# Coordinated control of connexin 26 and connexin 30 at the regulatory and functional level in the inner ear

Saida Ortolano[a,b,1], Giovanni Di Pasquale[c,1], Giulia Crispino[a], Fabio Anselmi[a], Fabio Mammano[a,b,2], and John A. Chiorini[c,2]

[a]Venetian Institute of Molecular Medicine, Foundation for Advanced Biomedical Research, 35129 Padua, Italy; [b]Department of Physics ''G.Galilei,'' University of Padua, 35129 Padua, Italy; and [c]Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892

Edited by Michael V. L. Bennett, Albert Einstein College of Medicine, Bronx, NY, and approved September 26, 2008 (received for review January 28, 2008)

**Connexin 26 (Cx26) and connexin 30 (Cx30) are encoded by two genes (*GJB2* and *GJB6*, respectively) that are found within 50 kb in the same complex deafness locus, DFNB1. Immunocytochemistry and quantitative PCR analysis of Cx30 KO mouse cultures revealed that Cx26 is downregulated at the protein level and at the mRNA level in nonsensory cells located between outer hair cells and the stria vascularis. To explore connexin coregulation, we manipulated gene expression using the bovine adeno-associated virus. Overexpression of Cx30 in the Cx30 KO mouse by transduction with bovine adeno-associated virus restored Cx26 expression, permitted the formation of functional gap junction channels, and rescued propagating $Ca^{2+}$ signals. Ablation of Cx26 by transduction of $Cx26^{loxP/loxP}$ cultures with a Cre recombinase vector caused concurrent downregulation of Cx30 and impaired intercellular communication. The coordinated regulation of Cx26 and Cx30 expression appears to occur as a result of signaling through PLC and the NF-κB pathway, because activation of $IP_3$-mediated $Ca^{2+}$ responses by stimulation of P2Y receptors for 20 min with 20 nM ATP increased the levels of Cx26 transcripts in Cx30 KO cultures. This effect was inhibited by expressing a stable form of the IκB repressor protein that prevents activation/translocation of NF-κB. Thus, our data reveal a $Ca^{2+}$-dependent control in the expression of inner ear connexins implicated in hereditary deafness as well as insight into the hitherto unexplained observation that some deafness-associated DFNB1 alleles are characterized by hereditable reduction of both *GJB2* and *GJB6* expression.**

adeno-associated virus | cochlea | gap junctions | gene transfer | hearing loss

In the cochlea, the connexin isotypes, connexin 26 (Cx26) and connexin 30 (Cx30) account for the majority of intercellular gap junction (GJ) channels (1–3). Cx26 and Cx30 share 77% amino acid identity and colocalize in supporting and epithelial cells of the organ of Corti, in basal and intermediate cells of the stria vascularis (SV), and in type 1 fibrocytes of the spiral ligament (4–7) [for anatomy, see ref. 8 and supporting information (SI) Fig. S1*A*]. Maintenance of ionic balance in the inner ear is crucial for sensory transduction (9–11), which is modulated by complex signaling pathways (12). Cochlear GJ channels delineate networks of supporting cells that have been thought to participate in buffering and recycling of $K^+$ following mechanotransduction by the sensory hair cells (9–11). Previous studies suggested that inner ear GJ channels contain both Cx26 and Cx30 subunits in heteromeric assemblies (13–15). Connexin channels exhibit remarkable selectivity among larger permeants, including second messengers (16, 17). Furthermore, permeability of heteromeric/heterotypic connexin channels may differ from that of the corresponding homomeric channels (18).

The genes encoding Cx26 (*GJB2*) and Cx30 (*GJB6*) are found within 50 kb in the DFNB1 complex deafness locus on chromosome 13. Deafness is a major cause of sensory deficit in humans, and mutations in the DFNB1 locus are almost as frequent as those causing cystic fibrosis (19, 20). Several studies have been carried out to understand the molecular mechanisms underlying DFNB1 pathogenesis, either by resorting to creation of murine models (21, 22) or by analyzing mutant Cx26 variants in heterologous expression systems (23). Interestingly, it has been proposed that deletions such as Δ(*GJB6*-D13S1830) and Δ(*GJB6*-D13S1854) may eliminate a putative, as yet unknown, *cis* regulatory element crucial for expression of *GJB2* (23–26). Recently, a potential coregulation of *GJB2* and *GJB6* has been postulated to underlie hearing loss in members of large kindred of German descent carrying a newly identified DFNB1 allele in trans with the 35delG allele of *GJB2*, resulting in dramatically reduced expression of both genes (27). A substantial decrease in Cx26 protein level (but not mRNA) was recently reported in cochlea of Cx30 KO mice (28).

The inability of one connexin isoform to compensate for loss (of function) of another in KO mouse models (21, 22) is a prevalent, albeit not universal, feature in connexin-related disorders (29, 30). Indeed, transgenic expression of extra copies of the Cx26 gene from a modified bacterial artificial chromosome in a Cx30 KO background restored cochlea development and hearing (28). Transcriptional regulation of connexin genes is altered during development as well as in several pathological conditions (31) and is related to ionic selectivity, distinct gating sensitivity to protein kinases, and selective permeability to second messengers (29).

In this article, we investigate the interaction between Cx26 and Cx30 in organotypic cochlear cultures from Cx30 KO and $Cx26^{loxP/loxP}$ mice (21, 22) exploiting a bovine adeno-associated viral (BAAV) vector (32). Previous use of this vector system demonstrated efficient gene transfer in the inner ear with a minimum of toxicity (33). Our findings demonstrate coordinated regulation of these genes in the region between outer hair cells and the SV (Fig. S1), which we shall broadly refer to as the outer sulcus (*os*), and suggest that their expression is sensitive to changes in activity of NF-κB (34, 35), a $Ca^{2+}$-sensitive transcription factor (36).

