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Mutations in Cockayne Syndrome-Associated Genes (*Csa* and *Csb*) Predispose to Cisplatin-Induced Hearing Loss in Mice

Robert N. Rainey,[1]* Sum-yan Ng,[1]* Juan Llamas,[1] Gijsbertus T. J. van der Horst,[2] and Neil Segil[1]

[1]Department of Stem Cell Biology and Regenerative Medicine, and Caruso Department of Otolaryngology, University of Southern California, Eli and Edythe Broad Center, Los Angeles, California 90033, and [2]Department of Genetics, Cancer Genomic Center, Erasmus MC, Rotterdam 3015 CN, The Netherlands

Cisplatin is a common and effective chemotherapeutic agent, yet it often causes permanent hearing loss as a result of sensory hair cell death. The causes of sensitivity to DNA-damaging agents in nondividing cell populations, such as cochlear hair and supporting cells, are poorly understood, as are the specific DNA repair pathways that protect these cells. Nucleotide excision repair (NER) is a conserved and versatile DNA repair pathway for many DNA-distorting lesions, including cisplatin-DNA adducts. Progressive sensorineural hearing loss is observed in a subset of NER-associated DNA repair disorders including Cockayne syndrome and some forms of xeroderma pigmentosum. We investigated whether either of the two overlapping branches that encompass NER, transcription-coupled repair or global genome repair, which are implicated in Cockayne syndrome and xeroderma pigmentosum group C, respectively, modulates cisplatin-induced hearing loss and cell death in the organ of Corti, the auditory sensory epithelium of mammals. We report that cochlear hair cells and supporting cells in transcription-coupled repair-deficient Cockayne syndrome group A ($Csa^{-/-}$) and group B ($Csb^{-/-}$) mice are hypersensitive to cisplatin, in contrast to global genome repair-deficient $Xpc^{-/-}$ mice, both *in vitro* and *in vivo*. We show that sensory hair cells in $Csa^{-/-}$ and $Csb^{-/-}$ mice fail to remove cisplatin-DNA adducts efficiently *in vitro*; and unlike $Xpc^{-/-}$ mice, $Csa^{-/-}$ and $Csb^{-/-}$ mice lose hearing and manifest outer hair cell degeneration after systemic cisplatin treatment. Our results demonstrate that *Csa* and *Csb* deficiencies predispose to cisplatin-induced hearing loss and hair/supporting cell damage in the mammalian organ of Corti, and emphasize the importance of transcription-coupled DNA repair in the protection against cisplatin ototoxicity.

Key words: cisplatin; Cockayne syndrome; DNA repair; hearing loss; ototoxicity; sensory hair cells

Significance Statement

The utility of cisplatin in chemotherapy remains limited due to serious side effects, including sensorineural hearing loss. We show that mouse models of Cockayne syndrome, a progeroid disorder resulting from a defect in the transcription-coupled DNA repair (TCR) branch of nucleotide excision repair, are hypersensitive to cisplatin-induced hearing loss and sensory hair cell death in the organ of Corti, the mammalian auditory sensory epithelium. Our work indicates that *Csa* and *Csb*, two genes involved in TCR, are preferentially required to protect against cisplatin ototoxicity, relative to global genome repair-specific elements of nucleotide excision repair, and suggests that TCR is a major force maintaining DNA integrity in the cochlea. The Cockayne syndrome mice thus represent a model for testing the contribution of DNA repair mechanisms to cisplatin ototoxicity.

Introduction

The utility of cisplatin in cancer chemotherapy remains limited due to serious side effects, including nephrotoxicity, ototoxicity, and neurotoxicity (Schacht et al., 2012). A dose-dependent sensorineural hearing loss is observed in patients who receive cisplatin treatment (Yeung et al., 2009), even though the hair cells and supporting cells of the inner ear are permanently postmitotic and

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*R.N.R. and S.-y.N. are co-first authors and contributed equally to this work.

Correspondence should be addressed to Dr. Neil Segil, 1425 San Pablo Street, BCC211, Los Angeles, CA 90033. E-mail: nsegil@med.usc.edu.

S.-y. Ng's present address: Department of Biology, Stanford University, Stanford, California 94305.

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it is commonly thought that DNA-damaging agents, such as cisplatin, kill dividing cells. At pharmacologically relevant doses, DNA is the major target of cisplatin, and the accumulation of cisplatin-DNA adducts is directly correlated with cell death (Kartalou and Essigmann, 2001). Cells eliminate cisplatin-DNA adducts mainly through nucleotide excision repair (NER) (Welsh et al., 2004), which has two overlapping branches, transcription-coupled repair (TCR) and global genome repair (GGR), suggesting NER's importance to cisplatin-induced hearing loss.

In humans, mutations in NER lead to a variety of DNA repair disorders, including Cockayne syndrome (CS), which is deficient in TCR, and xeroderma pigmentosum group C (XP-C), a photosensitive syndrome resulting from a defect in GGR. TCR-deficient CS patients (affected genes: *CSA* or *CSB*) exhibit photosensitivity, progeria, growth retardation, and neurodegeneration, including progressive sensorineural hearing loss (Weidenheim et al., 2009). In contrast, inactivating mutations in GGR-specific factors reportedly do not cause hearing loss, and XP-C patients display only mild neurodegenerative symptoms (Niedernhofer, 2008). TCR and GGR are partially overlapping pathways that remove transcription-blocking lesions in the transcribed strand of active genes and helix-distorting lesions throughout the genome, respectively. After the lesion recognition step, TCR of NER-type lesions (designated TC-NER) and GGR converge and use the same downstream factors in NER. Hearing loss is commonly observed in XP patients with mutations in the downstream repair process (Lai et al., 2013; Totonchy et al., 2013). Additionally, CSA and CSB are implicated in roles outside of NER, including the still poorly defined TCR of non–NER-type lesions associated with base excision repair (Marteijn et al., 2014), transcription (Vélez-Cruz and Egly, 2013), and mitochondrial maintenance (Scheibye-Knudsen et al., 2013).

