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Efnb2 haploinsufficiency induces early gap junction plaque disassembly and endocytosis in the cochlea

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ABSTRACT

Chromosome 13q deletions encompassing *EFNB2*, which encodes the transmembrane protein ephrin-B2, are likely to cause syndromic forms of sensorineural hearing loss of unclear origin. Thus, unravelling the pathogenic mechanisms could help to improve therapeutic strategies. In the cochlea, adjacent non-sensory epithelial cells are connected via gap junction channels, the activity of which is critical to maintain cochlear homeostasis. Here we show that ephrin-B2 promotes the assembly of connexin 30 (Cx30) gap junction plaques (GJPs) between adjacent non-sensory Deiters' cells. 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 of Cx30 GJPs. According to recent observations, early GJP degradation could certainly play a role in the pathogenic process leading to progressive sensorineural hearing loss due to *Efnb2/EFNB2* haploinsufficiency.

1. Introduction

Congenital hearing loss affects 1–3 in 1000 children and is the most prevalent childhood disability diagnosed through newborn screening, with at least half of all cases attributable to genetic causes (Mason and Herrmann, 1998; Korver et al., 2017). Approximately 30 % of the genetic cases of hearing loss are considered to be syndromic, i.e. accompanied by additional clinical features (Kalatzis and Petit, 1998). Among them are some patients suffering from a 13q deletion syndrome (Abdallah-Bouhjar et al., 2013; Bellucco et al., 2019; Dworschak et al., 2013; Haskins et al., 2014; Kirchhoff et al., 2009; Liao et al., 2011; Lévy et al., 2018). Recently, a terminal deletion at chromosome 13q33 encompassing *EFNB2* was shown to cause a syndromic neurodevelopmental disorder including intellectual disability, seizures, congenital heart defects and a progressive sensorineural hearing loss beginning in the first decades (Lévy et al., 2018). *EFNB2/Efnb2* encodes the transmembrane protein ephrin-B2, which belongs to the Eph/ephrin protein family. Eph receptors represent the largest family of receptors tyrosine kinase identified to date. 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 (Gale et al., 1996). One of the unique properties of the Eph/ephrin system is the fact that both receptors and ligands are competent to transduce forward and reverse signalling pathways, respectively (Kania and Klein, 2016; Kullander and Klein, 2002). Eph/ephrin interactions can trigger a wide array of cellular responses, including the control of axon guidance, intercellular junctions, cell adhesion and migration (Kania and Klein, 2016). There is now evidence proving that the Eph/ephrin system plays a leading role in the development and function of the cochlea in mammals (Defourny, 2019).

In this context, unravelling the molecular mechanisms causing hearing loss in *EFNB2/Efnb2* haploinsufficiency could help to improve therapeutic strategies. An auditory brainstem response (ABR) audiometry test revealed that mice lacking one *Efnb2* allele are hearing impaired, and their ABR wave 1 amplitude is significantly reduced (Miko et al., 2008). These data suggest that a defective activity of the cochlea in auditory processing is at least partly responsible for the hearing deficit. Hearing requires the conversion of sound-induced

Abbreviations: GJP, gap junction plaque; PLA, proximity ligation assay; WT, wild-type.

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vibrations into electrochemical signals by mechanosensory hair cells. In mammals, one row of inner hair cells, three rows of outer hair cells, and several types of non-sensory supporting cells are organized in a regular mosaic pattern to form the sensory epithelium of the cochlea (the organ of Corti). Hair cell mechanotransduction relies on ionic gradients, which allows the passive flow of K^+ from the endolymph into the cells. K^+ ions entering hair cells are brought back to the stria vascularis for secretion into the endolymph using a largely intracellular pathway, which involves a system of channels, transporters and gap junctions made of connexin 26 (Cx26) and connexin 30 (Cx30) (Zdebik et al., 2009). Epithelial non-sensory supporting cells are connected via two types of gap junctions, which form a syncytium extending from the spiral limbus to the spiral ligament. On one side, Cx30 is mostly expressed as homomeric (and homotypic) 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 side, Cx30 co-assembles with Cx26 to form heteromeric (and/or heterotypic) channels in other supporting cell types (Ahmad et al., 2003; Sun et al., 2005) (Fig. 1A). In humans, mutations in *GJB2* and *GJB6*, which encode CX26 and CX30, 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). Although recent advances have been made, several important questions remain about gap junction biogenesis and distribution in the cochlea. Among a large number of physiological and pathological processes, Eph and ephrin genes have been shown to control gap junction communication (Davy et al., 2006; Mellitzer et al., 1999; Trease et al., 2019). Since ephrin-B2 was found to be particularly expressed in non-sensory supporting cells (Defourny et al., 2015) (**Fig. S1**), this protein represents an attractive candidate for regulating gap junctions in the cochlear sensory epithelium. Here we show that ephrin-B2 specifically promotes the assembly of Cx30 gap junction plaques (GJPs) between adjacent Deiters' cells in a cell-autonomous manner.

