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Short communication

Tricellular adherens junctions provide a cell surface delivery platform for connexin 26/30 oligomers in the cochlea

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ABSTRACT

In the cochlea, connexins 26 (Cx26) and 30 (Cx30) largely co-assemble into heteromeric gap junctions, which connect adjacent non-sensory epithelial cells. These channels are believed to ensure the rapid removal of K^+ away from the base of sensory hair cells, resulting in K^+ recycling back to the endolymph to maintain cochlear homeostasis. Many of the mutations in *GJB2* and *GJB6*, which encode CX26 and CX30, impair the formation of membrane channels and cause autosomal hearing loss in humans. Although recent advances have been made, several important questions remain about connexin trafficking and gap junction biogenesis. Here we show that tricellular adherens junctions present at the crossroad between adjacent gap junction plaques, provide an unexpected cell surface delivery platform for Cx26/Cx30 oligomers. Using an *in situ* proximity ligation assay, we detected the presence of non-junctional Cx26/Cx30 oligomers within lipid raft-enriched tricellular junction sites. In addition, we observed that cadherin homophilic interactions are critically involved in microtubule-mediated trafficking of Cx26/Cx30 oligomers to the cell surface. Overall, our results unveil an unexpected role for tricellular junctions in the trafficking and assembly of membrane channels.

1. Introduction

Hearing loss is the most common congenital sensory deficit. About 1–3 in 1000 children are affected at birth or during early childhood by severe hearing loss, which is defined as prelingual deafness, with at least half of all cases attributable to genetic causes (Korver et al., 2017). 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 (del Castillo et al., 2002; Grifa et al., 1999; Kelsell et al., 1997). Beside 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 (Xu and Nicholson, 2013). 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 ensure 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 (Zdebik et al., 2009). However, gap junctions may serve additional roles in the cochlea. There is increasing evidence that intercellular fluxes of second messengers such as inositol phosphates and Ca^{2+} ions may regulate cochlear physiology. Indeed, the 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 (Beltramello et al., 2005). In addition, gap junctions within the cochlear sensory epithelium of immature mice are permeable to fluorescent analogues of D-glucose (Chang et al., 2008), pointing to a role for connexins in the transport of energy substrates. In the cochlea, Cx26 and Cx30 largely co-assemble into heteromeric channels, which form a syncytium extending from the spiral limbus to the cochlear spiral ligament (Ahmad et al., 2003; Sun et al., 2005). Although recent advances have been made towards understanding gap junction assembly mechanisms in the cochlea (Defourny et al., 2019b,c), some important questions remain about connexin trafficking pathways and gap junction biogenesis. Indeed, beside mutations that affect the Cx26/Cx30 channel function itself, many of the disease-causing mutations in *GJB2* or *GJB6* impair the trafficking and delivery of connexin oligomers to the cell surface, what prevents the formation of membrane channels (Ambrosi et al., 2013; Berger et al., 2014; Hoang Dinh et al., 2009; Xu and Nicholson, 2013). Thus, deciphering the trafficking pathway of cochlear Cx26/Cx30 oligomers