Previous studies have shown that mouse models for CS and XP reliably recapitulate the repair defects and UV sensitivity that are associated with the corresponding human NER syndromes, except for some neurological features (Jaarsma et al., 2011, 2013). TCR-deficient CS ($Csa^{-/-}$, $Csb^{-/-}$) mice develop age-related blindness at a high incidence (Gorgels et al., 2007), as well as severe hearing loss in the high frequencies and cochlear hair cell degeneration by 16 weeks of age (Nagtegaal et al., 2015). GGR-deficient $Xpc^{-/-}$ mice, which are otherwise unaffected in TCR, present increased UV radiation-dependent skin cancer as observed in XP-C patients (Cheo et al., 2000) but have unknown long-term hearing ability.

Sensory hair cells, like neurons, are nondividing, highly specialized, and terminally differentiated (Kelley, 2006). The cause of hair cell hypersensitivity to cisplatin and the mechanism(s) of DNA repair in these cells are unknown. We previously reported that TCR-deficient $Csa^{-/-}$ and $Csb^{-/-}$ mice manifest progressive high-frequency hearing loss and sensory hair cell degeneration starting after 6 weeks of age (Nagtegaal et al., 2015), suggesting that unrepaired DNA damage can induce age-related decline of the auditory system. To begin to understand the DNA repair mechanisms used by sensory hair cells following cisplatin assault, we investigated the effects of mutations in *Csa*, *Csb*, and *Xpc* on cisplatin-induced hearing loss and cochlear cell death in the mouse both *in vitro* and *in vivo*. Our work indicates that mutation of *Csa* or *Csb*, but not *Xpc*, renders sensory hair cells and supporting cells hypersensitive to cisplatin, and suggests that TCR is a major force maintaining DNA integrity in the mammalian organ of Corti.

Materials and Methods

Experimental animals. $Csa^{-/-}$ (van der Horst et al., 2002) and $Csb^{-/-}$ (van der Horst et al., 1997) mice were acquired from G.T.J.v.d.H. $Xpc^{-/-}$ (strain 010563) (Cheo et al., 1997) mice were purchased from The Jackson Laboratory. Mouse lines harboring *Csa* and *Csb* mutations were maintained on pure C57BL/6J background for all *in vitro* experiments, except those in Figure 6. To generate $Csa^{-/-}$ and $Csb^{-/-}$ mice with endogenous GFP protein in hair cells, *Csa* and *Csb* mice were crossed with Math1-GFP^{+} mice. The Math1-GFP transgenic line was obtained from Jane Johnson (Lumpkin et al., 2003). In Figure 6, the lines were outcrossed to the Math1-GFP reporter strain at different times, yielding a different mixed background for the *Csa* line (50% C57BL/6J/ 50% BALB/cJ) and *Csb* line (25% C57BL/6J/ 25% CBA/CaJ/50% CD-1). All controls for the experiments were from littermates with the same background. As C57BL/6J mice are known to develop high-frequency hearing loss due to a hypomorphic cadherin 23 allele leading to disorganized hair bundles known as age-related hearing loss (Noben-Trauth et al., 2003), *Csa* and *Csb* animals used for auditory brainstem response (ABR) testing were bred onto CBA/CaJ background (strain 000654, The Jackson Laboratory, N3 and N4 generations). Because age-related hearing loss susceptibility requires C57BL/6J-derived modifiers that are absent from the CBA/CaJ background in addition to the $Cdh23^{ahl}$ allele (Kane et al., 2012), the chance of the needed modifiers being present in *Csa* and *Csb* in CBA/CaJ mice was extremely low ($p \leq 0.073$ after N3 and N4 backcrosses, assuming the probability for occurrence for linked loci described by Silver, 1995). Mice of either sex were tested as available, with no obvious bias between male and female groups noted (data not shown), which is consistent with the absence of sex bias in Cockayne syndrome (Nance and Berry, 1992). The House Research Institute Institutional Animal Care and Use Committee approved all animal experiments.

DNA extraction and PCR genotyping. Standard DNA extraction was performed on tail clips from mice, and PCR genotyping has been described in detail previously: *Csa* (van der Horst et al., 2002); *Csb* and *Xpc* (Cheo et al., 1997; Berg et al., 2000).

Drug treatments. For *in vitro* experiments, cochleae from postnatal day (P) 1 mice were dissected in PBS (Invitrogen) and plated on polycarbonate membrane filters (SPI Supplies) in DMEM-F12 (Invitrogen) with penicillin (Sigma) and N2 supplement (Invitrogen). After overnight incubation to ensure survival, the explants were treated with cisplatin (Sigma; 8.43 mM stock dissolved in water) or gentamicin (Sigma; 50 mM stock dissolved in water) of various concentrations for the indicated times, washed 3 times in PBS, and incubated in drug-free culture medium for an additional specified time. All cultures were maintained in a 5% CO_2/5% O_2 humidified incubator (Forma Scientific). Selected cultures were treated with 20 ng/ml nuclear export inhibitor Leptomycin B (Sigma) or its solvent methanol (0.28% final concentration) for 16 h. At the desired endpoint, organs were fixed in 4% PFA for 20 min at room temperature and stored in 1× PBS at 4°C or embedded in OCT and cut into 10-μm-thick frozen sections until further processing. For *in vivo* cisplatin intraperitoneal injection, cisplatin was diluted and filtered in sterile saline solution to the indicated concentration. Animals were asphyxiated by CO_2 inhalation, followed by cervical dislocation and 4% PFA transcardiac perfusion. The cochleae were collected and stored in 4% PFA until immunohistochemical processing.