2. Materials and methods

2.1. Animals

Wild-type (WT) and *Efnb2*$^{+/H2BGFP}$ littermates were obtained as previously described (Davy and Soriano, 2007). 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 were fixed in 4% paraformaldehyde for 2 h at room temperature. Whole-mount cochleae or organotypic explants were incubated overnight at 4 °C with primary antibodies directed against Cx30 (rabbit monoclonal IgG clone 16H9L8; 1/100; Thermo Fisher Scientific; RRID: AB_2532309), caveolin-1 (goat polyclonal IgG; 1/100; Abcam; RRID: AB_725985), clathrin heavy chain 1 (goat polyclonal IgG; 1/50;

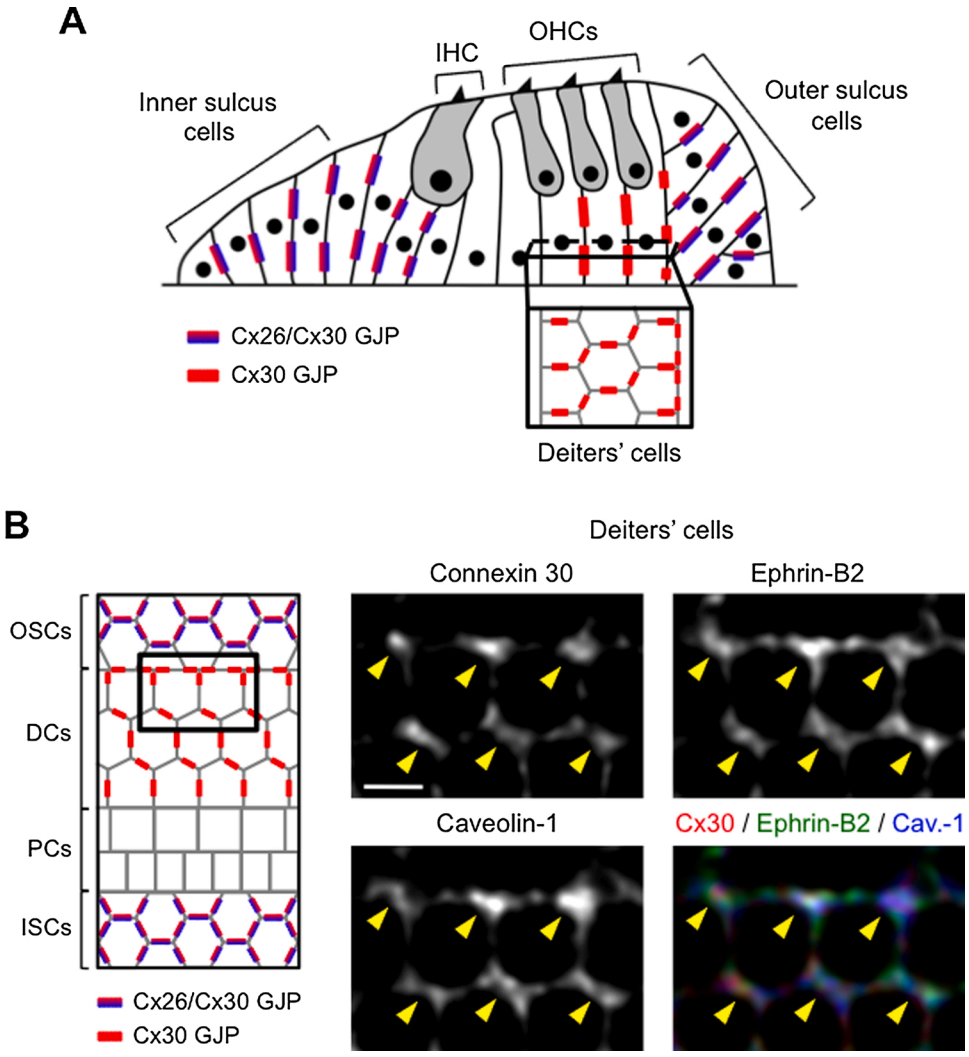

Fig. 1. Cx30 homomeric channels associate with ephrin-B2 into caveolae membrane domains. (**A**) Schematic cross-sectional view of the neonatal organ of Corti showing the distribution of Cx30 and Cx26/Cx30 GJPs between adjacent non-sensory supporting cells of the cochlea. (**B**) Left panel: schematic view from the top across the sensory epithelium of the cochlea showing the distribution of Cx30 and Cx26/Cx30 GJPs between adjacent non-sensory supporting cells. The black square indicates the region where adjacent immunofluorescence images come from. Right panels: Cx30 / ephrin-B2 / caveolin-1 co-immunolabelling of the whole-mount cochlea of a two-day-old mouse. Cx30 immunolabelling largely overlaps with ephrin-B2 and caveolin-1 between adjacent Deiters' cells (arrowheads). Scale bar = 5 µm. DCs = Deiters' cells; GJP = gap junction plaque; IHC = inner hair cell; ISCs = inner sulcus cells; OHCs = outer hair cells; OSCs = outer sulcus cells; PCs = pillar cells.

