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Cochlear connexin 30 homomeric and heteromeric channels exhibit distinct assembly mechanisms

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ABSTRACT

Many of the mutations in *GJB2* and *GJB6*, which encode connexins 26 and 30 (Cx26 and Cx30), impair the formation of membrane channels and cause autosomal syndromic and non-syndromic hearing loss. In cochlear non-sensory supporting cells, Cx26 and Cx30 form two types of homomeric and heteromeric gap junctions. The biogenesis processes of these channels occurring *in situ* remain largely unknown. Here we show that Cx30 homomeric and Cx26/Cx30 heteromeric gap junctions exhibit distinct assembly mechanisms in the cochlea. When expressed as homomeric channels, Cx30 preferentially interacts with β-actin in the peripheral non-junctional membrane region, called perinexus, and strongly relies on the actin network for gap junction plaque assembly. In contrast, we found that Cx26/Cx30 heteromeric gap junction plaques are devoid of perinexus and associated actin network, and resist to actin-depolymerizating drug. This supports that Cx26/Cx30 oligomers could be directly delivered from the interior of the cell to the junctional plaque. Altogether, our data provide a novel insight in homomeric and heteromeric gap junction plaque assembly in the cochlea.

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 allow 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 (Kikuchi et al., 2000). However, gap junctions may serve additional roles in the cochlea, such as providing networks for nutrient transfer (Chang et al., 2008; Jagger and Forge, 2015). In the cochlea, Cx26 and Cx30 assemble in two types of gap junctions, which form a syncytium extending from the spiral limbus to the cochlear spiral ligament. On the one hand, Cx30 is expressed as homomeric channels in Deiters' cells, *i.e.* the supporting cells which surround the outer hair cells (Jagger and Forge, 2015; Sun et al., 2005). On the other hand, Cx30 co-assembles with Cx26 as heteromeric channels in other supporting cell types (Ahmad et al., 2003; Sun et al., 2005) (Fig. 1). Although these two channel components are well characterized, the gap junction plaque assembly mechanisms occurring *in situ* remain largely unknown. Beside 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 (Berger et al., 2014; Hoang Dinh et al., 2009; Xu and Nicholson, 2013). Thus, deciphering the processes of gap junction biogenesis occurring *in situ* when Cx30 is expressed as homomeric or heteromeric channels with Cx26 should represent an advance in understanding the pathogenic significance of these mutations.

Gap junction assembly usually occurs in a kind of “two-step mechanism”. First, hexameric connexons assembled in the *trans*-Golgi network are trafficked to the non-junctional plasma membrane (Falk et al., 2016; Thévenin et al., 2013). Secondly, hemichannels associate with the cortical actin through actin-binding proteins zonula occludens (ZOs) which regulate delivery of connexins from the periphery to the

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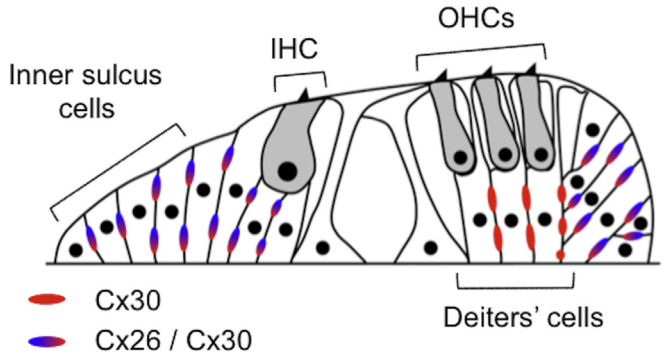

Fig. 1. Schematic distribution of Cx30 and Cx26/Cx30 GJPs in cochlear supporting cells.
IHC = inner hair cell; OHCs = outer hair cells.

gap junction plaque (GJP) (Hervé et al., 2014; Thévenin et al., 2013). This peripheral membrane region containing non-junctional hemichannels and surrounding the gap junction plaque is called "perinexus" (Rhett et al., 2011). Because of the relatively short half-life of connexins (usually 1–5 h), the junctional plaque is in a dynamic state, constantly remodeled through both recruitment of newly synthesized connexons to the periphery and endocytosis of older components from the center of the plaque (Gaietta et al., 2002).

