Brief Communications

# Hearing Is Normal without Connexin30

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***Gjb2* and *Gjb6,* two contiguous genes respectively encoding the gap junction protein connexin26 (Cx26) and connexin 30 (Cx30) display overlapping expression in the inner ear. Both have been linked to the most frequent monogenic hearing impairment, the recessive isolated deafness DFNB1. 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* both in human and in mouse. Thus, it is imperative that any role of Cx30 in audition be clearly evaluated. Here, we developed a new *Cx30* knock-out mouse model (*Cx30*$^{\Delta/\Delta}$) in which half of *Cx26* expression was preserved. Our results show that *Cx30* and *Cx26* coordinated expression is dependent on the spacing of their surrounding chromosomic region, and that *Cx30*$^{\Delta/\Delta}$ mutants display normal hearing. Thus, in deaf patients with *GJB6* deletion as well as in the previous *Cx30* knock-out mouse model, defective *Cx26* expression is the likely cause of deafness, and in contrast to current opinion, Cx30 is dispensable for cochlear functions.**

## Introduction

Cx30 and Cx26, which coassemble in gap junction (GJ) channels allowing the direct passage of ions and small molecules between the cytoplasms of cochlear non-sensory cells, are considered crucial for hearing (Forge et al., 2003; Martínez et al., 2009). In humans, >100 mutations in *GJB2,* nearly all affecting proper translation of Cx26, underlie the recessive non-syndromic hearing impairment DFNB1, the most common cause of prelingual inherited deafness (del Castillo and del Castillo, 2012). Additionally, *Cx26* inactivation results in profound deafness in mouse (Cohen-Salmon et al., 2002; Sun et al., 2009; Crispino et al., 2011). In contrast to *GJB2*, silencing point mutations in *GJB6* encoding Cx30 and located just 30 kb upstream from *GJB2* in the same DFNB1 locus on chromosome 13, have never been found in deafness cases, although three missense mutations are associated with dominantly inherited hearing loss (Grifa et al., 1999; Nemoto-Hasebe et al., 2009; Wang et al., 2011). Thus far, the only evidence supporting that the absence of Cx30 leads to deafness in human was provided by the discovery of two large deletions truncating *GJB6* and segregating in a double heterozygous state with a single *GJB2* recessive mutation, hence suggesting the possibility of digenic inheritance for DFNB1 (Lerer et al., 2001; Pallares-Ruiz et al., 2002; del Castillo et al., 2002, 2005). However, skin or buccal biopsies of these patients have revealed that not only Cx30 but also Cx26 expression was dramatically reduced, demonstrating that both genes are coregulated and suggesting the existence of a common *cis*-regulatory element in the DFNB1 locus (Common et al., 2005; Rodriguez-Paris and Schrijver, 2009). A comparable situation is found in mouse, in which Cx30 inactivation causes profound deafness (Teubner et al., 2003; Cohen-Salmon et al., 2007; Sun et al., 2009), but also strongly reduces the expression of Cx26 (Ortolano et al., 2008; Lynn et al., 2011). Thus, how exactly the lack of Cx30 translates into a deafness phenotype still remains unclear.

## Materials and Methods

*Animal experimentation.* Experiments on mice were performed according to Institut National de la Santé et de la Recherche Médicale welfare guidelines as well as in compliance with the European Community Council Directive of November 24, 1986 (86/609/EEC).

*Mice. Cx30*$^{fl/fl}$ and *Cx30*$^{-/-}$ mice were maintained on a pure C57BL/6 background. *Cx30*$^{\Delta/\Delta}$ and *Cx30*$^{+/+}$ mice used in all experiments were littermates. Mice of either sex were used.