Novus Biologicals; RRID: AB_11025060) and ephrin-B2 (mouse monoclonal IgG; 1/50; Santa Cruz Biotechnology; sc-398735). 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). Tissues were mounted using Vectashield HardSet Antifade Mounting Medium (Vector Laboratories).

2.3. In situ proximity ligation assay

Whole-mount cochleae were treated and handled as for immunolabelling (see above). Anti-Cx30 (rabbit monoclonal IgG clone 16H9L8; 1/100; Invitrogen; RRID: AB_2532309) and anti-ephrin-B2 (mouse monoclonal IgG; 1/50; Santa Cruz Biotechnology; sc-398735) primary antibodies were incubated with tissues overnight at 4 °C. Oligo-labelled anti-mouse plus and anti-rabbit minus probes (Duolink, Olink Biosciences) were then used as recommend by the manufacturer. Negative control was obtained by omitting one of the two primary antibodies. Cochleae were then labelled with FITC-conjugated anti-Cx30 antibody (mouse monoclonal IgG; 1/100; Santa Cruz Biotechnology; sc-514847 FITC) and mounted using Duolink In Situ Mounting Medium.

2.4. In vitro organotypic assay

Organs of Corti were isolated from two-day-old mice and cultured for 6 h in Dulbecco's Modified Eagle Medium (DMEM) onto Millicell Culture Insert (Millipore), as previously described (Defourny et al., 2013). Organotypic cultured were treated for 2 h with anti-mouse IgG-Fc (5 µg/mL; Abcam; RRID: AB_10681188), or with pre-clustered recombinant mouse EphA4-Fc (5 µg/mL; R&D systems; 641-A4). The monomeric form of EphA4-Fc receptor is not competent to induce a reverse signalling into ephrin-B2-expressing cells. Pre-clustering of EphA4-Fc by IgG-Fc is thus needed to trigger ephrin-B2 reverse signalling. This was achieved by incubating EphA4-Fc with anti-mouse IgG-Fc in a 1/10 ratio for 1 h at 37 °C.

2.5. Confocal microscopy, image analysis and quantifications

Confocal fluorescence images were acquired using the Olympus Fluoview FV1000 confocal system (Olympus Europa GmbH). For comparison between genotypes and culture conditions, all preparations were analysed at the same time, using the same acquisition parameters. For each genotype, the GJPs of 120 Deiters' cells and 120 inner sulcus cells of four WT and four *Efnb2*$^{+/H2BGFP}$ mice were measured and summed, and data were plotted. Within the same cells, the number of Cx30^{+} / clathrin^{+} spots was quantified, and data were plotted. For each culture condition (IgG-Fc vs pre-clustered EphA4-Fc), the GJPs of 90 Deiters' cells and of 90 inner sulcus cells of three independent experiments were measured and summed, and data were plotted. Within the same cells, the number of Cx30^{+} / ephrin-B2^{+} / clathrin^{+} or Cx30^{+} / clathrin^{+} spots was quantified, and data were plotted. Middle portions of the cochlea were considered for comparison between genotypes and culture conditions. In terms of frequency ranges, the middle portion is midway between the apex and the base of the cochlea. This portion is thus the most representative of the entire cochlea. Deiters' cells and inner sulcus cells were randomly chosen and GJPs were measured using ImageJ software. For PLA spot quantification, Cx30 GJPs were divided into four equal portions. The two exterior portions were jointly considered as the peripheral regions of the GJPs, whereas the two interior portions were jointly considered as the central region of the GJPs. PLA spots were quantified within each of these four portions of the GJPs, as well as within the regions adjacent to each lateral border of the GJPs, which are called "perinexus". 30 GJPs were divided and considered for PLA spot quantification. Data were summed and plotted within each of the three groups.

2.6. Statistics

All data are presented as mean ± SEM. Data were statistically analysed using one-Way ANOVA followed by Dunnett's test, or using a two-tailed Student's *t*-test. p-values < 0.05 were considered significant (**p < 0.01; ***p < 0.001).

3. Results and discussion

3.1. Cx30 homomeric channels associate with ephrin-B2 into caveolae membrane domains

At first, it should be remembered that most connexins interact with components of caveolae membrane domains, such as caveolin proteins, which in turn regulate gap junction communication (Langlois et al., 2008; Schubert et al., 2002). Caveolae represent a special type of lipid raft microdomains, which are enriched in cholesterol and sphingolipids (Fielding and Fielding, 2000). It is worth noting here that ephrin-B2 has been shown to associate with caveolin-1^{+} domains in vascular smooth muscle cells (Nakayama et al., 2013). We thus examined whether ephrin-B2 associates with caveolae components, with Cx30 homomeric and/or with Cx26/Cx30 heteromeric channels. We observed that ephrin-B2 does not co-localize with Cx30 in inner sulcus cells, i.e. where Cx30 co-assembles with Cx26 to form heteromeric GJPs (Fig. **S2**). These findings are consistent with previous data showing that Cx26 exhibits a low affinity for cholesterol (Hung and Yarovsky, 2011; Locke and Harris, 2009), and that Cx26/Cx30 GJPs do not associate with lipid rafts in the cochlea (Defourny et al., 2019a). In contrast, we found that ephrin-B2 immunolabelling overlaps with caveolin-1 and Cx30 homomeric GJPs between adjacent Deiters' cells (Fig. 1B).