Here we show that cochlear Cx30 exhibits distinct gap junction assembly mechanisms when expressed as homomeric or heteromeric channels with Cx26. In Deiters' cells, homomeric Cx30 preferentially interacts with the β-actin isoform in the perinexus of the GJP. Moreover, the assembly of Cx30 into GJPs is strongly disturbed in the presence of actin-depolymerizating drug. In inner sulcus cells, in contrast, Cx26/Cx30 heteromeric GJPs are devoid of adjacent perinexus and associated actin network, and resist to treatment with actin-depolymerizating drug. Altogether, our data provide a novel insight in homomeric and heteromeric GJP assembly in the cochlea.

2. Materials and methods

2.1. Animals

Mice of the BALB/c strain were group-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 EC laboratory animal care and use regulation (2010/63/UE, 22 September 2010).

2.2. Tissue processing and immunostainings

Cochleae of newborn 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 30 (mouse monoclonal antibody; 1:100; Santa Cruz Biotechnology; RRID: AB_2532309; rabbit monoclonal antibody; 1:500; Thermo Fisher Scientific), connexin 26 (RRID: AB_2533903; rabbit polyclonal antibody; 1:500; Invitrogen), ZO-1 (RRID: AB_628459; rat monoclonal antibody, 1:50; Santa Cruz Biotechnology) and ZO-2 (RRID: AB_2203577; mouse monoclonal antibody, 1:50; Santa Cruz Biotechnology). TRITC-conjugated phalloidin (1:500; Sigma-Aldrich) was used as an F-actin marker. Tissues were then incubated for 1 h with either Rhodamine Red X- or FITC-conjugated goat anti-mouse, anti-rabbit or anti-rat IgGs secondary antibodies (Jackson Immunoresearch Laboratories).

2.3. In situ proximity ligation assay

In order to characterize endogenous protein interactions, we used the Duolink *in situ* proximity ligation assay reagent (Olink Biosciences, Uppsala, Sweden). Whole-mount cochleae were treated and handled as for immunolabelling (see above). Oligo-labelled anti-mouse plus and anti-rabbit minus probes were then used as recommended by the manufacturer. Two combinations of primary antibodies were used for incubation overnight at 4 °C: anti-connexin 30 (RRID: AB_2532309; rabbit monoclonal antibody, 1:500; Thermo Fisher Scientific) and either anti-β-actin (RRID: AB_476692; mouse monoclonal antibody, 1:100; Sigma-Aldrich) or anti-γ-actin (RRID: AB_2289264; mouse monoclonal antibody, 1:100; Sigma-Aldrich). Negative controls were obtained by omitting one of the two primary antibodies. Cochleae were then labelled using FITC-conjugated anti-connexin 30 (mouse monoclonal antibody; 1:100; Santa Cruz Biotechnology) and FluoProbes 647H – Phalloidin (1:100, Cheshire Sciences, Chester, UK) then mounted using Duolink *In Situ* Mounting Medium. Proximity ligation assay images combined with connexin 30 and F-actin staining were acquired as for immunolabelling above.

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 cultures were incubated for 5 h with dimethyl sulfoxide (DMSO, vehicle) or cytochalasin D (10 µM; Sigma-Aldrich).

2.5. Confocal microscopy, image analysis and quantification

Confocal fluorescence images were acquired using the Olympus Fluoview FV1000 confocal system (Olympus Europa GmbH). For comparison between different culture conditions, all preparations were analysed at the same time, using the same acquisition parameters. For each culture condition, the GJPs of 90 Deiters' cells and 90 inner sulcus cells from three independent experiments were measured and summed, and data were plotted. Deiters' and inner sulcus cells were randomly chosen and gap junction plaques were measured using ImageJ software. Mean pixel intensities in GJPs and in adjacent perinexus 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 ($^{**}P < 0.01$, $^{***}P < 0.001$).