## Results

**Interrelated Expression of Cx26 and Cx30 in Mouse Cochlear Cultures.** We performed immunohistochemistry of Cx26 in cochlear cultures (Fig. S1 *B* and *C*) obtained from postnatal day (P) 5 Cx30

Author contributions: F.M. and J.A.C. designed research; S.O., G.D.P., G.C., and F.A. performed research; S.O., G.D.P., F.M., and J.A.C. contributed new reagents/analytic tools; S.O., F.A., and F.M. analyzed data; and F.M. and J.A.C. wrote the paper.

This article is the result of a joint collaboration between J.A.C., and F.M. to utilize gene transfer technology in the inner ear.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]S.O. and G.D.P. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: fabio.mammano@unipd.it or jchiorini@dir.nidcr.nih.gov.

This article contains supporting information online at www.pnas.org/cgi/content/full/0800831105/DCSupplemental.

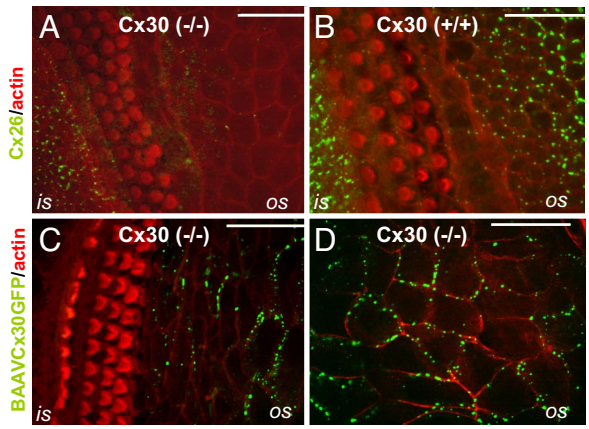


**Fig. 1.** Connexin expression patterns in organotypic cultures from WT and Cx30 KO mice. (*A*, *B*) Immunoassays of Cx26 (green; red, actin) in a representative culture from a Cx30 KO mouse [*A*, labeled Cx30(−/−)] and in a control culture from a WT littermate [*B*, labeled Cx30(+/+)]. (*C*, *D*) Representative cultures from Cx30 KO mice transduced with BAAVCx30GFP (green; red, actin) show recombinant Cx30GFP protein expression in ≈90% of the *os* cells at points of contact between adjacent cells; images were acquired 48 h after adding the viral vectors to the culture medium. (Scale bars: *A*–*C*, 50 μm; *D*, 30 μm.)

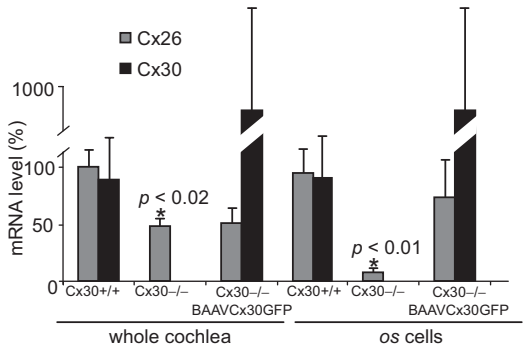


**Fig. 2.** Effect of transduction with BAAVCx30GFP. Histogram representation of Cx26 and Cx30 qPCR products amplified from reverse-transcribed mRNAs extracted from whole cochlea or microdissected *os* cells from indicated samples of Cx30 KO mice or WT littermates ($n = 4$ for each indicated condition, error bars represent SD, *P* value estimated by Student's *t* test). Cx30(+/+), mRNA from Cx30 WT cultures; Cx30(−/−), mRNA from Cx30 KO cultures; Cx30(−/−) BAAVCx30-GFP, mRNA from Cx30 KO cultures transduced with BAAV expressing GFP-tagged Cx30.

KO mice and determined that, compared with WT controls (Fig. 1*B*), Cx26 is remarkably downregulated in *os*-supporting and epithelial cells (Fig. 1*A*). However, Cx26 is still detectable in Deiters' cells as well as in nonsensory cells in the region between inner hair cells and spiral limbus (Fig. 1*A*), which we shall broadly refer to as the inner sulcus and which, at this developmental stage, is occupied by a transient structure known as the Kölliker organ or the greater epithelial ridge (37, 38) (Fig. S1).

To test if the level of expression of Cx26 was restored following replacement of the Cx30 gene into Cx30 KO mice, organ cultures from these mice were transduced with a BAAV vector encoding a Cx30GFP fusion protein (BAAVCx30GFP). Confocal fluorescence microscopy showed that recombinant Cx30GFP protein was expressed in ≈90% of the *os* cells and targeted to the cell plasma membrane at points of contact between adjacent cells (Fig. 1 *C* and *D*), suggesting that fusion of Cx30 to GFP did not interfere with the normal subcellular localization of Cx30. Indeed, the recombinant protein expression pattern closely resembled that of transduced WT controls in rats (Fig. S2*A*) or mice (Fig. S2*B*) as well as that of endogenous Cx30 in mouse cultures immunoassayed with a Cx30-specific antibody (Fig. S2*C*).

To establish whether the Cx30-dependent regulation of Cx26 observed at the protein level also occurs at the mRNA level, we extracted mRNA either from the whole cochlea or from microdissected *os* cells. In all samples we quantified Cx26 and Cx30 transcripts by quantitative PCR (qPCR) on cDNAs, obtained by reverse transcription, and compared the level of expression with that of actin transcripts (data are normalized to Cx26/actin ratio in cultures from WT control mice). Results, summarized in Fig. 2, show that, as expected, the expression of Cx26 and Cx30 mRNA was similar in whole cochlea samples. However, a statistically significant decrease in Cx26 mRNA was detected in whole cochlea samples from Cx30 KO mice. This reduction was more pronounced in microdissected *os* cells compared with corresponding controls. Following overexpression of Cx30 by transduction of Cx30 KO cultures with BAAVCx30GFP, Cx26 levels were not appreciably modified in whole cochlea samples. By contrast, similar overexpression of Cx30 in microdissected *os* cells restored Cx26 mRNA expression at levels similar to those in WT controls.