3.2. Cx30 homomeric channels mostly interact with ephrin-B2 in the peripheral region of the GJPs

To test whether ephrin-B2 interacts with junctional Cx30, we performed an *in situ* proximity ligation assay (PLA) aimed to detect *in situ* protein-protein interactions (Söderberg et al., 2006). We observed that the number of Cx30 / ephrin-B2 PLA positive spots is higher in the peripheral regions than in the central region of Cx30 GJPs. In addition, some PLA positive spots were also observed in the perijunctional region directly bordering the GJPs, which is called « perinexus » (Fig. 2). To ensure the specificity of PLA signals, an assay was performed by omitting the anti-ephrin-B2 primary antibody. In this case, no PLA signals were observed (Fig. 2). This particular distribution of PLA spots suggests that Cx30 / ephrin-B2 interactions could be transient rather than permanent. Indeed, it is commonly believed that the junctional plaque is in a dynamic state, constantly remodeled through recruitment of newly synthesized connexin hemichannels, which accrue to the periphery of the GJP (Gaietta et al., 2002). In this sense, recent data have shown the presence of actin-bound non-junctional Cx30 in the immediate vicinity of GJPs connecting adjacent Deiters' cells (Defourny et al., 2019b). Thus, our findings suggest that ephrin-B2 could be involved in the assembly of GJPs, and in the sequestration of Cx30 homomeric channels into caveolae membrane domains. These observations are thus similar to previous data showing that, in vascular smooth muscle cells, ephrin-B2 promotes the passive sequestration of PDGFRβ into caveolin-1^{+} membrane domains (Nakayama et al., 2013).

Of note here is that no Cx30 / ephrin-B2 PLA positive spots were observed between adjacent inner sulcus cells, i.e. where Cx30 co-assembles with Cx26 to form heteromeric GJPs (Fig. **S3**). These data thus reinforce the idea that Cx30 exhibits distinct GJP assembly mechanisms when it is expressed as homomeric or heteromeric channels in the cochlea (Defourny et al., 2019b).