2.7. Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3. Results

3.1. Cx30 homomeric and Cx26/Cx30 heteromeric channels exhibit distinct co-localization patterns with the cortical actin network

Cx26 and Cx30 are strongly expressed in cochlear supporting cells from two-day-old in mice (Sun et al., 2005). Once they have reached the plasma membrane, most connexins are linked to the submembrane actin network through ZOs. ZOs contain three PDZ domains and connexins interact with them *via* a PDZ-binding motif (Hervé et al., 2014; Thévenin et al., 2013). Interestingly, the genomic duplication and overexpression of TJP2/ZO-2 cause a progressive non-syndromic hearing loss in humans (Walsh et al., 2010). We thus examined whether

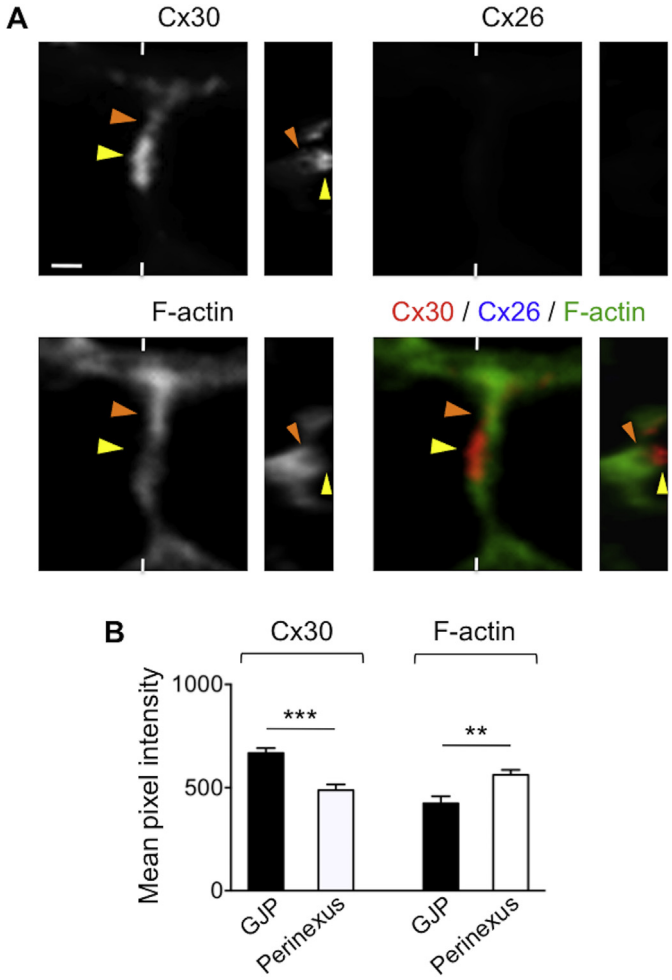

Fig. 2. Cochlear Cx30 exhibits dual co-localization patterns with the actin network.
(A) Cx30 immunolabelling and F-actin staining of whole-mount cochlea of a two-day-old mouse. In Deiters' cells, in which Cx30 is expressed as homomeric channels, the dense Cx30 labelling likely corresponding to the GJP is quite faintly co-localized with the cortical actin network (yellow arrowhead). In contrast, the weaker Cx30 labelling present in the membrane region adjacent to the GJP (perinexus) overlaps with a dense actin network (orange arrowhead). (B) Mean pixel intensity measurement showing that Cx30 immunolabelling is stronger in the GJP and weaker in the adjacent perinexus ($n = 10$). In contrast, the cortical actin network is weaker in the GJP itself, and enriched in the perinexus ($n = 10$). Statistical significance was determined using Student's *t*-test. Data are presented as mean ± SEM. n.s. = not significant, $^{**}P < 0.01$,$^{***}P < 0.001$. Scale bar in (A) represents 1 µm.

Cx30 associates with cortical actin and ZO proteins when expressed as homomeric channels in Deiters' cells or as heteromeric channels with Cx26 in inner sulcus cells at two-day-old. These latter non-sensory cells, located close to the inner hair cell layer, were previously considered to address the role of Cx26 in the assembly of the gap junction macromolecular complex in the cochlea (Kamiya et al., 2014). The capacity of Cx30 to bind to ZO proteins is experimentally controversial, although Cx30 does not contain any predicted PDZ-binding motif at its C-terminal extremity (Thévenin et al., 2013). In Deiters' cells, we observed that Cx30 gap junction plaques exhibit unclear, diffuse boundaries. The dense Cx30 immunolabelling, likely corresponding to the junctional plaque, quite faintly co-localizes with F-actin (yellow arrowhead in Fig. 2A). In contrast, the weaker Cx30 immunolabelling present adjacent to the plaque, which could correspond to non-junctional hemichannels accruing to the plaque, overlaps with a dense actin network (orange arrowhead in Fig. 2A). The mean pixel intensity of Cx30 immunolabelling is significantly stronger in the GJP and weaker in the adjacent perinexus. In contrast, mean pixel intensity measurement revealed that the cortical actin network is weaker at the level of the GJP itself, and enriched in the perinexus (Fig. 2B). Although Cx30 apparently co-localizes with cortical actin in Deiters' cells, it does not interact with ZO proteins (Fig. S1). In inner sulcus cells, where Cx30 is co-expressed with Cx26, the GJPs are devoid of cortical actin and exhibit sharp boundaries. Moreover, a gap is visible between each of the lateral boundaries of the GJP and the closest submembrane actin network, suggesting the absence of any adjacent perinexus (yellow arrowheads in Fig. 3).