To investigate whether the reciprocal is true and Cx26 levels affect Cx30 expression, we developed a strategy to downregulate Cx26 in cochlear cultures of the $Cx26^{loxP/loxP}$ mouse model (21). To catalyze site-specific recombination of the DNA between the *loxP* sites, cultures obtained from P5 $Cx26^{loxP/loxP}$ mice were incubated with BAAV vectors containing a Cre-IRESGFP expression cassette (BAAVCre-IRESGFP). Following vector transduction, Cre expression resulted in a recombination of the $Cx26^{loxP/loxP}$ gene and a drastic decrease of Cx26 protein in *os* cells (Fig. 3*A*) compared with nontransduced $Cx26^{loxP/loxP}$ controls (Fig. 3*B*). BAAV transduction preferentially targeted cells of the *os* vs. inner sulcus. Thus, Cre was poorly expressed in cells of the Kölliker organ, which disappear toward the onset of hearing (37), and Cx26 was still detectable in this region (Fig. 3*A*). We next examined the expression of Cx30 in $Cx26^{loxP/loxP}$ cochlear cultures and found decreased levels of Cx30 in *os* cells transduced with BAAVCre-IRESGFP (Fig. 3*C*), corresponding

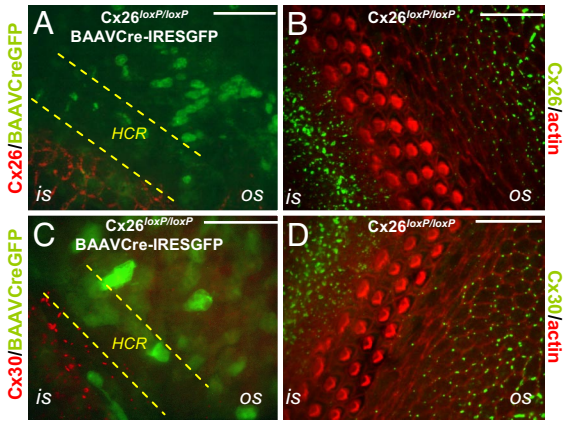


**Fig. 3.** Cx30 is downregulated in cochlear organotypic cultures from $Cx26^{loxP/loxP}$ mice transduced with BAAVCre-IRESGFP. (*A*) Immunoassay of Cx26 (red) in a cochlear culture from a $Cx26^{loxP/loxP}$ mouse transduced with BAAVCre-IRESGFP (green) to catalyze site-specific recombination of DNA between *loxP* sites. (*B*) Cx26 expression (green) in an untransduced $Cx26^{loxP/loxP}$ culture (red, actin). (*C*) Immunoassay of Cx30 (red) in a $Cx26^{loxP/loxP}$ culture transduced with BAAVCre-IRESGFP (green). (*D*) Immunoassay of Cx30 (green) in an untransduced culture from a $Cx26^{loxP/loxP}$ control (red, actin). All images are representative of their respective experimental conditions. The images in *A* and *C* were acquired 48 h after adding the viral vectors to the culture medium; dashed lines encompass the approximate position of the hair cell region. (Scale bars: 50 μm.)

CELL BIOLOGY

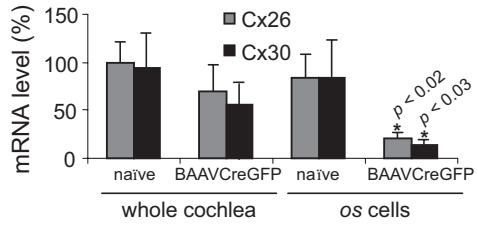


**Fig. 4.** Cx26 and Cx30 mRNA levels in Cx26$^{loxP/loxP}$ cochlear cultures transduced with BAAVCre-IRESGFP. Histogram representation of Cx26 and Cx30 qPCR products, amplified from reverse-transcribed mRNAs extracted from whole cochlea or microdissected *os* cells from Cx26$^{loxP/loxP}$ mice, either untransduced (naïve) or infected with BAAVCre-IRESGFP ($n = 4$ for each indicated condition, error bars represent SD, *P* value estimated by Student's *t* test). BAAVCreGFP indicates mRNA from Cx26$^{loxP/loxP}$ cultures transduced with BAAVCre-IRESGFP.

to the decrease in expression of Cx26 (Fig. 3*A*). Cx30 expression was normal in untransduced Cx26$^{loxP/loxP}$ control cultures (Fig. 3*D*) and similar to that of Cx26 (Fig. 3*B*). Likewise, Cx26 and Cx30 mRNA levels were normal in whole cochlea samples of untransduced Cx26$^{loxP/loxP}$ cultures (Fig. 4). Transduction of Cx26$^{loxP/loxP}$ cultures with BAAVCre-IRESGFP reduced mRNA levels of both connexins, although the changes we measured were statistically significant only in microdissected *os* cells (Fig. 4).

Taken together these results strongly suggest that Cx26 and Cx30 expression is interrelated both at the mRNA level and at the protein level in *os* cells of the immature cochlea.