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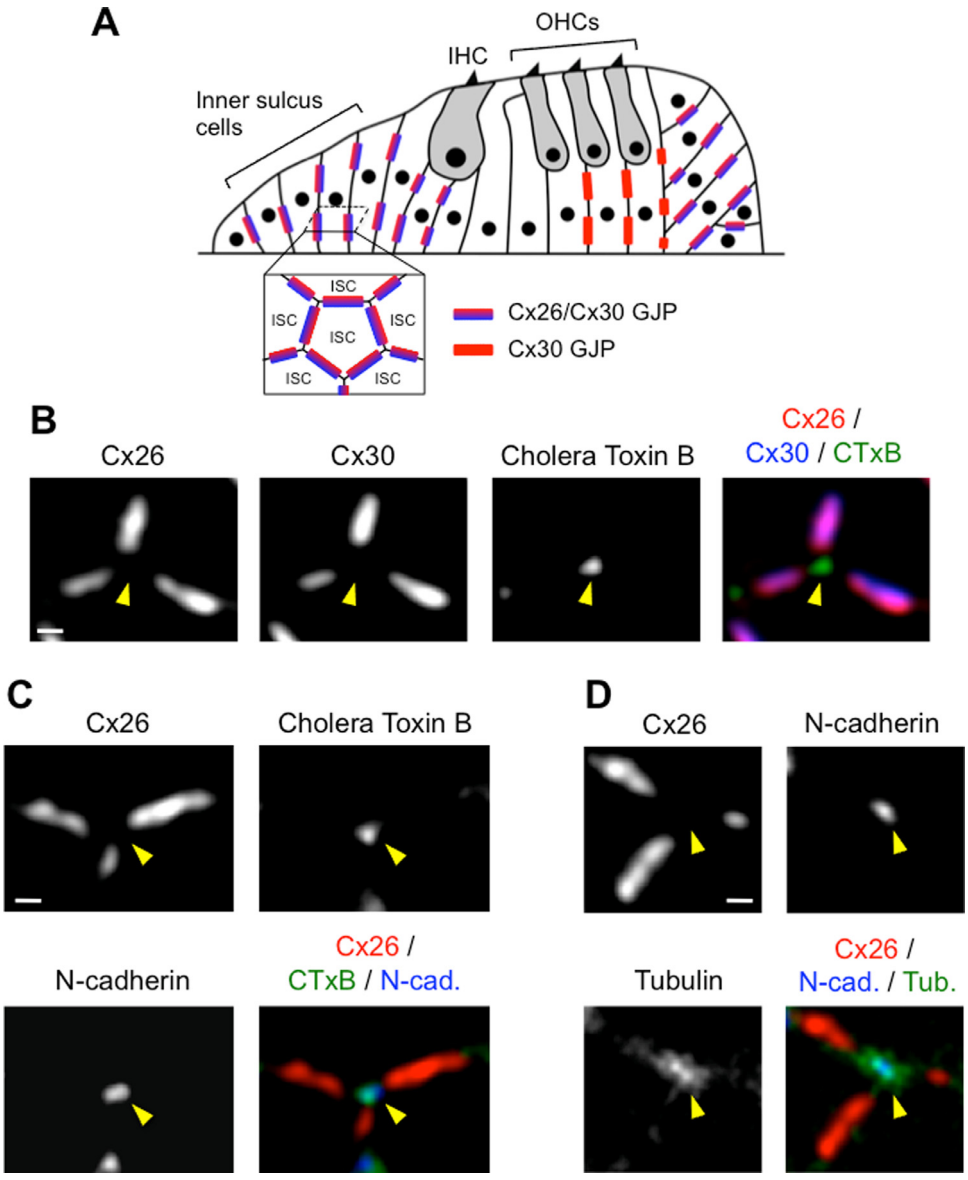

Fig. 1. N-cadherin and associated microtubules are exclusively present at the crossroad between three adjacent Cx26/Cx30 GJPs. (A) Schematic distribution of Cx26/Cx30 and Cx30 GJPs between adjacent non-sensory supporting cells of the cochlea. (B) Cx26 / Cx30 co-immunolabeling, combined with Cholera toxin B staining of whole-mount cochlea of a two-day-old mouse. Cholera toxin B-labeled lipid rafts are absent from Cx26/Cx30 heteromeric GJPs, but enriched at tricellular junction sites (yellow arrowhead). (C) Cx26 / N-cadherin co-immunolabeling, combined with Cholera toxin B staining of whole-mount cochlea of a two-day-old mouse. N-cadherin is exclusively present within lipid raft-enriched tricellular junction sites (yellow arrowhead). (D) Cx26 / N-cadherin / tubulin co-immunolabeling of whole-mount cochlea of a two-day-old mouse. N-cadherin and the associated submembrane microtubule network are exclusively present at tricellular junction sites (yellow arrowhead). GJP = gap junction plaque; IHC = inner hair cell; ISC = inner sulcus cell; OHCs = outer hair cells. Scale bars in (B), (C) and (D) = 1 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

should represent an advance in understanding the pathogenic significance of these mutations.