3.2. Homomeric Cx30 interacts with β-actin in the perinexus of the gap junction plaque

Several types of membrane channel proteins are believed to interact directly with the actin network, which regulate channel trafficking and function (Sasaki et al., 2014). Since Cx30 does not associate with ZO proteins in Deiters' cells, we tested whether homomeric Cx30 directly interacts with ubiquitously expressed actin isoforms β-actin and γ-actin. Both of these isoforms are strongly expressed in Deiters' cells (Furness et al., 2005). Of note, mutations in *ATCB* and *ATCG1* genes encoding human β-actin and γ-actin, respectively, cause a Baraitser-Winter craniofrontofacial syndrome frequently including sensorineural hearing loss (Rivière et al., 2012; Verloes et al., 2015). Although β-actin and γ-actin are nearly identical proteins that differ by only four biochemically similar amino acids, they might have specific protein binding affinities and perform distinct cellular functions (Perrin and Ervasti, 2010). Using a proximity ligation assay (PLA) aimed to detect *in situ* protein-protein interactions (Söderberg et al., 2006), we observed a strong Cx30/β-actin PLA signal in the region directly adjacent to the Cx30 GJP (orange arrowhead in Fig. 4A) and a weaker signal at the level of the dense junctional plaque itself (yellow arrowhead in Fig. 4A). A PLA negative control performed by omitting one of the primary antibodies did not show any positive signal (Fig. 4B). In contrast no Cx30/γ-actin PLA signals were observed neither adjacent nor within the GJP (Fig. S2). These findings suggest that β-actin rather than γ-actin especially promotes the recruitment of peripheral Cx30 from the perinexus to the GJP. Although it was shown that Cx30-GFP GJPs are replenished at the outer edges *in vitro* (Kelly et al., 2015), the asymmetry of the Cx30/β-actin PLA signal we observed in Deiters' cells suggests that peripheral Cx30 is preferentially recruited to only one side of the GJP. In inner sulcus cells, where Cx30 co-assembles with Cx26, no Cx30/β-actin PLA signals were observed (Fig. 4C), suggesting the absence of any perinexus and distinct gap junction assembly mechanisms.

3.3. Cx30 homomeric but not Cx26/Cx30 heteromeric gap junction assembly requires the actin network

To examine the requirement of cortical actin in homomeric and heteromeric gap junction assembly in the cochlea, we performed an *in vitro* organotypic assay aimed to disrupt the actin network. To this end, explants of organ of Corti isolated from two-day-old mice were treated with DMSO and cytochalasin D as vehicle and actin-depolymerizating drug, respectively. In Deiters' cells, where Cx30 is expressed as homomeric channels, cytochalasin D strongly disturbed gap junction assembly (Fig. 5A). In contrast, cytochalasin D treatment had no effect on Cx26/Cx30 GJPs in inner sulcus cells (Fig. 5B). The effect of cytochalasin D on actin networks was ascertained by the presence of actin aggregates throughout the cytoplasm (Schliwa, 1982) (yellow arrowheads in Fig. 5A, B). These data suggest a distinct requirement of actin networks in homomeric Cx30 and heteromeric Cx26/Cx30 gap junction assembly.