**Functional Properties of GJ Networks Assayed in Cochlear Organotypic Cultures.** To assess the functionality of GJ channels in *os* cells of cochlear cultures from Cx30 KO mice before and after transduction with BAAVCx30GFP, we used calcein in a fluorescence recovery after photobleaching assay (39, 40). Representative experiments illustrated in Fig. 5 show that organ cultures from WT littermates recovered the fluorescent tracer (Fig. 5*A*) over time, whereas the effect of photobleaching persisted for the whole recording time in cultures from Cx30 KO mice (no recovery; Fig. 5*B*). These results suggest that dye transfer is impaired in *os* cells of Cx30 KO cultures because of lack of Cx30 and low expression levels of Cx26. Similarly, GJ channel function in Cx26$^{loxP/loxP}$ mice transduced with BAAVCre-IRESGFP was significantly impaired compared with that in untransduced controls (Fig. S3). Recovery of fluorescence in cultures of Cx30 KO mice transduced with BAAVCx30-GFP was indistinguishable from that of WT cultures (Fig. 5 *C* and *D*).

Intercellular $Ca^{2+}$ signals (ICSs) and $Ca^{2+}$ oscillations mediated by a P2Y/PLC/$IP_3$ pathway have been reported in these cultures under various stimulation conditions (41–43). For the experiments in Fig. 6, we preloaded the cultures with the acetoxymethyl ester moieties of caged $IP_3$ (44) and fura red, a ratiometric $Ca^{2+}$ indicator. Photostimulation with caged $IP_3$ in WT controls (Fig. 6*A*) elicited ICSs that spread across *os* cells. By contrast, $Ca^{2+}$ signals failed to propagate in untransduced Cx30 KO cultures (Fig. 6*B*), as we report in detail in a companion article. Viral transduction with BAAVCx30-GFP restored the ICS propagation range (Fig. 6*C*) to levels comparable to those of WT controls (Fig. 6*D*).

Taken together, these results confirm that restoration of connexin expression reinstates cell-cell communication to levels that are statistically indistinguishable from those of WT controls.

**Purinergic Signaling and $Ca^{2+}$ Oscillations Activate NF-κB and Control Connexin Expression.** Our data suggest coordinated regulation of Cx30 and Cx26 in cochlear supporting and epithelial cells at both

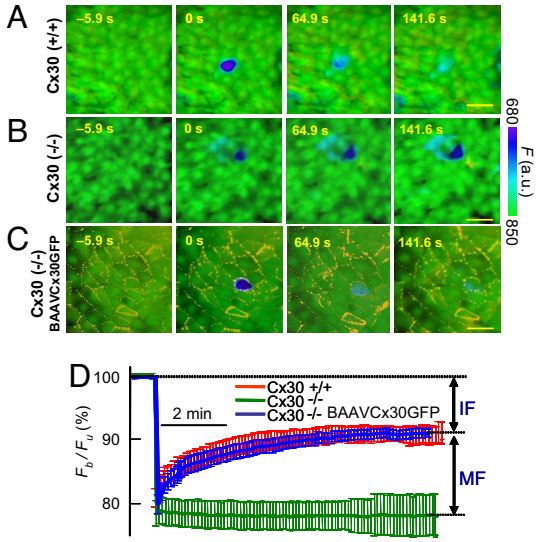


**Fig. 5.** Transduction of Cx30 KO cultures with BAAVCx30 restores GJ coupling. (*A–C*) Fluorescence image sequences representative of fluorescence recovery after photobleaching experiments in cochlear cultures from WT littermates [labeled Cx30(+/+)], Cx30 KO mice [labeled Cx30(−/−)], and in a Cx30 KO culture transduced with BAAVCx30GFP [labeled Cx30(−/−) BAAVCx30GFP]. Times of image capture, relative to stimulus offset, are indicated on each frame. Calcein fluorescence intensity (*F*), in arbitrary units (a.u.), is mapped to pseudocolors as indicated by the color-scale bar (bright green represents basal dye fluorescence). (Scale bars: 50 μm.) (*D*) Plot of calcein percent fluorescence ratio $F_b/F_u$ (bleached area/unbleached area) vs. time for the three types of cochlear cultures in *A* through *C*; ''mobile'' (MF) and ''immobile'' (IF) fractions (40) are indicated by black arrows. Each trace is the mean of $n = 6$ independent experiments for each condition; error bars on each averaged time point indicate SEM.

the mRNA level and the functional level. In nonexcitable cells, the dynamics of gene expression have been shown to depend on the cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) changes through NF-κB signaling (36, 45, 46). NF-κB is an important transcription factor in regulating cell responses to stress. In some cells, it is found in a constitutively active form, whereas it is complexed with an inhibitor protein (IκB) in others. The amplitude and duration of $Ca^{2+}$ signals control differential activation of NF-κB

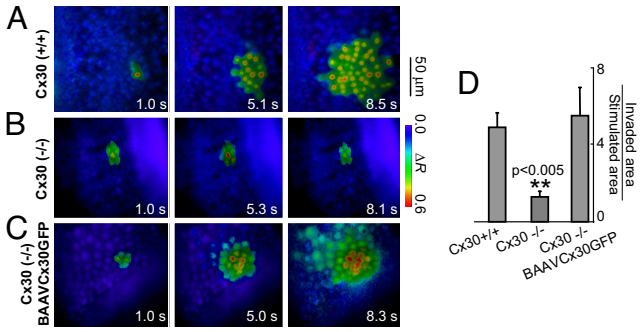


**Fig. 6.** Transduction of Cx30 KO cultures with BAAVCx30 restores intercellular $Ca^{2+}$ signaling. (*A–C*) Fluorescence image sequences highlight representative variations of $[Ca^{2+}]_i$ in *os* cell of mouse cochlear cultures photostimulated with caged $IP_3$. Shown are fura red fluorescence ratio changes (Δ*R*, encoded as shown by the color scale bar) from WT culture, labeled Cx30(+/+) (A); Cx30 KO culture, labeled Cx30(−/−) (B); and Cx30 KO culture transduced with BAAVCx30-GFP, labeled Cx30(−/−) BAAVCx30-GFP (*C*). Times of image capture, relative to stimulus offset, are indicated on each frame. (*D*) Pooled data show maximal ICS propagation range ($n = 8$ for each condition, error bars represent SD, *P* value estimated by Student's *t* test).