The biogenesis of gap junction channels usually relies on pre-existing cell-cell junctions, among which are adherens junctions. These are based on cadherin homophilic interactions, which promote the microtubule-mediated trafficking of connexin hemichannels to the cell surface (Shaw et al., 2007). Cadherins are well known as molecular links between two adjacent cells. However, these proteins are also constituents of tricellular junctions (Vanderleest et al., 2018; Yonemura, 2011). These junctions appear to connect three adjacent cells, and they contain a variety of adhesion proteins (Bosveld et al., 2018; Higashi and Miller, 2017). As such, they represent an attractive site for connexin trafficking and gap junction biogenesis in the cochlea.

Here we show that tricellular junctions present at the crossroad between adjacent gap junction plaques (GJPs), provide an unexpected cell surface delivery platform for Cx26/Cx30 oligomers. Using an *in situ* proximity ligation assay (PLA), we detected the presence of non-junctional Cx26/Cx30 oligomers within lipid raft-enriched tricellular junction sites. In addition, we found that the molecular machinery presumably involved in cell surface delivery of Cx26/Cx30 oligomers, including cadherins and associated microtubules, is specifically present within and in the immediate vicinity of tricellular junctions. Using a cadherin neutralizing antibody, we observed that Cx26/Cx30 oligomers fail to reach the cell surface and abnormaly accumulate in the cytoplasm. Overall, our results unveil an unexpected role for tricellular junctions in the trafficking and assembly of membrane channels in the cochlea.

2. Material and methods

2.1. Animals

Mice of the BALB/c strain were grouped-housed in the animal facility of the University of Liège under standard conditions with food and water *ad libitum* and were maintained on a 12-h light/dark cycle. All animals were taken care in accordance with the Declaration of Helsinki and following the guidelines of the Belgian ministry of agriculture in agreement with the EC laboratory animal care and use regulation (2010/63/UE, 22 September 2010).

2.2. Tissue processing and immunostaining

Cochleae of two-day-old mice were fixed for 2 h in 4% formaldehyde. Whole-mount cochleae or organotypic explants were incubated overnight at 4 °C with primary antibodies directed against connexin 26 (rabbit polyclonal antibody; 1:500; Invitrogen; RRID: AB_2533903), connexin 30 (mouse monoclonal antibody; 1:100; Santa Cruz Biotechnology; sc-514847), N-cadherin (mouse monoclonal antibody, clone GC-4; 1:250; Novus Biologicals; NBP2–21805) and alpha tubulin (rat monoclonal antibody, clone YOL1/34; 1:100; Abcam; RRID: AB_305329). Tissues were la-