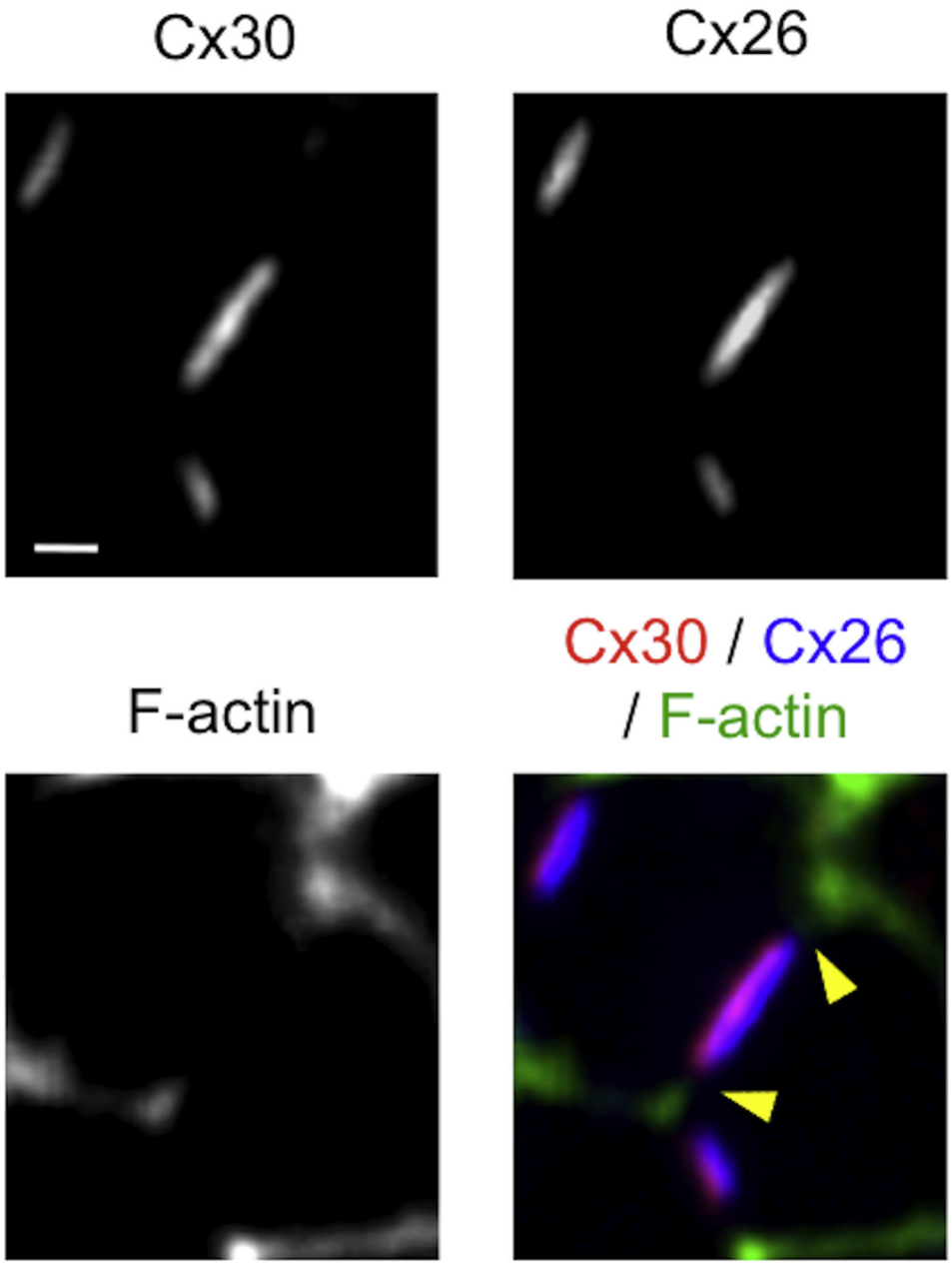

Fig. 3. Cx26/Cx30 heteromeric channels do not co-localize with the cortical actin network.
Cx26/Cx30 immunolabelling and F-actin staining of whole-mount cochlea of a two-day-old mouse. In inner sulcus cells, where Cx30 co-assembles with Cx26, the GJP is devoid of cortical actin and exhibit sharp boundaries. A space is visible between each of the lateral edges of the GJP and the closest submembrane actin network, suggesting the absence of any adjacent perinexus (yellow arrowheads). Scale bar in (A) represents 1 μm.