Immunohistochemistry. Primary antibodies used in this study were anti-parvalbumin clone PARV-19 (1:500, mouse; Sigma), anti-Prox1 (1:500, rabbit; Novus Biologicals), anti-caspase3 active (1:500, rabbit; R&D Systems), anti-p27^{Kip1} (1:500, mouse; NeoMarkers), anti-myosin VI (1:500, rabbit; Proteus), anti-CP9/19 (1:100, rat, gift from M.J. Tilby) (Tilby et al., 1991), and anti-CSA and anti-CSB (both 1:200, rabbit; Abcam). Secondary antibodies were Alexa-594 or Alexa-488 (1:1000; Invitrogen). The immunohistochemistry procedures followed standard protocols with some minor modifications. For anti-Prox1 staining, samples were permeabilized in 3% Triton X-100 (Sigma) for 15 min. For anti-p27^{Kip1}, anti-CSA, and anti-CSB staining, samples were boiled for 10 min in 10 mM citric acid (Sigma), pH 6.0. For anti-CP9/19 staining, samples were hydrolyzed in 2N HCl for 20 min, neutralized with 100 mM boric acid (Sigma), pH 8.5, for 10 min, and permeabilized in 3% Triton X-100 for

60 min. Cell nuclei were labeled with Hoechst (1:5000; Invitrogen). Adult cochlear boluses were decalcified in fresh 300 mM EDTA (Sigma) in 1× PBS daily until completion. Apical and basal regions of adult cochlear bolus were opened up before immunohistochemical processing.

Cell counts and staining quantification. Outer hair cell, inner hair cell, and supporting cell counts were performed on cochlear whole mounts imaged at high power using a Leica TCS SP5 confocal microscope (*Z*-stack projections were created at 10 μm intervals). ImageJ software (National Institutes of Health, Bethesda, Maryland) was used to calculate density (cells per 100 μm) in each of the four cochlear regions of equivalent length (base, mid-base, mid-apex, and apex). Cell counts in all 100 μm segments within each cochlear region were averaged and expressed as percentage of treated samples to untreated control. To measure relative cisplatin-DNA adduct levels, CP9/19$^+$ staining intensity within the nuclei of >100 outer hair cells (verified by coincidence with anti-myosin VI) in the basal region was measured using ImageJ. The offset and photomultiplier tube gain on the confocal microscope were adjusted such that the area expected to have least signal was set to intensity 0, and the area with maximum signal was set just below saturation as measured by readout from photomultiplier tube detector. A single image was used to determine optimal offset and gain at each cisplatin dose tested, and these parameters were maintained when imaging the remaining specimens. CP9/19 staining was calculated by subtracting background values from staining intensity (integrated intensity) of hair cell nuclei. For purposes of quantitation, baseline measures were taken 6 h after the removal of cisplatin (as this resulted in better signal-to-noise ratios compared with 0 h baseline measurements), and compared with the 48 h time point. The SEM was calculated from at least three animals (one cochlea from each mouse) per experimental group. Differences between groups were analyzed for significance with Student's *t* test.

ABR recording. Baseline ABR recordings were performed prior to cisplatin injection, at 3–4 weeks of age, in a soundproof room, and presented as average thresholds from both ears. Animals were anesthetized for 30–60 min with ketamine hydrochloride (50–80 mg/kg, i.p.) and xylazine hydrochloride (5–10 mg/kg, i.p.). Normal body temperature was maintained during the procedure with a heating pad at 37°C. Three subdermal electrodes were inserted into vertex of the head, under the ear as reference, and at the base of the tail for grounding. Animals were exposed to moderate to low levels of acoustic stimulation (105 to 20 dB SPL) delivered through inserted ear phones. Auditory thresholds were determined by the lowest sound intensity that generated recognizable and reproducible wave form (300 responses with artifacts of <30 μV were averaged). ABR recordings were performed weekly after cisplatin intraperitoneal injection up to 2 weeks after injection. Animals were monitored until they could assume a sternal position following the procedure. The presentation of stimuli and averaging of responses were controlled by BioSig software (Tucker-Davis Technologies). The mouse line carrying the *Xpc* deletion had slightly higher baseline ABR thresholds, as expected in a mixed C57BL/6J and CD-1 strain background (Willott and Erway, 1998), relative to the two lines carrying the *Csa* and *Csb* mutations, which were backcrossed on a CBA/CaJ background (N3 and N4 generations; data not shown). For this reason, ABR data are presented as threshold shift relative to baseline thresholds taken before cisplatin administration. ABR threshold shift was analyzed with one-way ANOVA at each of 5 frequencies separately, followed by pairwise comparison between strains when necessary. When pairwise comparisons were conducted, a Bonferroni correction with an adjusted α level of .0167 per test (0.05/3) was applied. Differences were considered significant at the $p < 0.05$ level.

Results

TCR-deficient *Csa*$^{-/-}$ and *Csb*$^{-/-}$ hair cells are hypersensitive to cisplatin *in vitro*, compared with GGR-deficient *Xpc*$^{-/-}$ hair cells

To measure the effect of cisplatin exposure on cochlear sensory hair cell survival *in vitro*, we used an established technique of organotypic culture of the mouse organ of Corti (Doetzlhofer et al., 2009). Neonatal wild-type (WT) organ of Corti explants were cultured and treated with varying concentrations of cisplatin for 2 h, followed by culture in cisplatin-free medium for an additional 48 h (Fig. 1*A*,*B*). Cell counts of whole-mount preparations indicated that outer hair cells were lost, starting in the base, in a dose-dependent manner [20 μM cisplatin = 4% (±8%) reduction of all outer hair cells; 40 μM = 35% (±3%); 80 μM = 63% (±4%); 160 μM = 91% (±4%)], and the degree of cisplatin-induced outer hair cell loss increased from base-to-apex at all doses (Fig. 1*C*). Inner hair cells remained intact <80 μM, and the degree of inner hair cell destruction was approximately equivalent along the length of the cochlear duct at 80 and 160 μM. In contrast to the near total loss of outer hair cells at 160 μM cisplatin, 58% (±7%) of the inner hair cells were lost at this dose (Fig. 1*C*). The observed sensitivity to cisplatin of hair cells recapitulates findings reported by others both *in vivo* and *in vitro*, with regard to the basal-to-apical gradient of outer hair cell sensitivity, and the relative resistance of inner hair cells (Anniko and Sobin, 1986; Cardinaal et al., 2000).