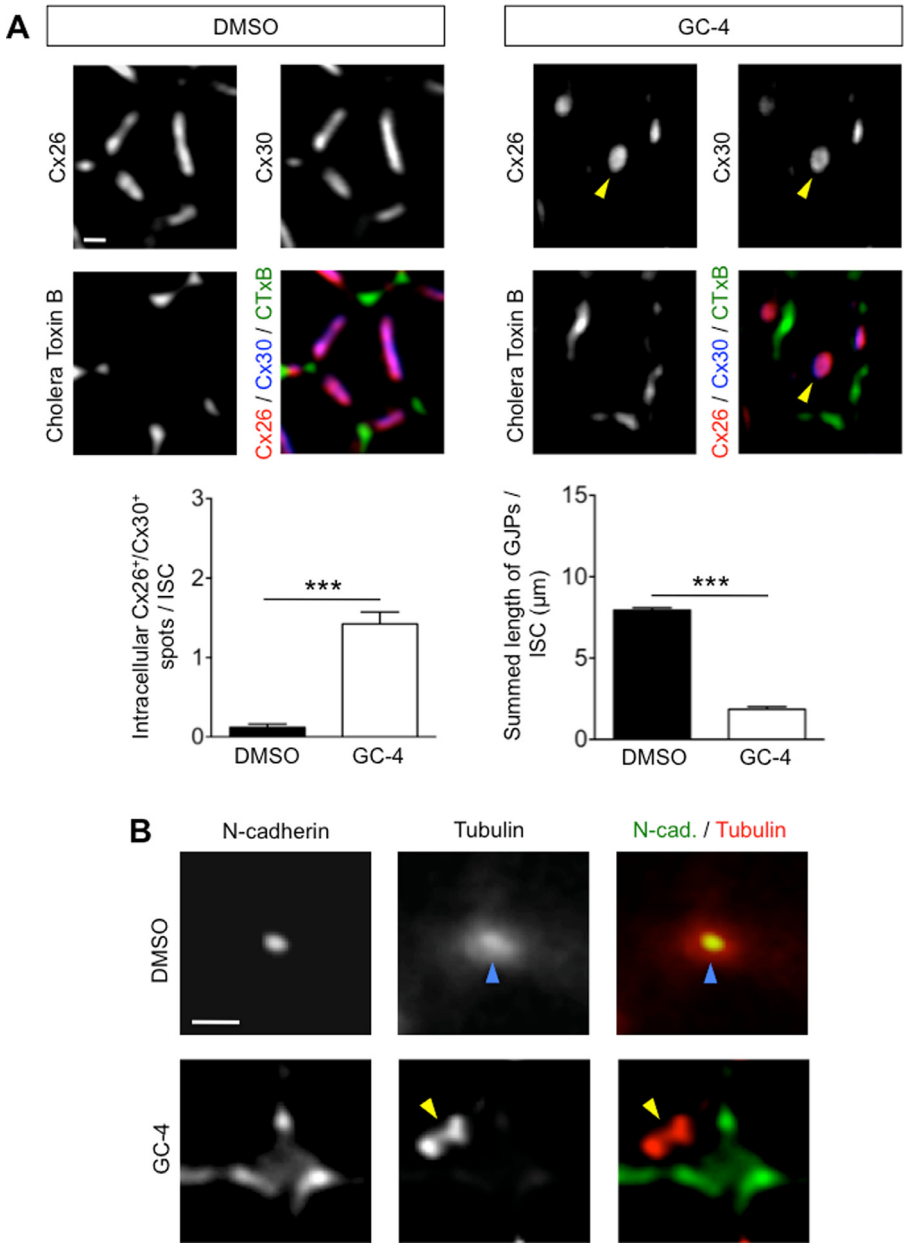

Fig. 3. Tricellular adherens junctions are required for Cx26/Cx30 oligomer trafficking to the cell surface and assembly into GJPs. Organotypic cultures of two-day-old organs of Corti were treated with DMSO (vehicle) or with N-cadherin neutralizing antibody (clone GC-4). (A) The treatment with GC-4 strongly disrupts the formation of GJPs and leads to intracellular accumulation of Cx26/Cx30 oligomers (yellow arrowhead). Left graph: the number of $Cx26^{+}/Cx30^{+}$ spots per inner sulcus cell is significantly increased in the presence of GC-4 antibody. Right graph: the summed length of GJPs per inner sulcus cell is significantly reduced in the presence of GC-4 antibody. Statistical significance was determined using Student's *t*-test. Data are presented as mean ± SEM. $^{***}P < 0.001$. $n = 90$ inner sulcus cells. (B) The disruption of cadherin-based tricellular junctions impairs the recruitment of microtubules to the cell surface. In control condition, microtubules are recruited to N-cadherin-based tricellular junction sites (blue arrowhead). In the presence of GC-4 antibody, the disruption of N-cadherin homophilic interactions leads to destabilization of tricellular junctions. Microtubules can not be recruited to N-cadherin-expressing membrane domains and instead accumulate in the cytoplasm (yellow arrowhead). Scale bar in (A) = 1 µm, in (B) = 0.5 µm. ISC = inner sulcus cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