4. Discussion

Although Cx26 and Cx30 belong to the same β-group of connexins and share 77% protein sequence identity in mouse (Dahl et al., 1996), our data show that Cx30 homomeric and Cx26/Cx30 heteromeric GJPs exhibit different assembly mechanisms in the cochlea. Just as Cx26 has been shown to be the key organizer of the heteromeric gap junction macromolecular complex in the cochlea (Kamiya et al., 2014), our data suggest that intrinsic Cx26 gap junction assembly features dominates the ones of Cx30 *in situ*. We observed that cochlear Cx26/Cx30 GJPs exhibit sharp boundaries and are apparently devoid of adjacent perinexus. In addition, the submembrane actin network is not required for GJP assembly and/or stability. This supports that Cx26/Cx30 oligomers could be directly delivered from the interior of the cell to the junctional plaque without trafficking through any surrounding perijunctional space. Although this model does not fit with the classical connexin oligomerization and trafficking pathway (Laird, 2006), it has been shown that Cx43 hemichannels can be directly delivered to junctional plaques from the interior of the cell (Shaw et al., 2007). This hypothesis of an unusual trafficking and delivery pathway of Cx26/Cx30 hexamers is supported by previous data. Whereas connexins usually oligomerize in the *trans*-Golgi network, Cx26 oligomers are already found in the endoplasmic reticulum (Diez et al., 1999) and can reach the cell surface *via* a route bypassing the Golgi complex (George et al., 1999; Martin et al., 2001). In this context, an *in vitro* cell-free transcription/translation system has shown that Cx26 exhibits a singular membrane-integration behaviour and integrates directly in a post-translational manner into plasma membranes. Protein-cleavage studies of Cx26 integrated into plasma membranes indicated a similar native transmembrane topography to that of Cx26 integrated co-translationally into