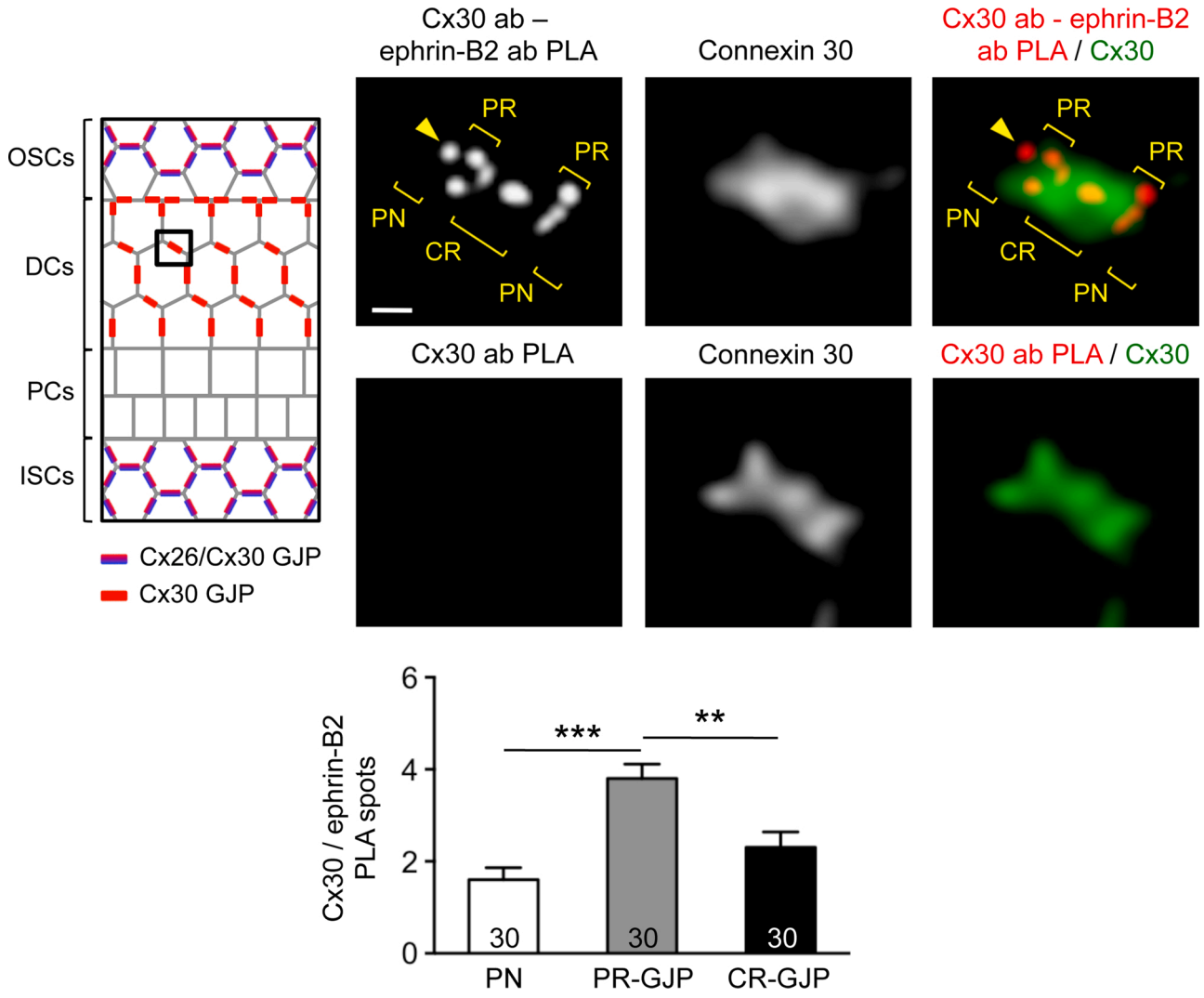

Fig. 2. Cx30 homomeric channels mostly interact with ephrin-B2 in the peripheral regions of the GJPs. Left panel: schematic view from the top across the sensory epithelium of the cochlea showing the distribution of Cx30 and Cx26/Cx30 GJPs between adjacent non-sensory supporting cells. The black square indicates the region where the adjacent immunofluorescence images come from. Right panels: *in situ* proximity ligation assay (PLA) using anti-Cx30 and anti-ephrin-B2 primary antibodies followed by a Cx30 immunolabelling performed on the whole-mount cochlea of a two-day-old mouse. PLA positive spots are mostly observed in the peripheral regions of Cx30 GJPs. Some spots are also present in the perinexus of the GJP (yellow arrowhead). An *in situ* PLA negative control was performed by omitting the anti-ephrin-B2 primary antibody. Lower panel: the number of Cx30 / ephrin-B2 PLA positive spots is significantly higher in the peripheral regions of the GJPs, as compared either to the perinexus or to the central region of the GJPs. $n = 30$ Cx30 GJPs. Data are presented as mean ± SEM. ** $p < 0.01$, *** $p < 0.001$. Scale bar =1 µm. CR / CR-GJP = central region of the GJP; DCs = Deiters' cells; GJP = gap junction plaque; ISCs = inner sulcus cells; PN = perinexus; PR / PR-GJP = peripheral regions of the GJP; OSCs = outer sulcus cells; PCs = pillar cells.

3.3. Efnb2 haploinsufficiency results in excessive endocytosis of Cx30 GJPs

To find out about how *Efnb2* haploinsufficiency could affect cochlear gap junctions, we used a mouse line encoding an *Efnb2*^{H2BGFP} null allele (Davy and Soriano, 2007). In order to compare the stability of Cx30 GJPs between adjacent Deiters' cells, we investigated putative gap junction degradation processes, which usually occur via a clathrin-mediated endocytic pathway (Falk et al., 2014). We observed that the summed length of Cx30 GJPs per Deiters' cell is, on average, about 40 % reduced in *Efnb2*$^{+/H2BGFP}$ as compared to WT mice. Moreover, the number of Cx30^{+} / clathrin^{+} spots per Deiters' cell is significantly increased in *Efnb2*$^{+/H2BGFP}$ as compared to WT mice (Fig. 3). In contrast, the summed length of Cx30 GJPs, as well as the number of Cx30^{+} / clathrin^{+} spots per inner sulcus cell remain unchanged between WT and *Efnb2*$^{+/H2BGFP}$ mice (Fig. **S4**). These data suggest that *Efnb2* haploinsufficiency induces Cx30 GJP disassembly and clathrin-mediated endocytosis in Deiters' cells. It is worth noting that our findings are similar to previous observations showing that lack of ephrin-B2 induces the redistribution of caveolae-associated proteins towards clathrin-coated vesicles (Nakayama et al., 2013). Recently, it has been shown that the degradation of GJPs in the cochlea is a key feature in the pathogenic process leading to progressive age-related hearing loss (Tajima et al., 2020). In this context, it is tempting to speculate that Cx30 GJP defects could be at least partly responsible for the progressive hearing loss observed in patients suffering from *EFNB2* haploinsufficiency. This assumption is supported by electrophysiological recordings showing that both *Gjb6* (which encodes Cx30) deficient and *Efnb2*$^{+/lacZ}$ heterozygous mice exhibit an impaired endolymph K^{+} homeostasis in the inner ear (Dravis et al., 2007; Teubner et al., 2003).