*Construction of Cx30 floxed mice.* We subcloned a 10,950-bp fragment of 129/Sv mouse genomic DNA, containing *Cx30* exon 3, the single *Cx30* coding exon, by using gap-repair homologous recombination (Lee et al., 2001) on BAC 166gt19 from the CITB-CJ7B mouse BAC library (Re-

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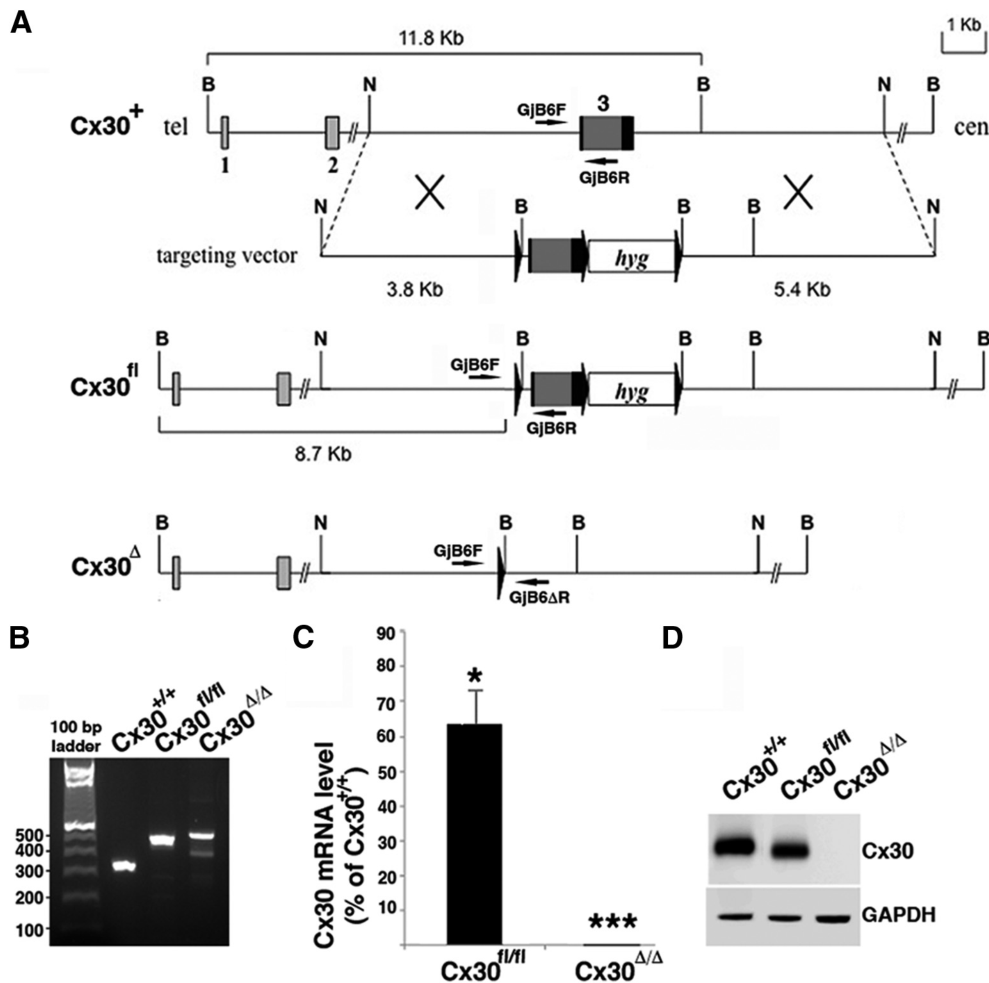