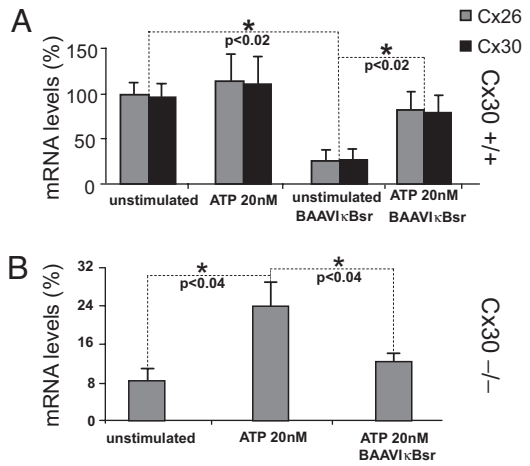


**Fig. 7.** Stimulation of cochlear organotypic cultures with 20 nM extracellular ATP. Histograms of Cx26 and Cx30 mRNA levels, measured by qPCR in *os* cells from WT [*A*, Cx30(+/+)] and Cx30 KO [*B*, Cx30(−/−)] cultures following stimulation with 20 nM ATP. Results are normalized to the Cx26/actin ratio in unstimulated WT controls. BAAVIκBsr indicates samples transduced with the BAAV construct expressing the IκB superrepressor protein ($n = 4$ for each indicated condition, error bars represent SD, *P* value estimated by Student's *t* test).

(36), which, when activated, is released from IκB and translocates to the nucleus, where it participates in the regulation of transcription (34, 35). Interestingly, a binding site for NF-κB has been identified in the promoter region of *GJB2* (47, 48), and NF-κB is reported to regulate the expression of at least one other connexin (connexin 43 [Cx43]) (49).

To investigate mechanisms potentially implicated in coregulation of Cx30 and Cx26, we examined the effect of altering $[Ca^{2+}]_i$ and signaling through the NF-κB pathway in WT and Cx30 KO cultures. To initiate $IP_3$-dependent changes in $[Ca^{2+}]_i$, we exposed either WT or KO cochlear cultures to 20 nM ATP for 20 min (41, 43), after the establishment of a baseline, while monitoring the ensuing $Ca^{2+}$ signals with the fura-2 indicator (Fig. S4*A*). After 20 min of continuous stimulation, *os* cells were microdissected and connexin transcript levels were estimated by qPCR on cDNA produced from pools of cultures for each condition (Fig. 7). ATP evoked similar patterns of intracellular $Ca^{2+}$ oscillations in WT and Cx30 KO cochlear cultures (Fig. S4 *B* and *C*), suggesting the P2Y receptors and the PLC/$IP_3$ signaling cascades are not affected by Cx30 ablation. Although no change in Cx26 expression was observed in WT cultures, stimulation with 20 nM ATP significantly increased Cx26 transcripts in *os* cells from Cx30 KO cultures (Fig. 7). Note that the unstimulated Cx26 mRNA level is very low in Cx30 KO culture compared with WT culture. To test the role of NF-κB in the ATP-induced change in Cx26 expression, we transduced both WT and Cx30 KO cultures with a viral construct (BAAVIκBsr) encoding the stable form of the IκB superrepressor protein (IκBsr), which prevents NF-κB activation/translocation. In agreement with NF-κB having an important role in regulation of Cx26 and Cx30 expression, IκBsr expression significantly decreased Cx26 and Cx30 transcription in unstimulated *os* cells from WT mice; however, this effect was overcome by stimulation with 20 nM ATP. IκBsr transduction coupled with ATP treatment yielded mRNA levels not significantly different from those of unstimulated WT controls. Furthermore, the stimulatory effect of ATP was reduced on both a ratio and absolute change basis (Fig. 7*A*). In contrast, transduction of Cx30 KO cultures with BAAVIκBsr diminished the ATP-dependent increase in Cx26 transcripts (Fig. 7*B*).

Taken together, these data strongly suggest that: (*i*) $Ca^{2+}$ signaling can regulate the expression of Cx26 and Cx30, (*ii*) both are regulated by NF-κB signaling, and (*iii*) they are mutually associated not only when they form channels (i.e., functionally) but at the regulatory level.

## Discussion

The inner ear expresses high levels of colocalized Cx26 and Cx30 (6). Regional specializations in the expression of these connexins have been reported both along the cochlear coils and across the organ of Corti, likely reflecting heterogeneity of homeostatic requirements (37, 50). Although the molecular mechanisms that determine the cell type-specific transcription of each connexin gene are still poorly understood, previous studies suggested a link between the expression of different connexins and implied mechanisms that regulate their coordinated expression accurately (31). Thus, the amount of Cx26 protein in liver cells of Cx32 KO mice is significantly lower than in controls (51); the Cx32 protein has been proposed to stabilize the amount of Cx26 in murine liver, where the two connexins form heteromeric channels (52). A previous study reported Cx26 downregulation at the protein level in the cochlea, but not the liver, of Cx30 KO mice and suggested that the level of Cx26 mRNA in the cochlea of Cx30 KO mice was not significantly changed by Cx30 gene deletion (28). Interestingly, hearing has been restored in Cx30 KO mice independently from Cx30 by overexpressing Cx26 (28), although the use of a bacterial artificial chromosome system evades the regulatory control at the chromosome level.