To assess whether mutations in the TCR/GGR pathways render the sensory hair cells more susceptible to cisplatin-induced cell death compared with WT controls, we used 20 μM cisplatin, which causes no inner hair cell loss in WT organ of Corti, and only minimal outer hair cell loss in the base of the cochlea (Fig. 1*C*). Treating TCR-deficient *Csa*$^{-/-}$ and *Csb*$^{-/-}$ organs at 20 μM cisplatin demonstrated their overt hypersensitivity, with significant loss of inner and outer hair cells throughout the entire length of the organ compared with WT explants (Fig. 2*A*,*B*). Additionally, unlike the situation in WT organs (Fig. 1*C*), TCR-deficient explants did not show the basal-to-apical gradient of outer hair cell destruction (Fig. 2*A*,*B*). Using parvalbumin immunostaining to quantify hair cell survival, we observed a 45% (±4%) and 41% (±6%) reduction of outer hair cells in *Csa*$^{-/-}$ and *Csb*$^{-/-}$ explants, respectively, relative to treated WT controls (Fig. 2*B*). In contrast, a differential reduction in surviving inner hair cells between *Csa*$^{-/-}$ (55 ± 4%) and *Csb*$^{-/-}$ (22 ± 3%) mutants was observed (Fig. 2*B*), indicating that, at this dose, *Csa*$^{-/-}$ inner hair cells are more susceptible than *Csb*$^{-/-}$ inner hair cells to cisplatin. Similar results were obtained using the Math1-GFP transgenic mouse reporter (Fig. 1*A*) (Lumpkin et al., 2003) to identify viable hair cells (see Fig. 6*A*; and data not shown). Histological sections through treated TCR-deficient organ of Corti explants showed severe disorganization and loss of hair cells (see Fig. 6*A*; and data not shown), consistent with the quantitative data from the whole cochlear preparations.

In contrast to the TCR mutants, GGR-deficient *Xpc*$^{-/-}$ organs of Corti showed no significant outer or inner hair cell loss compared with WT littermates after 20 μM cisplatin (Fig. 2) or 40 μM cisplatin (data not shown) treatment. These data indicate that TCR, but not GGR, is essential for sensory hair cell survival in response to cisplatin *in vitro*.

TCR-deficient hair cells remove cisplatin adducts at a much reduced rate

DNA damage caused by cisplatin can be visualized using an antibody specific to cisplatin-DNA adducts (CP9/19) (Tilby et al., 1991; Rocha et al., 2014). We applied 20 μM cisplatin to WT organ of Corti cultures for 2 h and quantified the relative disappearance of immunohistochemical staining over a 48 h period. The relative difference in cisplatin-DNA adduct staining is indicative of the cisplatin-DNA repair capacity of the cell. WT organ of Corti explants, double-labeled with the cisplatin adduct-specific antibody CP9/19 and a hair cell-specific marker, myosin VI (Fig. 3*A*), displayed markedly reduced staining of cisplatin-DNA