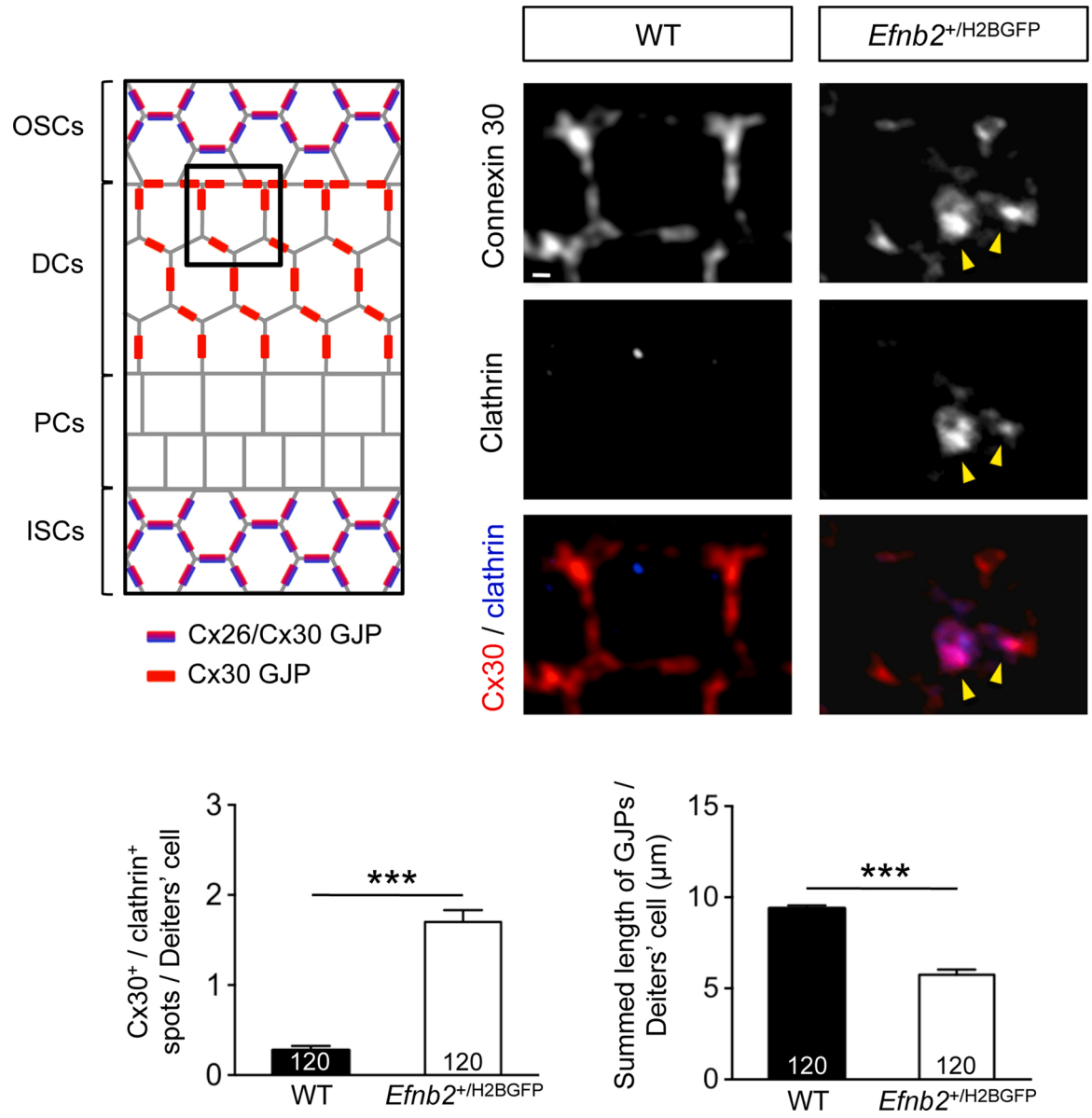

Fig. 3. Efnb2 haploinsufficiency results in excessive endocytosis of Cx30 GJPs. Left panel: schematic view from the top across the sensory epithelium of the cochlea showing the distribution of Cx30 and Cx26/Cx30 GJPs between adjacent non-sensory supporting cells. The black square indicates the region where the adjacent immunofluorescence images come from. Right panels: Cx30 / clathrin co-immunolabelling of whole-mount cochleae of four-day-old WT and *Efnb2*$^{+/H2BGFP}$ mice. Deiters' cells of *Efnb2*$^{+/H2BGFP}$ mice display intracellular Cx30^{+} / clathrin^{+} endocytic vesicles (yellow arrowheads). Lower panels: the number of Cx30^{+} / clathrin^{+} spots per Deiters' cell is significantly increased in *Efnb2*$^{+/H2BGFP}$ mice as compared to WT mice. The summed length of Cx30 GJPs per Deiters' cell is significantly reduced in *Efnb2*$^{+/H2BGFP}$ mice as compared to WT mice. $n = 120$ Deiters' cells from four WT and four *Efnb2*$^{+/H2BGFP}$ mice. Data are presented as mean ± SEM. *** $p < 0.001$. Scale bar = 1 µm. DCs = Deiters' cells; GJP = gap junction plaque; ISCs = inner sulcus cells; OSCs = outer sulcus cells; PCs = pillar cells.