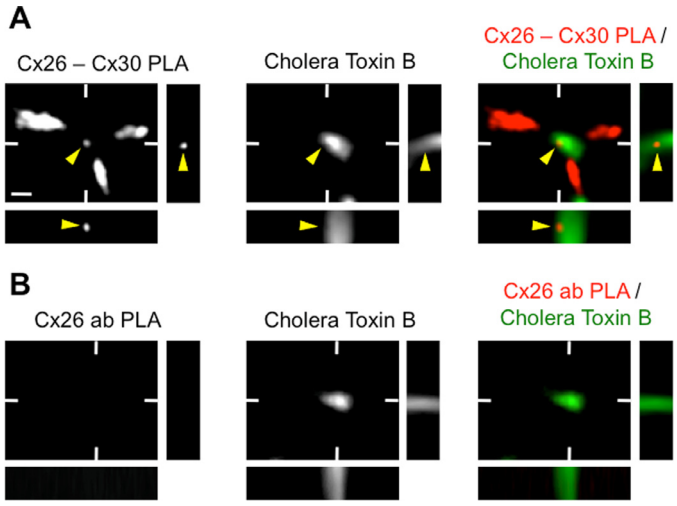

Fig. 2. Non-junctional Cx26/Cx30 oligomers are present within lipid raft-enriched tricellular junction sites. (A) *In situ* proximity ligation assay (PLA) using anti-Cx26 and anti-Cx30 antibodies, combined with Cholera toxin B staining on whole-mount cochlea of a two-day-old mouse. In addition to the GJPs themselves, PLA signals are also detected within lipid raft-enriched tricellular junction sites (yellow arrowhead). (B) The corresponding PLA negative control was performed by omitting the anti-Cx30 primary antibody. Scale bar in (A) = 1 µm in (A) and (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

beled for lipid rafts using FITC-conjugated Cholera toxin B subunit (1 µg/mL; Sigma-Aldrich; C1655). Tissues were then incubated for 1 h with either Rhodamine Red X- or FITC- or Cy5-conjugated goat anti-mouse, anti-rabbit or anti-rat IgGs secondary antibodies (Jackson Immunoresearch Laboratories).

2.3. In situ *proximity ligation assay*

In situ PLA (Söderberg et al., 2006) has been previously proven to work on cochlear tissues, and especially with membrane proteins (Defourny et al., 2019a). Whole-mount cochleae were treated and handled as for immunolabeling (see above). Anti-connexin 26 (rabbit polyclonal antibody; 1:500; Invitrogen; RRID: AB_2533903) and anti-connexin 30 (mouse monoclonal antibody; 1:100; Santa Cruz Biotechnology; sc-514847) primary antibodies were incubated with tissues overnight at 4 °C. Oligo-labeled anti-mouse plus and anti-rabbit minus probes (Duolink, Olink Biosciences; DUO92101) were then used as recommend by the manufacturer. Negative control was obtained by omitting one of the primary antibody (anti-connexin 30). Tissues were finally labeled for lipid rafts using FITC-conjugated Cholera toxin B subunit (1 µg/mL; Sigma-Aldrich; C1655).

2.4. In vitro *organotypic assay*

Organs of Corti were isolated from two-day-old mice and cultured onto Millicell Culture Insert (Millipore) as previously described (Defourny et al., 2015). Organotypic cultured were incubated for 2 h with dimethyl sulfoxide (DMSO, Sigma-Aldrich; 276855) or nocodazole (100 μM; Sigma-Aldrich; SML1665), and for 6 h with DMSO or anti-N-cadherin antibody, clone GC-4 (50 µg/mL; Novus Biologicals; NBP2–21805).

2.5. *Confocal microscopy, image analysis and quantifications*

Confocal fluorescence images were acquired using the Olympus Fluoview FV1000 confocal system (Olympus Europa GmbH) with objective LUCPLFLN 40X numerical aperture 0.60, working distance 2.7–4 mm, oil immersion. For comparison between different culture conditions, all preparations were analysed at the same time, using the same acquisition parameters. For each culture condition, intracellular $Cx26^{+}/Cx30^{+}$ spots in 90 inner sulcus cells from three independent experiments were quantified, and data were plotted. For each culture condition, the GJPs of 90 inner sulcus cells from three independent experiments were measured and summed, and data were plotted. Inner sulcus cells were randomly chosen and GJPs were measured using ImageJ software.

2.6. *Statistics*

All data are presented as mean ± SEM. Data were statistically analysed using two-tailed Student's *t*-test. *P*-values < 0.05 were considered significant ($^{***}P < 0.001$).