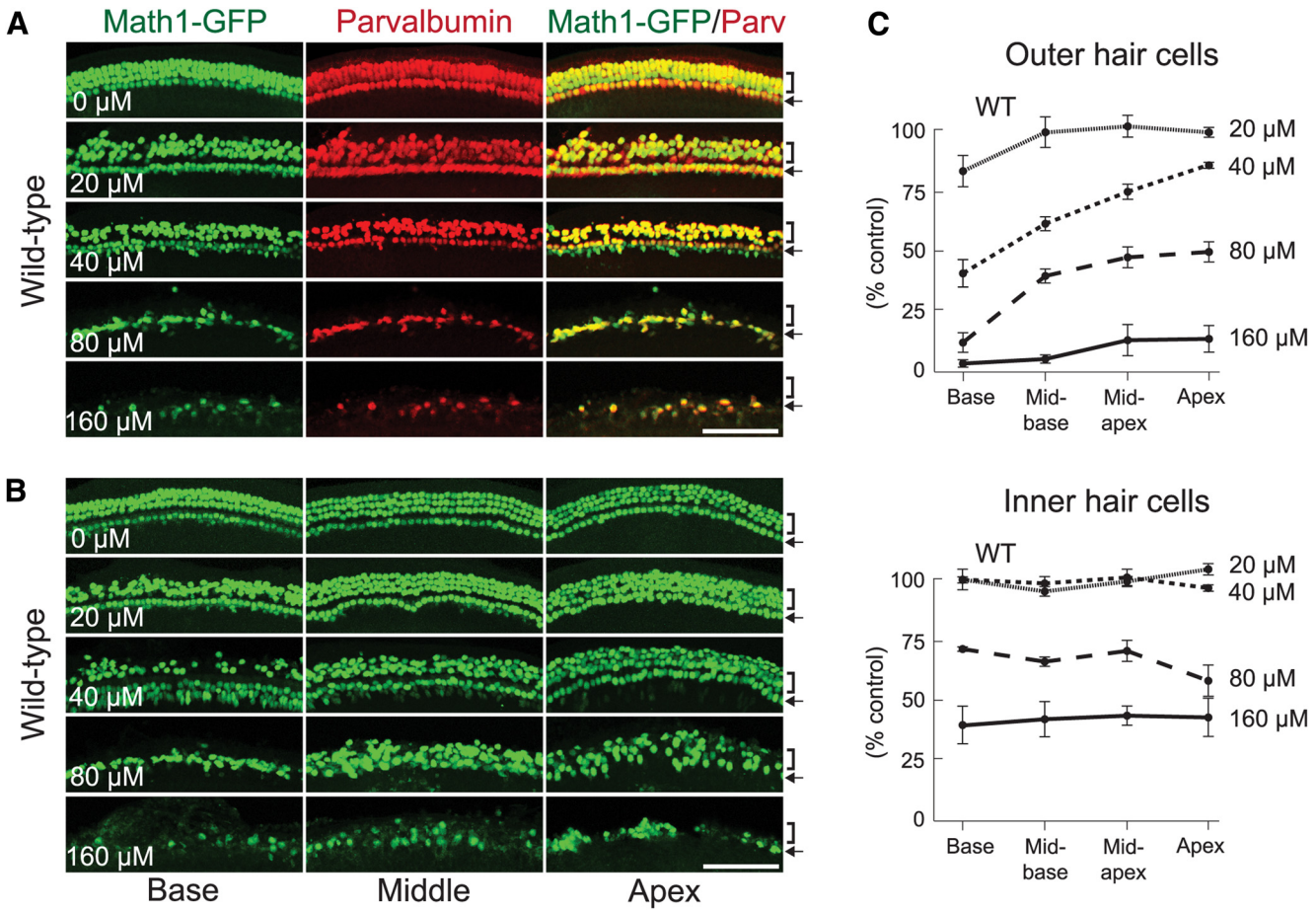

Figure 1. Cochlear sensory outer hair cells, but not the more resistant inner hair cells, are lost in a basal-to-apical gradient in cisplatin-exposed WT organ of Corti *in vitro*. ***A–C***, Organ of Corti explants from P1 Math1-GFP $^{+}$ mice were treated with cisplatin at the indicated doses for 2 h and incubated in cisplatin-free medium for 48 h. Whole-mount preparations of 48 h post-treated explants were visualized using Math1-GFP (green), which labels hair cells, and an antibody against parvalbumin (red), a hair cell marker. ***A***, Representative pictures from the basal region show the coincidence of Math1-GFP $^{+}$ cells with parvalbumin $^{+}$ hair cells at each cisplatin dose. At 0 μM cisplatin, the explants have three rows of outer hair cells (brackets) and one row of inner hair cells (arrows). ***B***, Representative pictures from basal, middle, and apical regions labeled with Math1-GFP are shown. ***C***, Quantification of the change in outer hair cells and inner hair cells in ***B*** along the length of the cochlear explant (base to apex) relative to 48 h untreated control (0 μM cisplatin in ***B***). $n = 3$ animals for each dose. Error bars indicate SEM. Scale bars: ***A***, ***B***, 100 μm.

adducts in outer hair cells, inner hair cells, and supporting cells in the apical turn at 48 h-post treatment relative to baseline control, indicating an effective clearing of cisplatin-DNA adducts. The basal turn, in contrast, presented relatively stronger CP9/19 staining intensity in outer hair cells at the 48 h time point (Fig. 3*A*), suggesting that basal outer hair cells are less efficient in removing cisplatin-DNA adducts than apical outer hair cells.

To better quantify the removal of cisplatin-DNA adducts and to overcome the observed hypersensitivity of TCR-deficient organs of Corti to 20 μM cisplatin treatment (Fig. 2), we used a 20-fold lower dose (1 μM cisplatin; Fig. 3*A*), which results in negligible cell loss in $Csa^{-/-}$ or $Csb^{-/-}$ explants. Organ of Corti explants from $Csa^{-/-}$, $Csb^{-/-}$, $Xpc^{-/-}$, and WT littermates were treated with 1 μM cisplatin for 2 h and incubated for a further 48 h in cisplatin-free medium. Change in adduct level was quantified by measuring CP9/19 staining intensity in the nuclei of myosin VI-positive outer hair cells relative to baseline controls (Fig. 3*B*). At 48 h after 1 μM cisplatin treatment, there was a 44% reduction (±7%) of adducts in WT hair cells from $Csa^{+/+}$ and $Csb^{+/+}$ littermates and a 51% reduction (±7%) in WT hair cells from $Xpc^{+/+}$ littermates. GGR-defective $Xpc^{-/-}$ hair cells had 32% lower levels of cisplatin-DNA adduct staining after 48 h of recovery time in drug-free medium. No statistically significant difference in cisplatin-DNA adduct staining intensity between $Xpc^{-/-}$ and WT littermates was observed (p = not significant; Fig. 3*B*). In contrast, TCR-deficient hair cells from $Csa^{-/-}$ and $Csb^{-/-}$ mutants were less effective at removing cisplatin-DNA adducts. $Csa^{-/-}$ and $Csb^{-/-}$ hair cells accumulated 132% (±16%; $p = 0.0004$) and 64% (±15%; $p = 0.0122$) more cisplatin-DNA adducts, respectively, relative to WT controls (Fig. 3*B*). This indicates a slower overall cisplatin adduct removal rate in $Csa^{-/-}$ hair cells, compared with those of the $Csb^{-/-}$ mutants. Together, the data show that TCR-deficient cochlear sensory cells fail to remove cisplatin-DNA adducts efficiently, suggesting a diminished cisplatin-DNA repair capacity in TCR-deficient cochleae.

TCR-deficient hair cells are not hypersensitive to gentamicin

Gentamicin is an aminoglycoside antibiotic that, similar to cisplatin, causes hair cell loss, but whose major cytotoxic action is believed to result from the generation of ROS (Schacht et al., 2012). To test whether TCR/GGR also plays a role in gentamicin-induced hair cell death, we measured the effect of mutations on gentamicin-induced hair cell loss. In contrast to the situation with cisplatin ototoxicity, quantitation of hair cells in 0.02 mM gentamicin-treated $Csb^{-/-}$ and $Xpc^{-/-}$ explants revealed no hypersensitivity to gentamicin, relative to those of WT littermates (p = not significant; Fig. 4*A*,*B*). Surprisingly, at the higher (0.1