Here, we have shown that in the selected subpopulation of cochlear nonsensory *os* cells, Cx26 is dramatically downregulated in Cx30 KO mice compared with WT controls both at the protein level and at the mRNA level. The difference is less pronounced (but still significant) when mRNA levels are averaged over whole cochlea samples (Fig. 2) and may have been overlooked in the report by Ahmad *et al.* (28). In addition, we have shown that Cx30 is downregulated in parallel with Cx26 in $Cx26^{loxP/loxP}$ mouse cochlear cultures transduced with BAAVCre-IRESGFP, resulting in impairment of GJ communication. qPCR analysis of Cx26 and Cx30 mRNA levels in the skin and liver of P5 mice gave comparable levels of Cx26 in the skin of WT and Cx30 KO mice (Fig. S5). We also found comparable levels of Cx26 in the liver of WT and Cx30 KO mice, whereas Cx30 mRNA was not detectable in WT mouse liver, as previously reported (53). Likewise, Western blot analysis in skin raw extracts from P5 mice gave comparable Cx26 protein expression levels in WT and Cx30 KO mice (Fig. S6).

Regional specification has been recently highlighted in the vestibular system, where deletion of the Cx30 gene causes little change in the Cx26 expression pattern (but results in a significant and age-related loss of vestibular hair cells in the sacculus) (54). Likewise, the presence of Cx26 in SV basal and intermediate cells of Cx30 KO mice (22) may account for the persistence of GJ coupling even in the absence of Cx30 (55). Indeed, (*i*) neurobiotin or Lucifer yellow injections as well as freeze fracture microscopy reveal similar coupling patterns in SV basal and intermediate cells of WT and Cx30 KO mice and (*ii*) microarray results show that Cx26 is only slightly downregulated in the SV of Cx30 KO mice compared with WT mice (55). Thus, it appears that the coregulation explored in this study is a feature of supporting and epithelial cells in the *os* region.

Our demonstration of Cx26 and Cx30 gene coregulation offers crucial insight into the hitherto unexplained observations that some deafness-associated DFNB1 alleles are characterized by hereditable significant reduction of both *GJB2* and *GJB6* (27) and that *GJB6* deletions in *trans* with recessive mutations of *GJB2* cause deafness in humans (24–26). How exactly the lack of connexin transcription translates into a deafness phenotype remains to be determined. Thus, deafness in Cx30 KO mice is

correlated with disruption of the SV endothelial barrier attributable to increased levels of homocysteine but with no obvious link to connexin channel function (55); therefore, the gene network contributing to the appearance of the disease is likely to be even more complex than predicted.

Our characterization of the coregulation mechanisms implicates intracellular $Ca^{2+}$ and activation of NF-$\kappa$B as important players. Because these are unlikely to be the sole mechanisms at play, future research will clarify further this important matter; nonetheless, their ability to modulate the expression of the connexins points to an important role in the inner ear. Furthermore, although GFP expression is not detected in every cell, the results we obtained in Cx30 KO cultures transduced with BAAVCx30GFP unequivocally show that the newly formed channels are functional and rescue intercellular communication in the cell network. As a marker of transduction, GFP has its limitations, and our findings may suggest that expression of the GFP surrogate marker does not exactly represent the amount, or possibly the extent of expression, of the connexins required to restore function in our Cx30 mice. In an accompanying article, we analyze the mechanisms underlying intercellular $Ca^{2+}$ signaling and show that connexins support a propagation mechanism based on regenerative release of ATP through connexin hemichannels paralleled by $IP_3$ diffusion across GJ channels.

Our results suggest that gene delivery to *os* cells of cochlear tissue can be performed with great ease and high efficiency using BAAV as a vehicle for gene transfer. BAAV is a member of the *Dependovirus* genus of the Parvoviridae family; a group of small single-stranded replication defective parvoviruses whose genomes can be easily manipulated to produce recombinant proteins. Several other members of this genus are currently being evaluated for use as gene transfer vehicles in clinical studies for the treatment of both genetic and acquired diseases. Although our work has focused on organ cultures, BAAV-mediated gene transfer may also be useful *in vivo* for functional genomics studies in whole animals. Indeed, gene transfer vectors based on BAAV have several attributes that make it an attractive candidate for use in the inner ear, such as strong tropism for neuroepithelia, minimal impact on normal cellular activity, and the ability to penetrate barrier epithelial cell layers via a transcytosis pathway (56).

## Materials and Methods

**BAAV Vector Production and Quantification.** Recombinant BAAVCx30GFP, BAAVCre-IRESGFP, and BAAVI$\kappa$Bsr were produced by transfecting 293T cells with the following plasmids: an adeno virus helper plasmid (pAd12) encoding the viral-associated RNA, E2, and E4; AAV helper plasmids containing AAV2-Rep and BAAV-Cap separately; and a vector plasmid containing inverted terminal repeats flanking the CMVCx30GFP, CMV-Cre-IRESGFP, or CMV-I$\kappa$Bsr gene cassette. The detailed production and quantification procedure has been previously described (57), and details are listed in *SI Methods*.

**Cochlear Cultures and Transduction with BAAV.** Cochleae were dissected from P5 mouse pups in ice-cold Hepes-buffered (10 mM, pH 7.2) HBSS (Sigma) and placed onto glass coverslips coated with 10 $\mu$g/ml Cell Tak (Becton Dickinson), as described in by Gale *et al.* (41). Cultures were incubated in DMEM/F12 (Invitrogen), supplemented with 5% vol/vol FBS and maintained at 37 °C for 1 day. Transduction with viral constructs was performed by adding purified vector at a final titer of $10^{11}$ particles/ml in culture medium devoid of FBS; cultures were kept in this medium at 37 °C for the first 24 h to favor viral transduction. Thereafter, cultures were maintained in DMEM/F12 supplemented with FBS up to 48 h before experiments.