**Figure 1.** Conditional *Cx30* inactivation model. ***A***, Homologous recombination resulting in the $Cx30^{fl}$ allele where *Cx30* transcript sequence (coding sequence, dark gray box; untranslated 5′ and 3′ sequences, black boxes) is flanked by LoxP sites (arrowheads) followed by the hygromycin resistance gene (*hyg*) and a LoxP site. Crossing $Cx30^{fl/fl}$ mice with *Pgk*-Cre mice generated $Cx30^{\Delta/\Delta}$ mice. Arrows indicate genotyping primers. ***B***, PCR genotyping: GjB6 R and F produce a 350 bp band in $Cx30^{+/+}$ mice, and a 488 bp band in $Cx30^{fl/fl}$ mice. GjB6F and GjB6ΔR produce a 521 bp band in $Cx30^{\Delta/\Delta}$ mice. ***C***, ***D***, *Cx30* expression was quantified by qPCR (***C***) and immunoblot (***D***) in whole inner ear. Mean ± SEM; 3 independent experiments; 3 mice of each genotype per experiment; *t* test. $^*p < 0.05$, $^{***}p < 0.0001$.

search Genetics). To prepare the targeting vector, we introduced a *loxP* site upstream and a floxed *PGK-hyg*-resistance cassette downstream of *Cx30* exon 3, by means of bacterial recombinogenic engineering techniques (Liu et al., 2003). Embryonic stem (ES) cells derived from a 129/Sv embryos were electroporated with the purified, linearized targeting vector and plated on hygromycin selective medium. Two independently obtained ES cell clones, in which one *Cx30* wild-type allele had been replaced with the *Cx30* floxed allele, were used to generate chimeric mice by injecting C57BL/6 host blastocysts. All chimaeras tested were fertile and transmitted the transgene to their offspring. *Cx30* floxed mice ($Cx30^{fl/fl}$) from both lines were born at the expected mendelian frequency and showed no differences in growth, development or fertility compared with their $Cx30^{+/+}$ littermates.

*Genotyping.* Genotyping of the $Cx30^{fl}$ allele was performed by PCR analysis using a primer binding in the Cx30 transcribed sequence Gjb6R 5′-TTCCCTATGCTGGTAGAGTGCTTGT-3′ and a primer binding upstream of the first loxP site Gjb6F 5′-GCAGTAACTTATTGAAACCC TTCACCT-3′. Genotyping of the $Cx30^{\Delta}$ allele was performed using the Gjb6F and a primer binding downstream of the third loxP site Gjb6ΔR 5′-CCCACCATCAAGGTTGAACT-3′.

*Quantitative PCR analyses of Cx26 and Cx30 transcription.* Postnatal day 30 (P30) mice were anesthetized by lethal injection of pentobarbital and decapitated. Total RNA was extracted from whole dissected inner ear using the RNeasy kit (Qiagen). Reverse transcription was performed using 1 μg of RNA. Quantitative PCR (qPCR) was conducted on cDNAs using Cx26 and Cx30 primers previously designed (Ortolano et al., 2008) (working concentration 300 nM) and Hprt primers: *Hprt*.f 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and *Hprt*.r 5′-GATTCA ACTTGCGCTCATCTTAGGC-3′ (working concentration 300 nM), using SYBR Green PCR master kit (Applied Biosystems). PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were analyzed in triplicate on a LC480 Roche LightCycler. The relative abundance of amplified Cx30 or Cx26 cDNA was calculated as $2^{-\Delta Ct}$, where ΔCt (change in cycle threshold) equals Ct of Cx30 or Cx26 in mutants minus wild-type mice.

*Western blot analysis of Cx26 and Cx30 expression.* P30 mice were anesthetized by lethal injection of pentobarbital and decapitated. Inner ears were dissected, reduced to powder at −80°C, and immediately dissolved in PBS with 2% SDS, and 1× EDTA-free Complete Protease Inhibitor (Roche). Lysates were sonicated 3 times at 10 Hz (Vibra-Cell VCX130) and centrifuged 20 min at 10,000 × *g* at 4°C. Supernatants were boiled in 5× Laemmli loading buffer. Protein content was measured using the Pierce 660 nm protein assay reagent (Thermo Scientific). Equal amounts of proteins were separated by denaturing electrophoresis in NuPAGE gel (Invitrogen), electrotransferred to nitrocellulose membranes, first analyzed using either the primary rabbit anti-Cx30 antibody (Invitrogen, 1:500) or the rabbit anti-Cx26 antibody (Invitrogen, 1:500), and HRP-conjugated secondary antibodies. HRP activity was visualized by enhanced chemiluminescence using Western Lightning Plus enhanced ECL system (PerkinElmer). Blots were reprobed with mouse monoclonal