3. Results and discussion

3.1. *N-cadherin and associated microtubules are exclusively present at tricellular junction sites between adjacent Cx26/Cx30 gap junction plaques*

Gap junction channels are present between the precursor cells of the sensory epithelia in the embryonic cochlea and persist in those cells which differentiate as non-sensory supporting cells. At early postnatal stages, the cells of the nascent inner sulcus display a columnar shape, which progressively evolves to a cuboidal shape with time (Lim and Anniko, 1985). Along the gap junction biogenesis pathway, connexin oligomers are usually delivered from the interior of the cell to non-lipid raft membrane domains. Then, connexin hemichannels diffuse laterally from the peripheral non-junctional region to assemble into lipid raft GJPs (Hunter et al., 2005; Musil and Goodenough, 1991). The situation is strikingly different concerning the formation of Cx26/Cx30 heteromeric channels, which connect most non-sensory supporting cell types of the cochlear sensory epithelium, including the inner sulcus cells located medially to the inner sensory hair cell layer (Fig. 1A) (Ahmad et al., 2003; Sun et al., 2005). Cx26/Cx30 GJPs are mostly present basally down the cells, *i.e.* at some distance away from the apical membrane of these cells (Fig. 1A). We recently observed that Cx26/Cx30 oligomers assemble into non-lipid raft GJPs between adjacent inner sulcus cells, whereas lipid raft microdomains (revealed by Cholera toxin B-subunit labeling) are only found at tricellular junctions, *i.e.* at the crossroad between three adjacent GJPs (yellow arrowhead in Fig. 1B) (Defourny et al., 2019a). Since lipid rafts are frequently the sites of cell surface delivery of membrane proteins (Bagnat et al., 2000), we wondered whether 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 (Bosveld et al., 2018; Higashi and Miller, 2017), among which are cadherin proteins (Vanderleest et al., 2018; Yonemura, 2011), which are known to regulate microtubule dynamics and to promote microtubule anchoring at the cell-cell border (Chausovsky et al., 2000; Shaw et al., 2007; Stehbens et al., 2006). The combined activity of cadherins and microtubules would be consistent with recent findings showing that Cx26/Cx30 GJP formation in the cochlea strongly relies on intact microtubule network (Defourny et al., 2019a). Cadherin proteins are broadly, but differently, expressed in non-sensory supporting cells of the cochlea. Among the latter, inner sulcus cells especially express neural cadherin (N-cadherin) (Etournay et al., 2010), making this protein a candidate for regulating the targeted delivery of Cx26/Cx30 oligomers to specific membrane domains. At inner sulcus cell-cell borders, we observed that N-cadherin is exclusively present within lipid raft domains, which are confined to