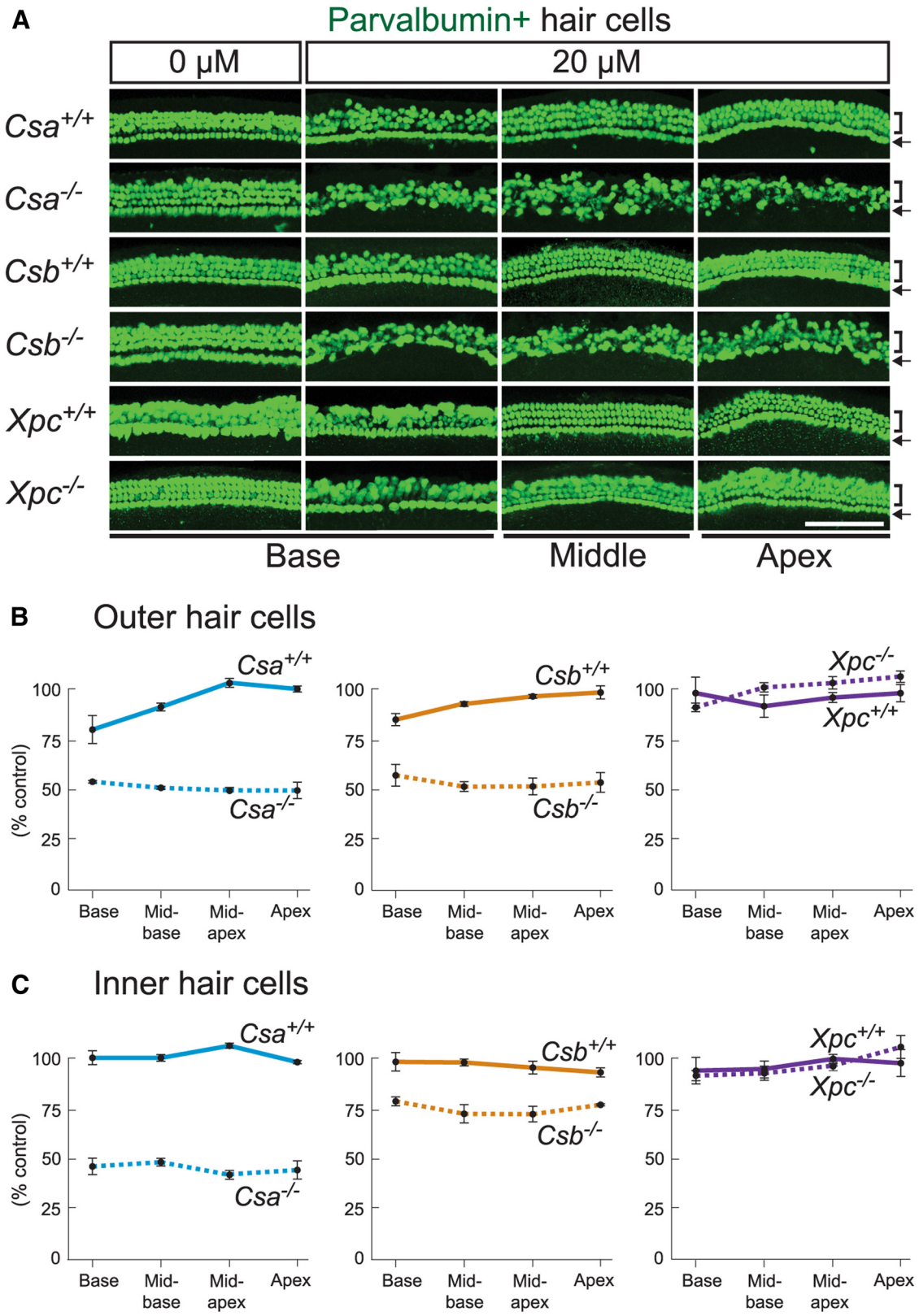

Figure 2. Transcription-coupled repair, but not global genome repair, is crucial for cochlear hair cell survival in response to cisplatin *in vitro*. ***A***, Organ of Corti explants from P1 mice defective in TCR ($Csa^{-/-}$, $Csb^{-/-}$) or GGR ($Xpc^{-/-}$) and WT littermates were treated with 20 μM cisplatin for 2 h and incubated in cisplatin-free medium for 48 h. Whole-mount preparations of 48 h post-treated explants were stained with an antibody against parvalbumin (green), a marker of hair cells. Representative pictures from basal, middle, and apical regions are shown. Outer hair cells (brackets) and inner hair cells (arrows) in $Csa^{-/-}$ and $Csb^{-/-}$ mice are hypersensitive to cisplatin throughout the cochlear turn. Scale bar, 100 μm. ***B***, ***C***, Quantification of the change in outer hair cells (***B***) and inner hair cells (***C***) along the length of the cochlear explant (base to apex) relative to 48 h untreated control of each genotype (0 μM cisplatin in ***A***). $n = 3$ animals per experimental group of each genotype. Error bars indicate SEM.