**qPCR.** mRNA was extracted from freshly isolated P5 whole cochleae or microdissected *os* cells using RNAeasy kit (Qiagen). The same procedure was applied to organotypic cultures 48 h after *in vitro* transduction with BAAV constructs. cDNA was obtained by reverse transcription of mRNA with Oligo$(dT)_{12}$-18 (Invitrogen) and Omniscript Reverse Transcriptase (Qiagen) for 1 h at 37 °C. qPCR was performed on cDNA (obtained as described previously) to amplify Cx26 and Cx30 and was normalized to actin expression. Connexin expression relative to actin was estimated according to the method described by Pfaffl (58). For primers and reaction conditions, see *SI Methods*.

For further methodological details, see *SI Methods* online.

**ACKNOWLEDGMENTS.** We thank Jinfang Zhu for the CMV-Cre-IRESGFP gene cassette, Klaus Willecke for the Cx26$^{loxP/loxP}$ and Cx30 KO mice, Anke Seydel for mouse genotyping, Tullio Pozzan, Pierluigi Nicotera, Roberto Bruzzone, and the editor of this article, Michael V. L. Bennett, for helpful discussions and constructive criticism. This work was funded by grants from Fondazione Cariparo, Padua, Italy (Progetti di Eccellenza 2006), Telethon Italy Grant GGP05131, and European Commission FP6 Integrated Project EuroHear Grant LSHG-CT-20054-512063 under the Sixth Research Frame Program of The European Union (to F.M.) and by National Institutes of Health/National Institute of Dental and Craniofacial Research intramural support (to J.A.C.).

1. Forge A, *et al.* (2003) Gap junctions in the inner ear: Comparison of distribution patterns in different vertebrates and assessment of connexin composition in mammals. *J Comp Neurol* 467:207–231.
2. Cohen-Salmon M, *et al.* (2004) Expression of the connexin43- and connexin45-encoding genes in the developing and mature mouse inner ear. *Cell Tissue Res* 316:15–22.
3. Suzuki T, Takamatsu T, Oyamada M (2003) Expression of gap junction protein connexin43 in the adult rat cochlea: Comparison with connexin26. *J Histochem Cytochem* 51:903–912.
4. Kikuchi T, Adams JC, Paul DL, Kimura RS (1994) Gap junction systems in the rat vestibular labyrinth: Immunohistochemical and ultrastructural analysis. *Acta Otolaryngol* 114:520–528.
5. Kikuchi T, Kimura RS, Paul DL, Adams JC (1995) Gap junctions in the rat cochlea: Immunohistochemical and ultrastructural analysis. *Anat Embryol* 191:101–118.
6. Lautermann J, *et al.* (1998) Expression of the gap-junction connexins 26 and 30 in the rat cochlea. *Cell Tissue Res* 294:415–420.
7. Lautermann J, Frank HG, Jahnke K, Traub O, Winterhager E (1999) Developmental expression patterns of connexin 26 and 30 in the rat cochlea. *Dev Genet (Amsterdam)* 25:306–311.
8. Krstic RV (1997) *Human Microscopic Anatomy* (Springer, Berlin).
9. Hibino H, Kurachi Y (2006) Molecular and physiological bases of the $K^+$ circulation in the mammalian inner ear. *Physiology (Bethesda)* 21:336–345.
10. Wangemann P (2006) Supporting sensory transduction: Cochlear fluid homeostasis and the endocochlear potential. *J Physiol* 576:11–21.
11. Zhao HB, Kikuchi T, Ngezahayo A, White TW (2006) Gap junctions and cochlear homeostasis. *J Membr Biol* 209:177–186.
12. Mammano F, Bortolozzi M, Ortolano S, Anselmi F (2007) $Ca^{2+}$ signaling in the inner ear. *Physiology (Bethesda)* 22:131–144.
13. Zhao HB, Santos-Sacchi J (2000) Voltage gating of gap junctions in cochlear supporting cells: Evidence for nonhomotypic channels. *J Membr Biol* 175:17–24.
14. Forge A, Marziano NK, Casalotti SO, Becker DL, Jagger D (2003) The inner ear contains heteromeric channels composed of cx26 and cx30 and deafness-related mutations in cx26 have a dominant negative effect on cx30. *Cell Commun Adhes* 10:341–346.
15. Sun J, *et al.* (2005) Cochlear gap junctions coassembled from Cx26 and 30 show faster intercellular $Ca^{2+}$ signaling than homomeric counterparts. *Am J Physiol* 288:C613–C623.
16. Harris AL (2001) Emerging issues of connexin channels: Biophysics fills the gap. *Q Rev Biophys* 34:325–472.
17. Hernandez VH, *et al.* (2007) Unitary permeability of gap junction channels to second messengers measured by FRET microscopy. *Nat Methods* 4:353–358.
18. Harris AL (2007) Connexin channel permeability to cytoplasmic molecules. *Prog Biophys Mol Biol* 94:120–143.
19. Zelante L, *et al.* (1997) Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. *Hum Mol Genet* 6:1605–1609.
20. Snoeckx RL, *et al.* (2005) GJB2 mutations and degree of hearing loss: A multicenter study. *Am J Hum Genet* 77:945–957.
21. Cohen-Salmon M, *et al.* (2002) Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. *Curr Biol* 12:1106–1111.
22. Teubner B, *et al.* (2003) Connexin30 (Gjb6)-deficiency causes severe hearing impairment and lack of endocochlear potential. *Hum Mol Genet* 12:13–21.
23. Cohen-Salmon M, del Castillo FJ, Petit C (2005) Connexins responsible for hereditary deafness—The tale unfolds. *Gap Junctions in Development and Disease*, ed Winterhager E (Springer, Berlin), pp 111–134.
24. del Castillo I, *et al.* (2002) A deletion involving the connexin 30 gene in nonsyndromic hearing impairment. *N Engl J Med* 346:243–249.
25. del Castillo FJ, *et al.* (2005) A novel deletion involving the connexin-30 gene, del(GJB6–d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with DFNB1 non-syndromic hearing impairment. *J Med Genet* 42:588–594.
26. del Castillo I, *et al.* (2003) Prevalence and evolutionary origins of the del(GJB6–D13S1830) mutation in the DFNB1 locus in hearing-impaired subjects: A multicenter study. *Am J Hum Genet* 73:1452–1458.
27. Wilch E, *et al.* (2006) Expression of GJB2 and GJB6 is reduced in a novel DFNB1 allele. *Am J Hum Genet* 79:174–179.