anti-GAPDH-peroxidase (Sigma, 1:10,000) to check the protein load. Chemiluminescence imaging was performed on a LAS4000 (Fujifilm). Semiquantitative densitometric analysis was performed with ImageJ software.

*Assessment of hearing impairment.* Auditory brainstem response (ABR) reflects the electrical response of the cochlear ganglion neurons and the nuclei of the central auditory pathway to sound stimulation. Their threshold assesses the cochlear sensitivity. The distortion product otoacoustic emissions (DPOAEs) are a noninvasive measure of outer hair cell amplification activity. ABRs and DPOAEs were recorded and analyzed as described previously (Verpy et al., 2008).

## Results

We developed a novel knock-out mouse model ($Cx30^{\Delta/\Delta}$) in which, in contrast to the previous model (referred as $Cx30^{-/-}$ in the literature) (Teubner et al., 2003), *Cx30* was removed without perturbing the surrounding sequences. Indeed, in $Cx30^{-/-}$ mice, the Cx30 coding exon was both removed and replaced by a *lacZ* reporter gene and a *neo* resistance cassette, and we suspected that such strong manipulation may impact on the expression of the nearby Cx26 encoding gene and on the phenotypic outcome. In our model, the Cx30-coding exon was replaced with a *Cx30* floxed allele by homologous recombination and the $Cx30^{fl/fl}$ mouse strain was crossed with *Pgk*-Cre mice which ubiquitously express the Cre recombinase (Lallemand et al., 1998), allowing for the global deletion of the *Cx30* transcript sequence (see Materials and Methods; Fig. 1*A*,*B*). To test for *Cx30* inactivation, we measured the level of *Cx30* expression in the inner ear by qPCR (see Materials and Methods; Fig. 1C). As expected, no Cx30 transcript was detected in $Cx30^{\Delta/\Delta}$ mice, whereas $Cx30^{fl/fl}$ displayed reduced expression of Cx30 mRNA to 64 ± 3% of $Cx30^{+/+}$ mice used as controls, suggesting an effect of the 3′ hygromycin gene insertion on *Cx30* expression. Consistent with the mRNA results, Western blot analysis of protein levels in the inner ear of $Cx30^{fl/fl}$ mice showed a reduced level (64 ± 13%) of Cx30, which was undetectable in $Cx30^{\Delta/\Delta}$ mice (Fig. 1D).

To investigate the issue of *Cx30* and *Cx26* coregulation, we quantified the mRNA level of *Cx26* in the inner ear of $Cx30^{\Delta/\Delta}$, $Cx30^{fl/fl}$ and $Cx30^{-/-}$ mice relative to that of $Cx30^{+/+}$ controls (see Materials and Methods; Fig. 2). In $Cx30^{-/-}$ *mice*, we found a rather drastic reduction to 27 ± 6%. In $Cx30^{fl/fl}$ mice, Cx26 mRNA was decreased to 54 ± 6%, a level comparable to that of Cx30 mRNA (Fig. 2A). Of note, in $Cx30^{\Delta/\Delta}$ mice, *Cx26* transcription was 62 ± 5% of that measured in $Cx30^{+/+}$ (Fig. 2B), a level comparable to $Cx30^{+/-}$ mice (Rodriguez et al., 2012). These results were confirmed by Western blot analysis (Fig. 2B). Compared with $Cx30^{+/+}$ mice, Cx26 protein was significantly reduced to 10 ± 3% in $Cx30^{-/-}$ mice, to 35 ± 13% in $Cx30^{fl/fl}$ mice, and to 52 ± 4% in $Cx30^{\Delta/\Delta}$ mice. Thus, the level of residual Cx26 protein is 5 times higher in $Cx30^{\Delta/\Delta}$ than in $Cx30^{-/-}$ mice.