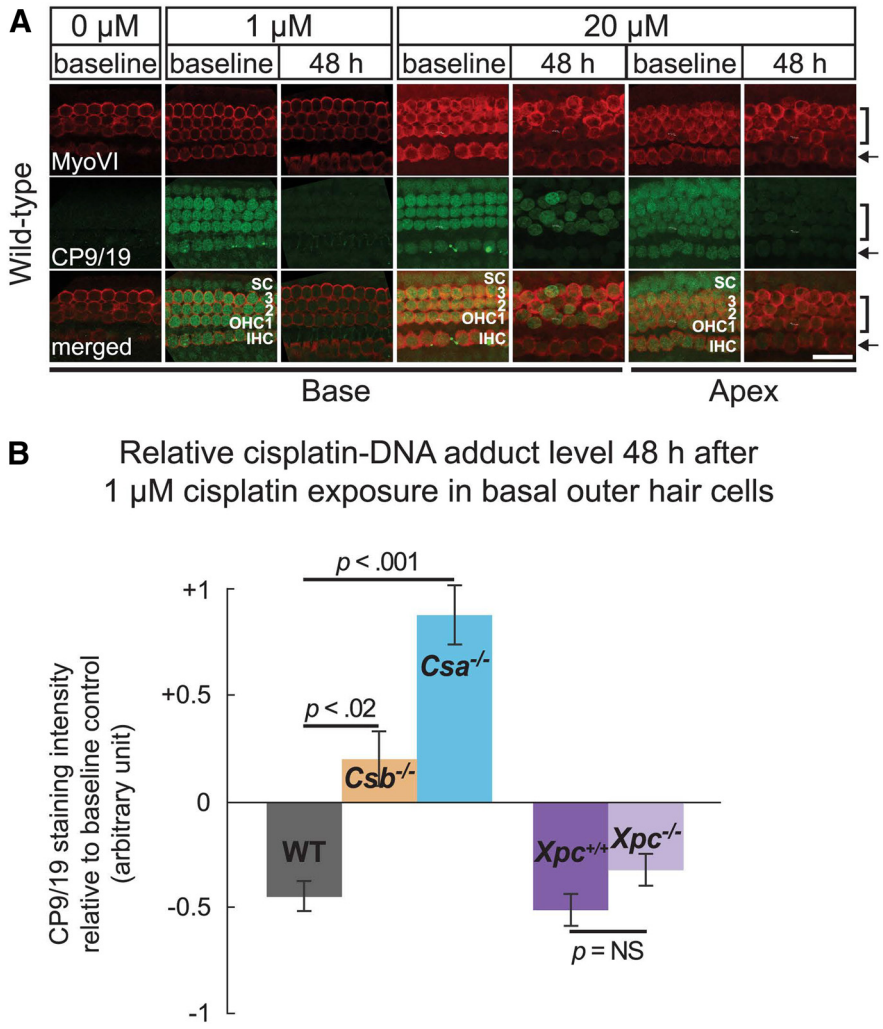

Figure 3. Transcription-coupled repair-deficient sensory hair cells are less efficient at removing cisplatin-DNA adducts *in vitro*. ***A***, Organ of Corti explants from P1 WT mice (C57BL/6J) were treated with 1 or 20 μM cisplatin for 2 h and incubated in cisplatin-free medium for 48 h. Whole-mount preparations were double-labeled with the cisplatin-DNA adduct specific antibody CP9/19 (green) and myosin VI (red), which marks hair cells. Representative pictures of explants fixed at 0 h (20 μM cisplatin) or 6 h (1 μM cisplatin) after the removal of cisplatin are shown as baseline controls (see Materials and Methods). OHC, Outer hair cells (brackets); IHC, inner hair cells (arrows); SC, supporting cells. Scale bar, 25 μm. ***B***, Quantification of staining intensity of cisplatin-DNA adduct antibody in outer sensory hair cells from the basal region of explants defective in TCR (*Csa*$^{-/-}$, *Csb*$^{-/-}$) or GGR (*Xpc*$^{-/-}$) and WT controls treated with 1 μM cisplatin for 2 h at 48 h after treatment relative to baseline (established at 6 h after treatment). WT littermates of *Csa* and *Csb* mutant mice were combined and are shown as WT. $n \geq 3$ animals in each experimental group. Error bars indicate SEM.

mM) dose of gentamicin, TCR-deficient *Csb*$^{-/-}$ explants appeared to be more resistant to gentamicin compared with WT explants ($p = 0.0014$; Fig. 4*A*,*C*). In contrast, no statistically significant difference between GGR-deficient *Xpc*$^{-/-}$ explants and WT explants was detected at this higher dose (p = not significant; Fig. 4*C*). We also observed a similar response to gentamicin in *Csa*$^{-/-}$ explants in a pilot study (data not shown). The reason(s) for the apparent increased resistance of TCR-deficient explants when treated at the higher gentamicin dose are unclear; however, CSA and CSB are implicated in mitochondrial maintenance (Scheibye-Knudsen et al., 2013) and oxidative stress response (Stevnsner et al., 2008; D'Errico et al., 2013). As such, mutating CS factors may lead to delayed cell death signals, which may implicate CSA and CSB factors as damage sensors. Our data show that, in contrast to cisplatin ototoxicity, TCR-deficient hair cells are not hypersensitive to gentamicin.

TCR-deficient supporting cells are also hypersensitive to cisplatin *in vitro*

Like sensory hair cells, supporting cells of the organ of Corti are terminally differentiated and maintain a lifelong postmitotic state (Kelley, 2006). However, little is known about differences in DNA repair mechanisms between the different specialized cell types of the inner ear. To this end, we also analyzed the role of *Csa*, *Csb*, and *Xpc* in the sensitivity of cochlear supporting cells to cisplatin treatment *in vitro*. Staining with an antibody to the transcription factor Prox1, which labels a defined subset of supporting cells (Deiters' and pillar cells), indicated that supporting cells were also sensitive to cisplatin in WT explants in the basal half of the turn in a dose-dependent manner (Fig. 5*A*). Supporting cells in the apical half of the cochlea displayed slightly less susceptibility to 40 μM cisplatin but exhibited greater vulnerability at the higher doses (80 and 160 μM cisplatin) relative to supporting cells in the basal half (Fig. 5*A*). That supporting cells are also affected in our *in vitro* system is consistent with the morphological and molecular evidence that cisplatin affects supporting cells of the inner ear (Anniko and Sobin, 1986; Cardinaal et al., 2000; Endo et al., 2002; van Ruijven et al., 2005; Slattery et al., 2014; Monzack et al., 2015). TCR-deficient supporting cells were hypersensitive to cisplatin (Fig. 5*B*,*C*), manifesting a 53% (±3%) and 52% (±3%) reduction in *Csa*$^{-/-}$ and *Csb*$^{-/-}$ explants, respectively, relative to treated WT controls after exposure to 20 μM cisplatin (Fig. 5*C*). In contrast, GGR-deficient *Xpc*$^{-/-}$ supporting cells were not sensitive to 20 μM cisplatin (Fig. 5*B*,*C*). Together, our results indicate that supporting cells have increased sensitivity to cisplatin in TCR-deficient, but not GGR-deficient, mice.