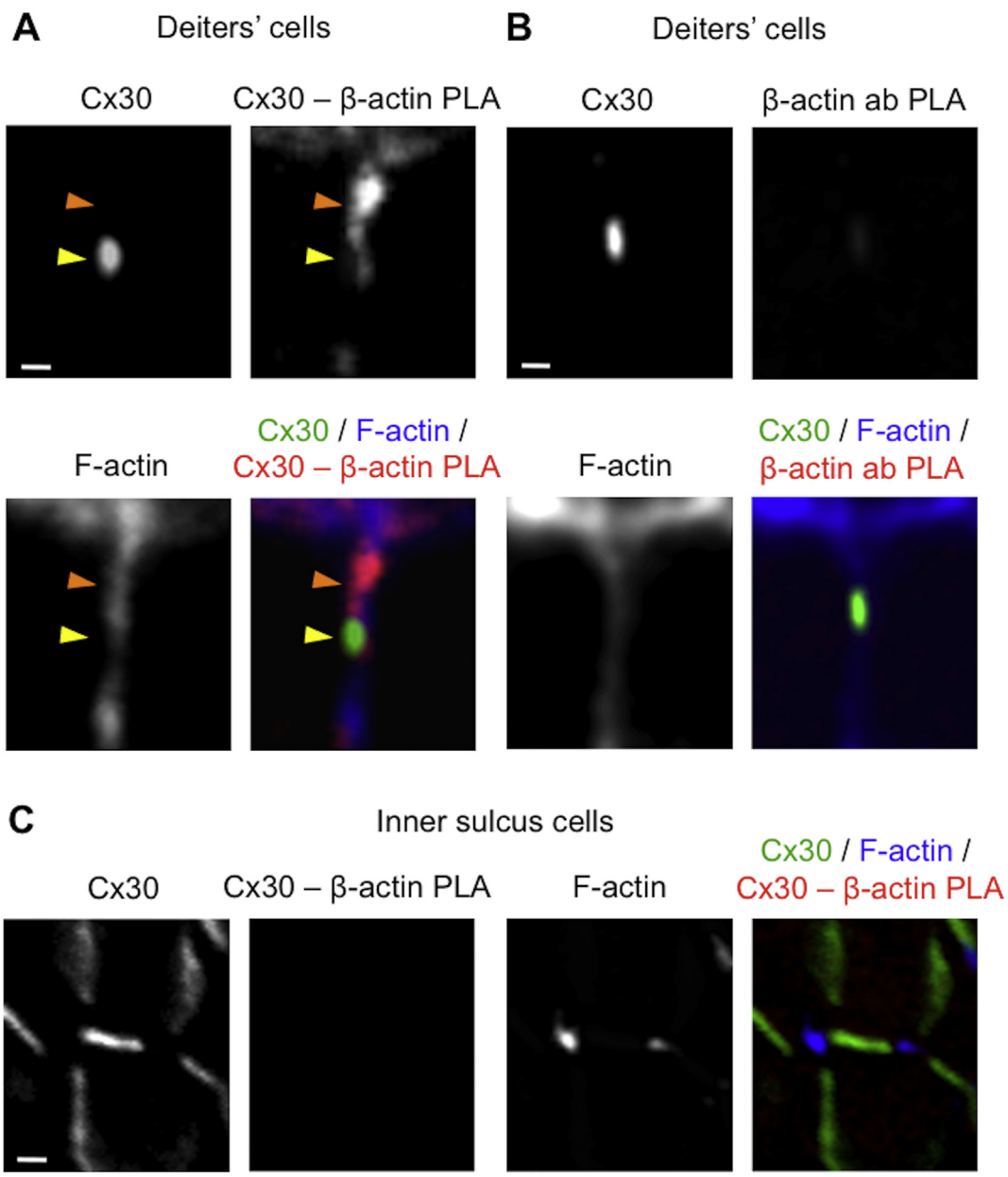

Fig. 4. Homomeric Cx30 interacts with β-actin in the perinexus of the GJP.
In situ proximity ligation assay (PLA) using anti-Cx30 and anti-β-actin antibodies, combined with FITC-conjugated Cx30 immunolabelling and F-actin staining on whole-mount cochlea of a two-day-old mouse. (A) In Deiters' cells, a strong Cx30/β-actin PLA signal is observed in the perinexus adjacent to the GJP (orange arrowhead), whereas a weaker signal overlaps with the Cx30 GJP itself (yellow arrowhead). (B) The corresponding PLA negative control was performed by omitting the anti-Cx30 primary antibody. (C) In inner sulcus cells, in which Cx26 co-assembles with Cx30, no Cx30/β-actin PLA signals are observed within or adjacent to the GJP. Scale bars in (A–C) represent 1 μm.

microsomes (Ahmad and Evans, 2002; Zhang et al., 1996). Cx26 oligomerization and assembly into hemichannels thus occurs independently of the conventional biogenesis of gap junctions involving connexin trafficking and oligomerization *via* membrane components of the secretory pathway (Ahmad and Evans, 2002).

When expressed as homomeric channels in Deiters' cells, Cx30 likely assembles into junctional plaques in a more classical way. Although Cx30 was previously found to associate with the actin cytoskeleton *in vitro* (Qu et al., 2009), we observed that cochlear Cx30 preferentially interacts with the β-actin isoform in the perinexus adjacent to the GJP. This suggests that F-actin especially promotes the recruitment of peripheral Cx30 from the perinexus to the GJP and, to a lesser extent, contributes to the stability of the junctional plaque. Of note, the sensitivity of Cx30 GJPs to actin-depolymerizating drugs varies among cell types. Cx30 GJPs are severely disrupted by cytochalasin B in transfected HeLa cells, whereas they resist to the same treatment in REK cells (Kelly et al., 2015; Qu et al., 2009). Given that Cx30-mediated intercellular communication promotes epithelial repair (Forge et al., 2013), our findings should be of interest beyond the hearing system, especially regarding the role of Cx30 in skin physiology and disease.

4.1. Conclusion

Altogether, our data provide a novel insight in homomeric and heteromeric gap junction assembly mechanisms in the cochlea. Since many of the disease-causing mutations in *GJB2* and *GJB6* impair the formation of GJPs (Berger et al., 2014; Hoang Dinh et al., 2009; Xu and Nicholson, 2013), our findings should help further research aimed to

