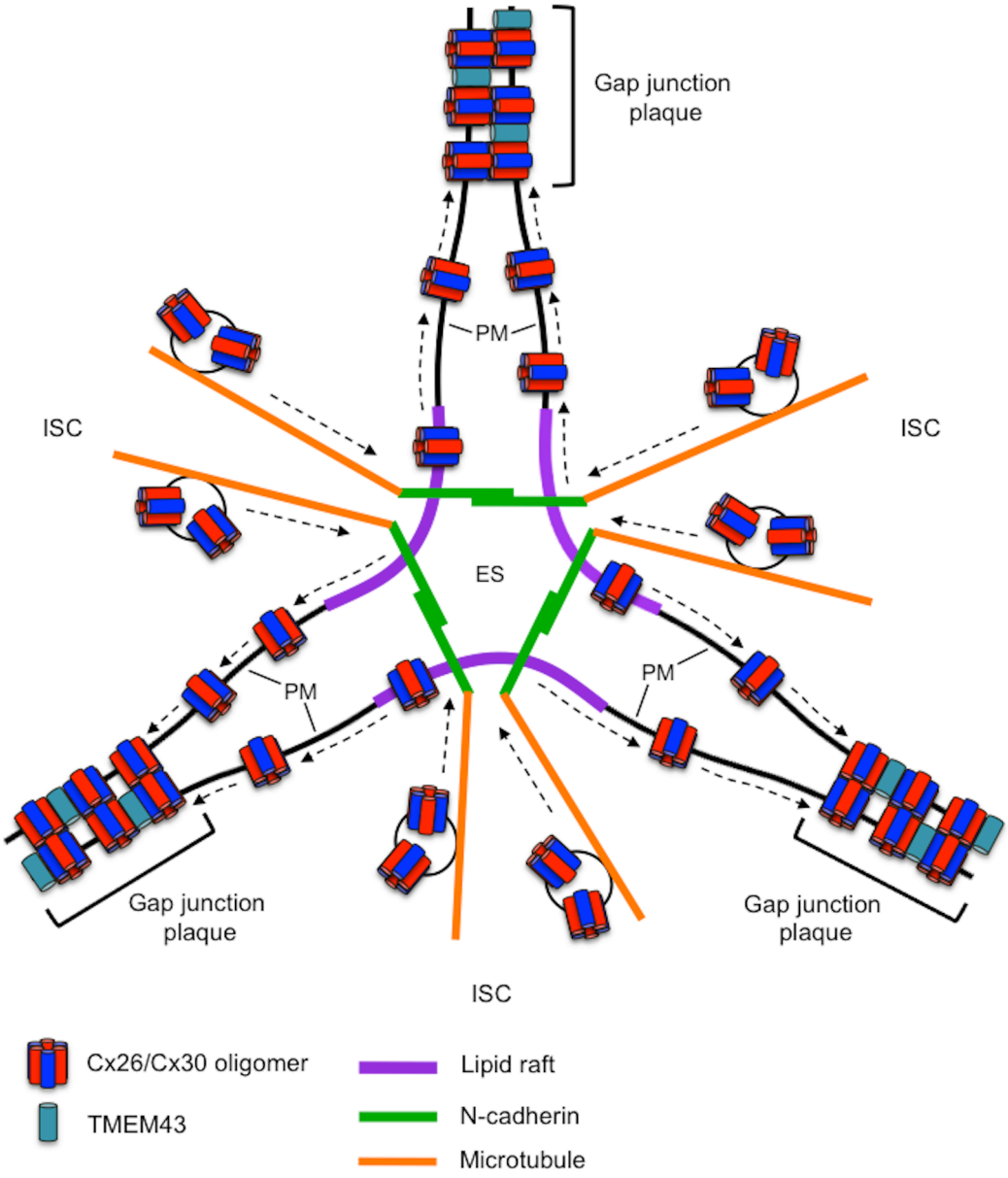

Figure 2. A model for how Cx26/Cx30 oligomers co-assemble into GJPs between adjacent inner sulcus cells. Cx26/Cx30 oligomers travel along microtubules and are delivered to the cell surface at lipid raft-enriched tricellular adherens junction sites. Owing to low affinity of Cx26 for cholesterol abundantly present within lipid rafts, Cx26/Cx30 hemichannels likely rapidly diffuse laterally out of lipid raft-enriched tricellular junction sites and accrue along the outer periphery of a pre-existing GJP, where each hemichannel docks with another from the neighbouring cell to form a new heteromeric channel. Within GJPs, the transmembrane protein TMEM43 interacts with Cx26 and Cx30. ES = extracellular space; ISC = inner sulcus cell; PM = plasma membrane.

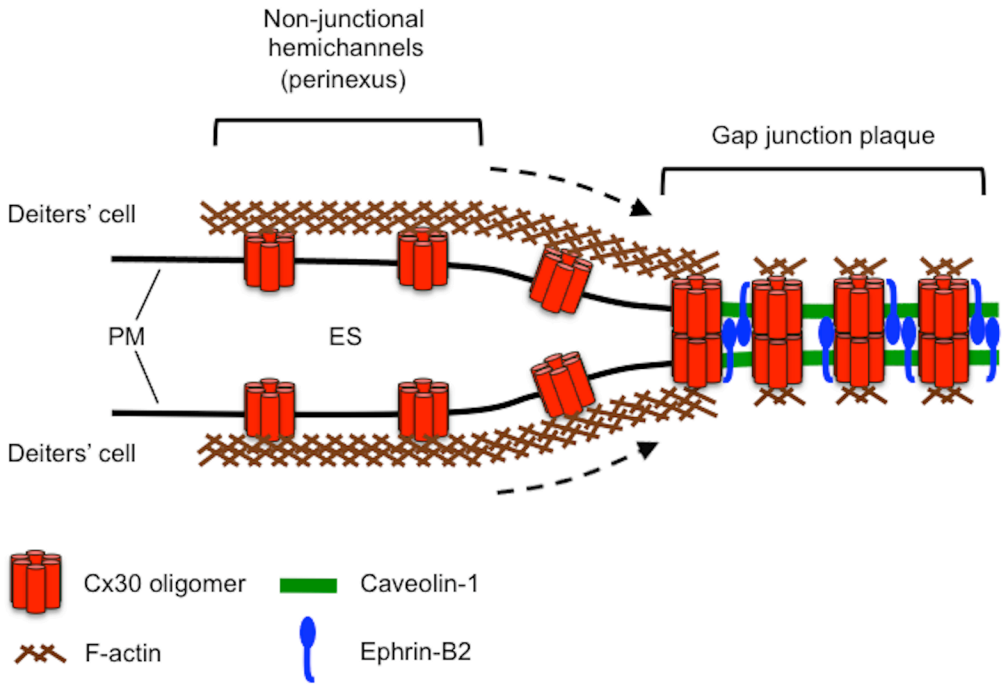

Figure 3. A model for how Cx30 oligomers co-assemble into GJPs between adjacent Deiters' cells. Cx30 hemichannels associate with the F-actin network in the perijunctional region of the pre-existing GJP. The F-actin network likely promotes the lateral diffusion and the recruitment of hemichannels from the periphery to the lateral edge of the pre-exisiting GJP. At the borders and within the GJP, ephrin-B2 interacts with Cx30 channels to promote their sequestration into caveolae membrane domains. ES = extracellular space; PM = plasma membrane.