3.4. Ephrin-B2 reverse signalling promotes the clathrin-dependent endocytosis of Cx30 GJPs

Although ephrin-B2 usually exerts biological effects in a non-cell-autonomous manner, some observations have shown that ephrin-B2 can also affect the cell behaviour independently of Eph receptor binding (Bochenek et al., 2010; Foo et al., 2006). To examine whether ephrin-B2 cell-autonomously promotes the assembly of Cx30 into GJPs, we performed an organotypic assay in which ephrin-B2 is activated using soluble pre-clustered EphA4-Fc. The endocytosis of ephrin-B ligands and gap junctions share common mechanisms. Upon binding to Eph receptor, the reverse endocytosis of transmembrane ephrin-B usually occurs via a clathrin-mediated pathway (Parker et al., 2004). In a similar fashion, the internalization of double-membrane GJPs also occurs via a clathrin-dependent endocytic process (Piehl et al., 2007). In agreement, we observed that ectopic activation of ephrin-B2 reverse signalling by EphA4-Fc leads to disassembly of GJPs through clathrin-dependent endocytosis of Cx30 (Fig. 4). Thus, these data argue in favour of a cell-autonomous role of ephrin-B2 in the assembly of Cx30 GJPs into caveolae membrane domains. These observations are consistent with previous data showing that gap junction communication is promoted at ephrin-ephrin interface within a cell compartment, whereas it is inhibited at Eph-ephrin interface (Davy et al., 2006; Mellitzer et al., 1999). In this sense, adjacent ephrin-B2^{+} Deiters' cells are largely coupled via gap junction channels (Jagger and Forge, 2006). In contrast, gap junction communication is non-existant between Deiters' cells and adjacent outer hair cells, which strongly express EphA4, but only few ephrin-B2 (Defourny et al., 2015; Jagger and Forge, 2006).

It is worth noting here that incubation of pre-clustered EphA4-Fc with organotypic cultures does not induce clathrin-mediated