28. Ahmad S, *et al.* (2007) Restoration of connexin26 protein level in the cochlea completely rescues hearing in a mouse model of human connexin30-linked deafness. *Proc Natl Acad Sci USA* 104:1337–1341.
29. White TW, Bruzzone R (2000) Gap junctions: Fates worse than death? *Curr Biol* 10:R685–R688.
30. Gerido DA, White TW (2004) Connexin disorders of the ear, skin, and lens. *Biochim Biophys Acta* 1662:159–170.
31. Oyamada M, Oyamada Y, Takamatsu T (2005) Regulation of connexin expression. *Biochim Biophys Acta* 1719:6–23.
32. Schmidt M, Katano H, Bossis I, Chiorini JA (2004) Cloning and characterization of a bovine adeno-associated virus. *J Virol* 78:6509–6516.
33. Di Pasquale G, *et al.* (2005) A novel bovine virus efficiently transduces inner ear neuroepithelial cells. *Mol Ther* 11:849–855.
34. Stancovski I, Baltimore D (1997) NF-kappaB activation: The I kappaB kinase revealed? *Cell* 91:299–302.
35. Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S (1995) Rel/NF-kappa B/I kappa B family: Intimate tales of association and dissociation. *Genes Dev* 9:2723–2735.
36. Dolmetsch RE, Xu K, Lewis RS (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 392:933–936.
37. Jagger DJ, Forge A (2006) Compartmentalized and signal-selective gap junctional coupling in the hearing cochlea. *J Neurosci* 26:1260–1268.
38. Tritsch NX, Yi E, Gale JE, Glowatzki E, Bergles DE (2007) The origin of spontaneous activity in the developing auditory system. *Nature* 450:50–55.
39. Wade MH, Trosko JE, Schindler M (1986) A fluorescence photobleaching assay of gap junction-mediated communication between human cells. *Science* 232:525–528.
40. Rabut G, Ellenberg J (2005) Photobleaching techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, iFRAP, and FLIP. *Live Cell Imaging*, eds. Goldman RD, Spector DL (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY) pp. 101–126.
41. Gale JE, Piazza V, Ciubotaru CD, Mammano F (2004) A mechanism for sensing noise damage in the inner ear. *Curr Biol* 14:526–529.
42. Beltramello M, Piazza V, Bukauskas FF, Pozzan T, Mammano F (2005) Impaired permeability to Ins(1,4,5)P3 in a mutant connexin underlies recessive hereditary deafness. *Nat Cell Biol* 7:63–69.
43. Piazza V, Ciubotaru CD, Gale JE, Mammano F (2007) Purinergic signalling and intercellular $Ca^{2+}$ wave propagation in the organ of Corti. *Cell Calcium* 41:77–86.
44. Li W, Llopis J, Whitney M, Zlokarnik G, Tsien RY (1998) Cell-permeant caged InsP3 ester shows that Ca2+ spike frequency can optimize gene expression. *Nature* 392:936–941.
45. Hu Q, Deshpande S, Irani K, Ziegelstein RC (1999) $[Ca^{2+}]_i$ oscillation frequency regulates agonist-stimulated NF-kappaB transcriptional activity. *J Biol Chem* 274:33995–33998.
46. Nelson DE, *et al.* (2004) Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science* 306:704–708.
47. Hennemann H, Kozjek G, Dahl E, Nicholson B, Willecke K (1992) Molecular cloning of mouse connexins 26 and 32: Similar genomic organization but distinct promoter sequences of two gap junction genes. *Eur J Cell Biol* 58:81–89.
48. Tu ZJ, Kiang DT (1998) Mapping and characterization of the basal promoter of the human connexin26 gene. *Biochim Biophys Acta* 1443:169–181.
49. Zhao Y, Rivieccio MA, Lutz S, Scemes E, Brosnan CF (2006) The TLR3 ligand polyI: C downregulates connexin 43 expression and function in astrocytes by a mechanism involving the NF-kappaB and PI3 kinase pathways. *Glia* 54:775–785.
50. Zhao HB, Yu N (2006) Distinct and gradient distributions of connexin26 and connexin30 in the cochlear sensory epithelium of guinea pigs. *J Comp Neurol* 499:506–518.
51. Nelles E, *et al.* (1996) Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin32-deficient mice. *Proc Natl Acad Sci USA* 93:9565–9570.
52. Valiunas V, Niessen H, Willecke K, Weingart R (1999) Electrophysiological properties of gap junction channels in hepatocytes isolated from connexin32-deficient and wild-type mice. *Pflügers Arch* 437:846–856.
53. Dahl E, *et al.* (1996) Molecular cloning and functional expression of mouse connexin-30, a gap junction gene highly expressed in adult brain and skin. *J Biol Chem* 271:17903–17910.
54. Qu Y, *et al.* (2007) Analysis of connexin subunits required for the survival of vestibular hair cells. *J Comp Neurol* 504:499–507.
55. Cohen-Salmon M, *et al.* (2007) Connexin30 deficiency causes intrastrial fluid-blood barrier disruption within the cochlear stria vascularis. *Proc Natl Acad Sci USA* 104:6229–6234.
56. Di Pasquale G, Chiorini JA (2006) AAV transcytosis through barrier epithelia and endothelium. *Mol Ther* 13:506–516.
57. Kaludov N, Handelman B, Chiorini JA (2002) Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography. *Hum Gene Ther* 13:1235–1243.
58. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.