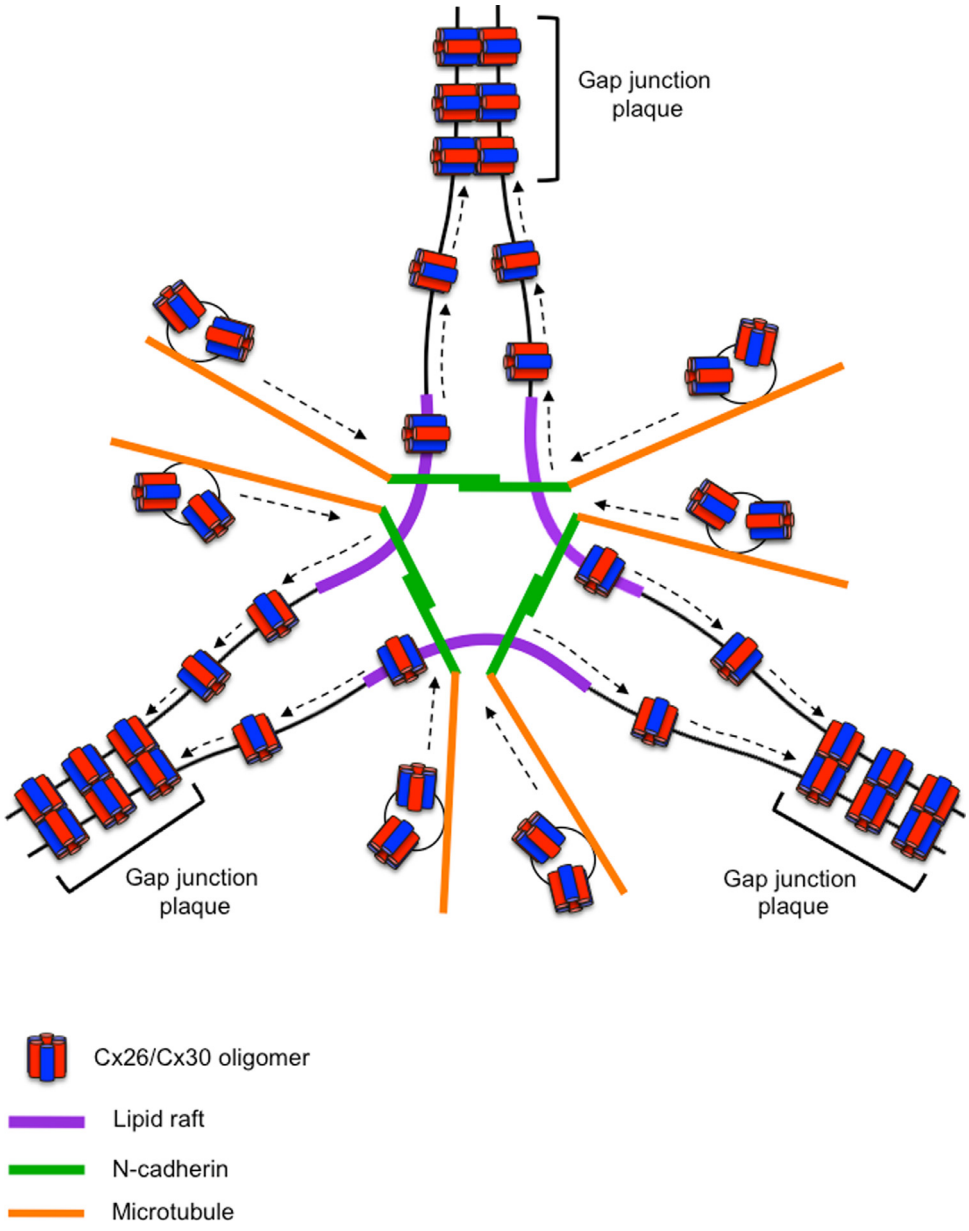

Fig. 4. A model for how tricellular adherens junctions promote cell surface delivery of Cx26/Cx30 oligomers and assembly into GJPs in the cochlear sensory epithelium. Cx26/Cx30 oligomers travel along microtubules and are delivered to the cell surface at lipid raft-enriched tricellular adherens junction 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.

tricellular junction sites (yellow arrowhead in Fig. 1C). This preferential association of N-cadherin with lipid raft fractions is consistent with previous observations (Nakai and Kamiguchi, 2002). In addition, the cadherin-associated submembrane microtubule network, presumably involved in delivering Cx26/Cx30 oligomers to the cell surface, was only observed in the immediate vicinity of N-cadherin-containing tricellular junctions (yellow arrowhead in Fig. 1D). The presence of microtubules at tricellular junction sites was further confirmed using the microtubule-depolymerizing drug nocodazole, which in turn affects the formation of N-cadherin-based adherens junctions (Fig. S1). This finding is consistent with previous observations that dynamic microtubules regulate the local cadherin concentration at cell-cell contacts (Stehbens et al., 2006). Of note is that Cx26/Cx30 GJPs themselves, or their immediate perijunctional regions are devoid of N-cadherin and tubulin, meaning that Cx26/Cx30 oligomers could not be directly delivered from the interior of the cell to the GJP.

3.2. Non-junctional Cx26/Cx30 oligomers are present within lipid raft-enriched tricellular junction sites

To detect the presumed transient presence Cx26/Cx30 oligomers within lipid raft-enriched tricellular junction sites of inner sulcus cells, we used an *in situ* PLA. Owing to signal amplification, this method should make it possible to reveal the presence of small amounts of proteins that could not be detected using traditional immunofluorescence, such as connexin oligomers in transit on the way to their final destination. Using anti-Cx26 and anti-Cx30 primary antibodies, we detected PLA signals within lipid raft-enriched tricellular junction sites, suggesting the presence of Cx26/Cx30 oligomers (yellow arrowhead in Fig. 2A). Such PLA signals were observed in 56% of the Cholera Toxin B-positive sites considered ($n = 50$). To ensure the specificity of PLA signals, an assay was performed by omitting the anti-Cx30 primary antibody. In this case, no PLA signals were observed (Fig. 2B).