To examine the physiological consequences of these reduced connexin levels, we next examined hearing in $Cx30^{\Delta/\Delta}$ mutants by measuring ABR thresholds as well as DPOAEs (see Materials and Methods). To our surprise, $Cx30^{\Delta/\Delta}$ mutants showed ABR thresholds and DPOAEs indistinguishable from $Cx30^{+/+}$ mice or $Cx30^{fl/fl}$ mice (Fig. 3). Thus, micromechanics and transduction, the two basic functions underpinning normal hearing (Ashmore and Gale, 2000) are maintained without Cx30, provided that the Cx30 coding exon is deleted without introducing other perturbation of the surrounding sequences. Accordingly, the simple insertion of *Hyg* gene into the 3′ end of *Cx30* in $Cx30^{fl/fl}$ mice decreased the expression of both *Cx30* and *Cx26* (Figs. 1, 2), although their residual level was sufficient to generate normal ABR and DPOAEs (Fig. 3).

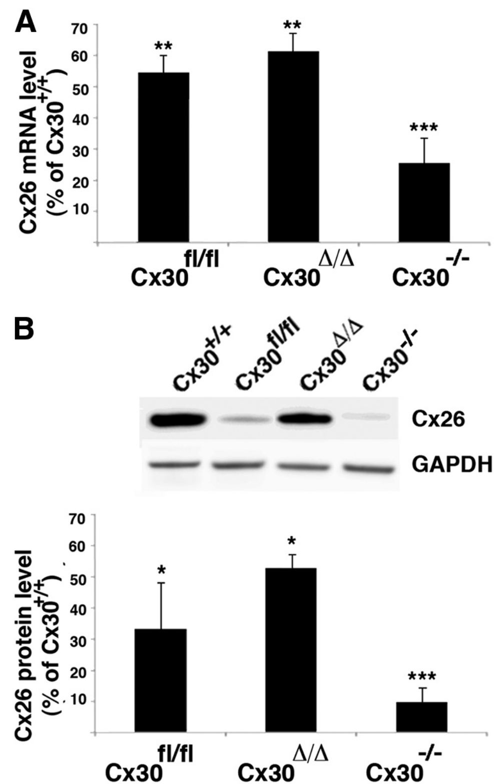


**Figure 2.** Cx26 expression in the inner ear of $Cx30^{+/+}$, $Cx30^{fl/fl}$, $Cx30^{\Delta/\Delta}$, and $Cx30^{-/-}$ mice. Cx26 expression quantified by qPCR (***A***) and quantitative immunoblot (***B***). Mean ± SEM; 3 independent experiments; 3 mice of each genotype per experiment; *t* test. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$.