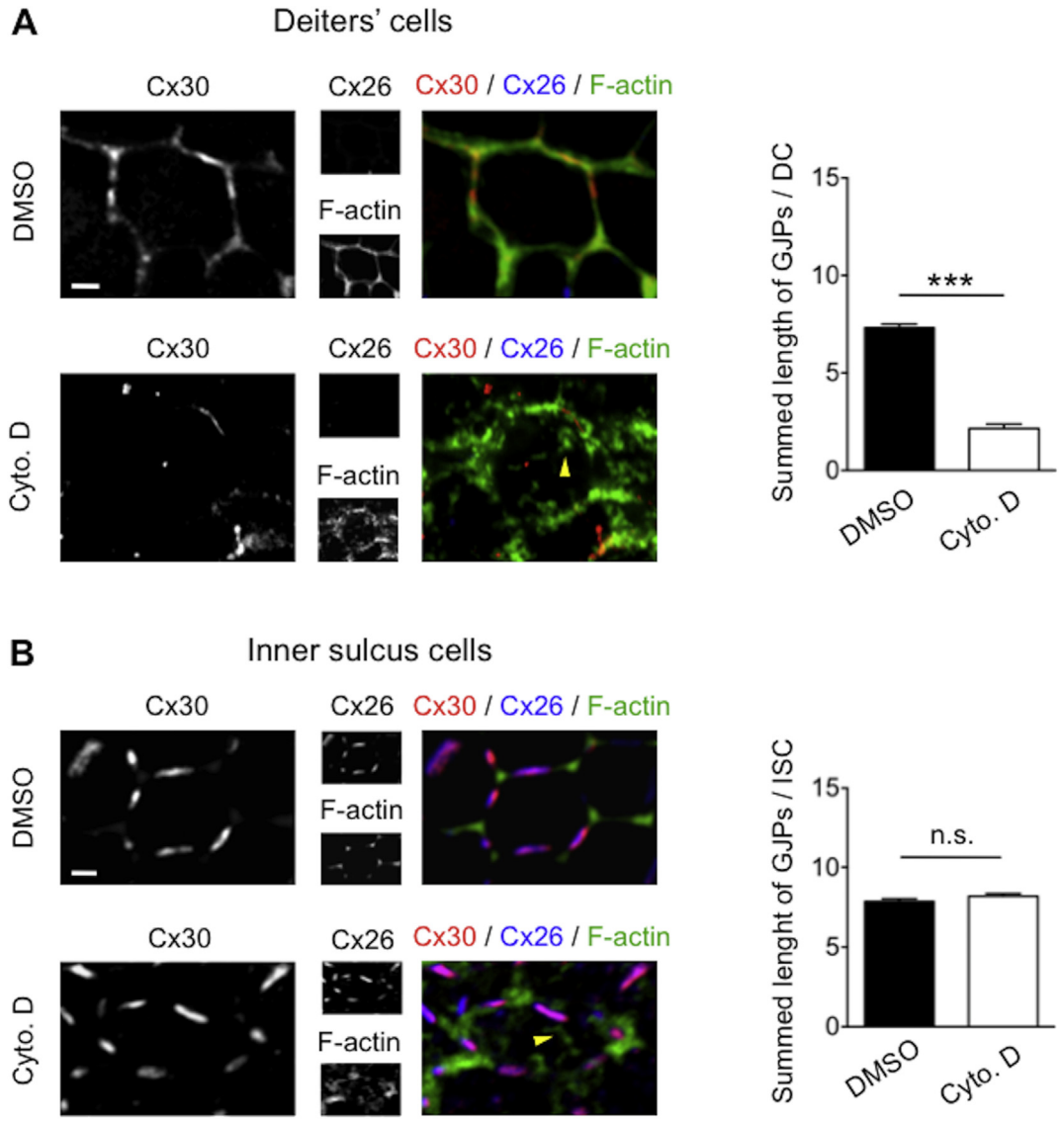

Fig. 5. Actin depolymerization differently affects Cx30 homomeric and Cx26/Cx30 heteromeric GJPs.
Organotypic cultures of two-day-old organs of Corti were treated with DMSO (vehicle) or cytochalasin D and labelled for Cx26, Cx30 and F-actin. (A) In Deiters' cells, cytochalasin D treatment significantly disrupts the formation of Cx30 homomeric GJPs ($n = 90$). (B) In inner sulcus cells, cytochalasin D treatment does not affect the Cx26/Cx30 heteromeric GJPs ($n = 90$). The effect of cytochalasin D on actin networks was ascertained by the presence of actin aggregates throughout the cytoplasm (yellow arrowheads in A and B). Statistical significance was determined using Student's *t*-test. Data are presented as mean ± SEM. n.s. = not significant, $^{***}P < 0.001$. Scale bars in (A) and (B) represent 2 µm. DC = Deiters' cell; ISC = inner sulcus cell.

decipher the pathogenic significance of these mutations.

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Competing interests

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mod.2018.10.001.

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