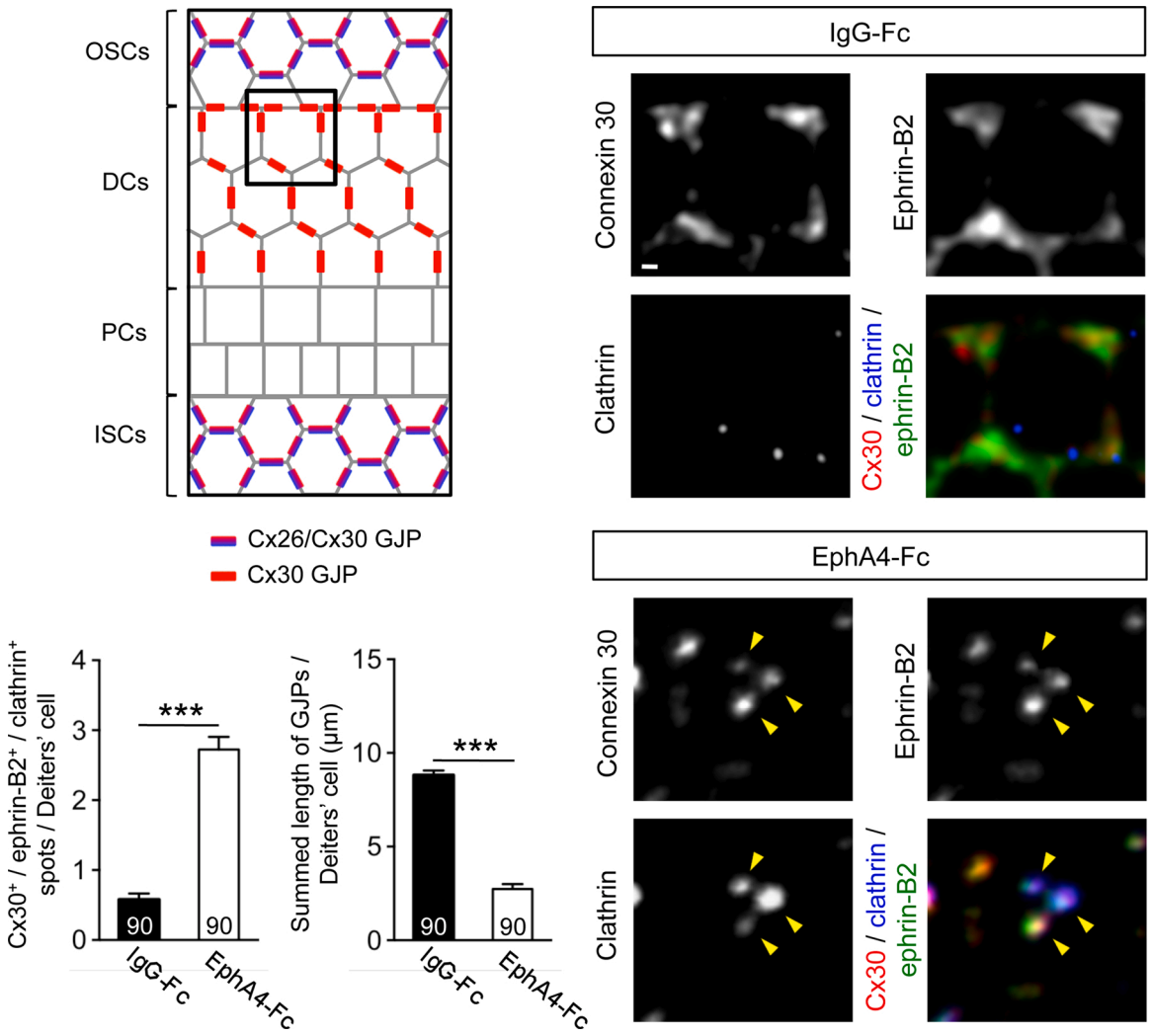

Fig. 4. Ephrin-B2 reverse signalling induces clathrin-mediated endocytosis of Cx30 GJPs. Upper left panel: schematic view from the top across the sensory epithelium of the cochlea showing the distribution of Cx30 and Cx26/Cx30 GJPs between adjacent non-sensory supporting cells. The black square indicates the region where the adjacent immunofluorescence images come from. Right panels: Cx30 / ephrin-B2 / clathrin co-immunolabelling of mouse cochlear organotypic cultures treated with IgG-Fc or with pre-clustered EphA4-Fc. Deiters' cells treated with pre-clustered EphA4-Fc display intracellular $Cx30^{+}$ / ephrin-$B2^{+}$ / $clathrin^{+}$ endocytic vesicles (arrowheads). Lower left panels: the number of $Cx30^{+}$ / ephrin-$B2^{+}$ / $clathrin^{+}$ spots per Deiters' cell is significantly increased in organotypic cultures treated with pre-clustered EphA4-Fc as compared to cultures treated with IgG-Fc. The summed length of Cx30 GJPs per Deiters' cell is significantly reduced in organotypic cultures treated with pre-clustered EphA4-Fc as compared to cultures treated with IgG-Fc. $n = 90$ Deiters' cells from three cultures treated with IgG-Fc or with pre-clustered EphA4-Fc. Data are presented as mean ± SEM. *** $p < 0.001$. Scale bar = 1 µm. DCs = Deiters' cells; GJP = gap junction plaque; ISCs = inner sulcus cells; OSCs = outer sulcus cells; PCs = pillar cells.

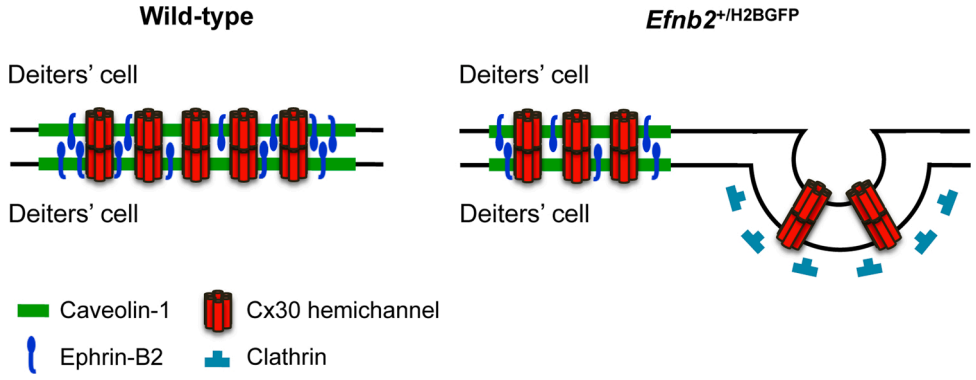

Fig. 5. A model for how *Efnb2* haploinsufficiency induces GJP disassembly and endocytosis in the cochlea. Schematic representation of Cx30 GJP assembly and degradation processes in Deiters' cells of WT and *Efnb2*$^{+/H2BGFP}$ mice. In WT mice, ephrin-B2 promotes the assembly of Cx30 channels into caveolae membrane domains. In contrast, *Efnb2* haploinsufficiency induces clathrin-mediated endocytosis of Cx30 GJPs.

internalization of GJPs in inner sulcus cells, i.e. where Cx30 is co-expressed with Cx26 (Fig. S5).

4. Conclusion

Overall, we describe here a novel role for ephrin-B2 in the assembly of Cx30 GJPs in the cochlear sensory epithelium. We observed that mice encoding an *Efnb2* null allele display excessive clathrin-mediated endocytosis of Cx30 GJPs in non-sensory Deiters' cells (Fig. 5). According to recent observations (Tajima et al., 2020), this early GJP degradation could certainly play a role in the pathogenic process leading to progressive sensorineural hearing loss due to *Efnb2/EFNB2* haploinsufficiency.

CRediT authorship contribution statement

Jean Defourny: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Funding acquisition. **Christophe Audouard:** Resources. **Alice Davy:** Resources, Writing - review & editing. **Marc Thiry:** Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.brainresbull.2021.06.008.

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