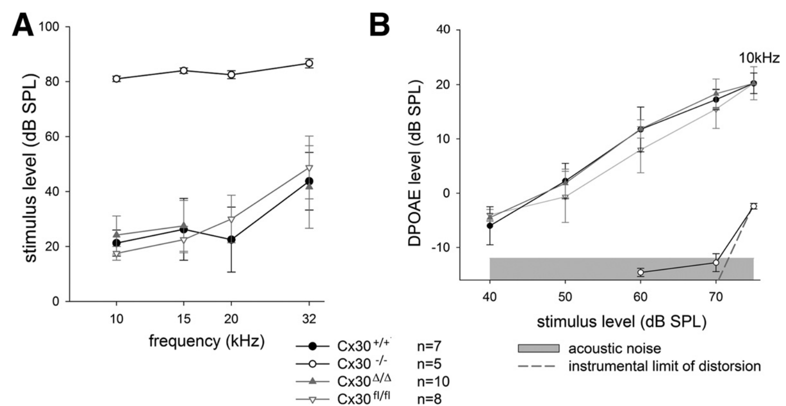


**Figure 3.** Assessment of hearing in $Cx30^{\Delta/\Delta}$ mice. ***A***, ABR thresholds (mean ± SEM) recorded at 20 – 100 dB SPL across a frequency range of 10 – 32 kHz. No significant difference in threshold was found between $Cx30^{+/+}$ and $Cx30^{\Delta/\Delta}$ or $Cx30^{fl/fl}$ mice. In contrast, ABR waveforms showed up only above 80 dB in the $Cx30^{-/-}$ mice. ***B***, DPOAEs at 10 kHz for equilevel stimuli (mean ± SEM), increasing stepwise from 30 to 75 dB SPL. No significant difference in threshold or level was found between $Cx30^{+/+}$ and $Cx30^{\Delta/\Delta}$ or $Cx30^{fl/fl}$ mice. In contrast, $Cx30^{-/-}$ mice had no DPOAE up to 75 dB SPL. ***A***, ***B***, Kruskal–Wallis nonparametric test, $p > 0.1$.

## Discussion

Together, our results demonstrate that hearing loss phenotype exhibited by *Cx30*$^{-/-}$ mice depends on the cumulative effect of deletion of *Cx30* and 3′ insertion of the *lacZ* and *neo* genes, which are associated with dramatically reduced *Cx26* levels (Figs. 1, 2). They also suggest that further investigation of two recent knock-in mouse models for connexin mutations linked to human deafness (Schütz et al., 2010, 2011) is needed, as both models present with *lacZ* insertion downstream of the mutated exon. Our data also support the notion of a *cis*-acting element coregulating *Cx30* and *Cx26,* whose efficiency depends on the spacing of the surrounding sequences. Similarly, large deletions in the DFNB1 locus in humans may perturb the functionality of such element, leading to the downregulation of both *GJB6* and *GJB2* and to profound deafness (del Castillo and del Castillo, 2012). This hypothesis is further supported by the recent discovery of deletions causing DFNB1 that exclude both *GJB2* and *GJB6* (Wilch et al., 2006, 2010).

In conclusion, our results show the following. (1) They demonstrate that Cx30 is dispensable for hearing. In this light, what role does Cx30 play in the cochlea? First, Cx30 itself modulates the expression of *Cx26*. Indeed, reexpression of *Cx30* in organotypic *Cx30*$^{-/-}$ cochlear cultures partially restores *Cx26* expression (Ortolano et al., 2008). Second, Cx26 and Cx30 coassemble in gap junctions in the cochlea, and their coexpression results in channels with dye-transfer properties that differ from those of homomeric Cx26 channels (Forge et al., 2003). Therefore, deafness linked to the three known Cx30 dominant missense mutations (Grifa et al., 1999; Nemoto-Hasebe et al., 2009; Wang et al., 2011) might reflect functional alterations in heteromeric channels comprising normal Cx26 and mutated Cx30. (2) Our results indicate that defective *Cx26* expression is the likely cause of hearing loss previously reported in *Cx30*$^{-/-}$ mice, which may explain why overexpression of *Cx26* rescues hearing in these mice (Ahmad et al., 2007). Similarly, the hearing loss of patients harboring one of the known large deletions encompassing *GJB6* would be linked to abnormal expression of *GJB2*. In this case, the current digenic inheritance hypothesis including cooperative functions between Cx30 and Cx26 would no longer be tenable. Of note, *Cx30*$^{\Delta/\Delta}$ and *Cx30*$^{+/-}$ mice (Rodriguez et al., 2012) display normal hearing with Cx26 expression reduced by half. Thus, it remains to be determined at which level of Cx26 expression hearing is lost. (3) Our results explain why no *GJB6* silencing mutations have been found in human deafness cases so far.

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