These findings are 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 already suggested that lipid rafts may be involved in trafficking non-junctional membrane Cx26 to non-lipid raft GJPs (Locke et al., 2005). Because of the relatively short half-life of connexins (usually 1 – 5 h), 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 (Gaietta et al., 2002; Lauf et al., 2002). In agreement with the latter model, we could suggest that, owing to low affinity of Cx26 for cholesterol abundantly present in lipid rafts (Hung and Yarovsky, 2011; Locke and Harris, 2009), 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.

3.3. Tricellular adherens junctions are required for Cx26/Cx30 oligomer trafficking and assembly into gap junction plaques

To test the role of cadherin-based tricellular adherens junctions in Cx26/Cx30 oligomer trafficking and assembly into GJPs, we aimed to disrupt N-cadherin homophilic interactions using an anti-N-cadherin neutralizing antibody (clone GC-4) (Puch et al., 2001) in organotypic culture. In this condition, inner sulcus cells display a significant accumulation of Cx26/Cx30 oligomers in the cytoplasm (yellow arrowhead in Fig. 3A). Moreover, we observed a significant decrease in the summed Cx26/Cx30 GJP length per inner sulcus cell (Fig. 3A). These concomitant defects support the hypothesis that the disruption of N-cadherin-based adherens junctions impairs the trafficking of Cx26/Cx30 oligomers to the cell surface, which instead accumulate in the cytoplasm. Interestingly, similar defects were previously observed using the microtubule-depolymerizing drug nocodazole (Defourny et al., 2019b), suggesting a cooperation between microtubules and N-cadherin-based adherens junctions in delivering Cx26/Cx30 oligomers from the interior of the cell to the cell surface. In support of this hypothesis, we found that microtubules can not be anchored at the plasma membrane anymore when N-cadherin homophilic interactions are disrupted with GC-4 neutralizing antibody (yellow arrowhead in Fig. 3B). These observations are consistent with previous data showing that cadherin homophilic interactions recruit microtubules to cell-cell borders (Chausovsky et al., 2000; Shaw et al., 2007; Stehbens et al., 2006). Thus, our results suggest that cadherin-based tricellular adherens junctions promote the microtubule-mediated trafficking of Cx26/Cx30 oligomers to the cell surface and further assembly into GJPs (Fig. 4). These findings are similar to previous observations showing that Cx40 and Cx43 fail to assemble into membrane channels in N-cadherin-depleted cardiac myocytes (Li et al., 2005; Luo and Radice, 2003). As a consequence, the cardiac-specific loss of N-cadherin leads to conduction slowing and arrhythmogenesis (Li et al., 2005).

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 *in vitro* data obtained in cell culture with fusion proteins (Cx26-YFP, Cx30-GFP,…) (Kelly et al., 2015; Stout et al., 2015; Thomas et al., 2005). 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 vivo*. Nevertheless, our model of Cx26/Cx30 GJP assembly in the cochlea is rather consistent with previous live imaging observations, showing that Cx30-GFP GJPs are replenished at their outer edges (Kelly et al., 2015).

4. Conclusion

Until now, tricellular junctions have mainly been considered to provide a mechanical support for epithelial development and morphogenesis (Bosveld et al., 2018; Higashi and Miller, 2017), as well as to maintain epithelial barrier function (Ikenouchi et al., 2005; Nayak et al., 2013). Our data further suggest that they also represent critical organizers of gap junction biogenesis in the cochlea. Indeed, we have shown that tricellular junctions provide an essential cell surface delivery platform for Cx26/Cx30 oligomers. Overall, our results unveil an unexpected role for tricellular junctions in membrane protein trafficking, and highlight the key dependence of bicellular junctions towards tricellular junctions. However, it has been proposed that active connexin hemichannels in the apical membrane of nascent inner sulcus cells have an important physiological activity during development (Verselis, 2019). In this case, we cannot rule out the possibility that these connexin hemichannels could be delivered to the cell surface by a different way. This question could be the subject of further investigations.

Declaration of Competing Interest

The authors declare no competing financial interests.

CRediT authorship contribution statement

Jean Defourny: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Funding acquisition. **Marc Thiry:** Writing - review & editing, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.heares.2020.108137.

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