To assess the mechanism of cisplatin-induced cell loss, whole-mount preparations and sections through the organ of Corti were stained with an antibody against the active form of Caspase 3 (ActCasp3), a marker of apoptosis. Similar numbers of ActCasp3-positive hair cells (double-labeled with the Math1-GFP transgene) and supporting cells (double-labeled by anti-p27^{Kip1}) were observed 48 h after a 2 h exposure to 20 μM cisplatin in TCR-deficient explants (Fig. 6*A*,*B*), indicating that they died through apoptosis. The time course of outer hair cell and supporting cell loss was similar at 24 and 48 h (Fig. 6*C*). In comparison, no ActCasp3-positive hair or supporting cells were ever detected in 20 μM cisplatin-exposed WT whole mounts or sections (Fig. 6*A*,*B*), in contrast to only a few apoptotic hair and supporting cells observed in WT controls treated with 40 μM cisplatin (data not shown). The data indicate that TCR-deficient

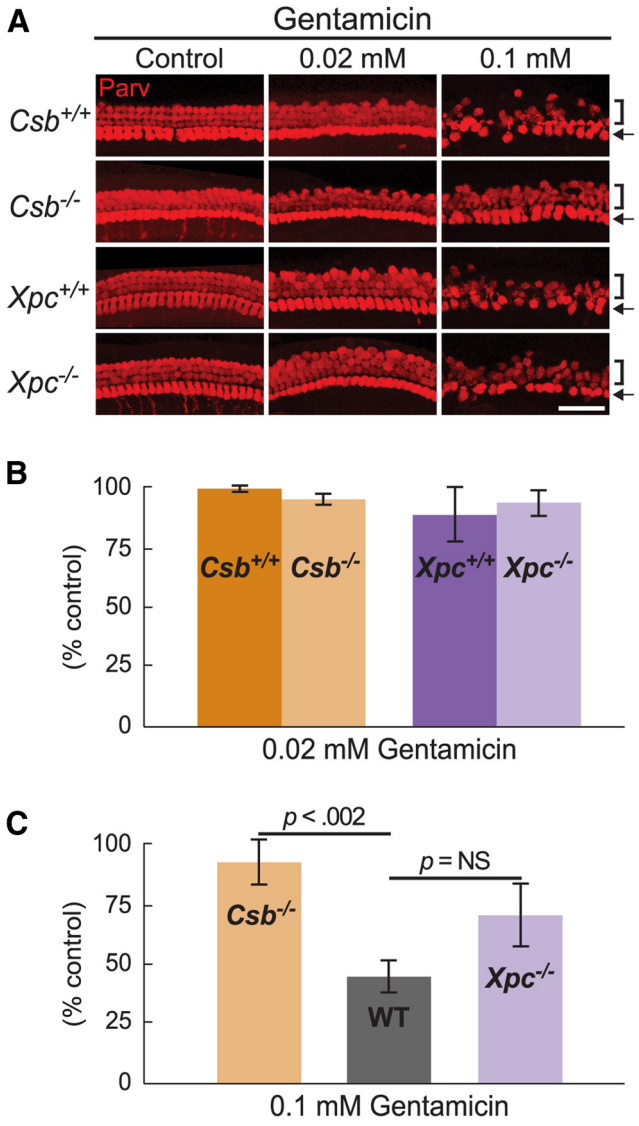

Figure 4. Transcription-coupled repair-deficient hair cells are not hypersensitive to gentamicin *in vitro*. ***A***, Organ of Corti explants from P1 mice defective in TCR ($Csb^{-/-}$) or GGR ($Xpc^{-/-}$) and WT littermates were treated with 0.02 mM gentamicin or 0.1 mM gentamicin for 3 h and incubated in gentamicin-free medium for 48 h. Representative pictures from the basal region stained with the hair cell marker parvalbumin are shown. Brackets indicate outer hair cells. Arrows indicate inner hair cells. Scale bar, 50 μm. ***B***, ***C***, Quantification of the change in all hair cells within the basal region of explants treated with 0.02 mM gentamicin (***B***) or 0.1 mM gentamicin (***C***) relative to 48 h untreated control of each genotype. WT littermates of *Csb* and *Xpc* mutant mice were combined and are shown as WT in ***C***. $n = 3$ animals per experimental group of each genotype. Error bars indicate SEM.

sensory hair cells and supporting cells are lost concomitantly through apoptosis upon exposure to cisplatin *in vitro*.

Given that outer hair cells are more vulnerable than supporting cells to the corresponding cisplatin doses in WT explants (compare Fig. 1*C* with Fig. 5*A*), and this differential susceptibility to cisplatin is lost in TCR-deficient explants (compare Fig. 2*B* with Fig. 5*C*), we asked whether there are cell-intrinsic differences in CSA and CSB protein expression in WT cochlea by immunostaining with antibodies to CSA or CSB. Whereas CSB appears equivalently expressed in the nucleus of hair cells and supporting cells in P1 WT explants, CSA is more highly expressed in supporting cells than in hair cells (Fig. 6*D*). Among supporting cells, CSA appears to be localized primarily in the cytoplasm of Deiters' cells, pillar cells, and Hensen's (tectal/undertectal) cells (Fig. 6*D*). However, blocking nuclear export with Leptomycin B, a protein export inhibitor, revealed more robust nuclear CSA staining in supporting cells than in hair cells (Fig. 6*E*). CSA contains a putative nuclear export signal at the N terminus (amino acids ^{24}TRRVLGLEL^{32}, as predicted by NetNES 1.1 Server (la Cour et al., 2004). The differential expression of CSA between hair cells and supporting cells could contribute to the difference in cell type sensitivity to DNA-damaging agents.

Hypersensitivity to cisplatin of TCR-deficient hair cells is also observed *in vivo*

To test whether the hypersensitivity to cisplatin seen in TCR mutants, but not GGR mutants, is observed *in vivo*, we compared hearing and hair cell loss following systemic cisplatin injection. We measured hearing thresholds using ABR starting at 1 month of age after a single intraperitoneal dose of cisplatin. ABR thresholds of *Csa*, *Csb*, and *Xpc* mutant mice were assessed at 4 weeks of age before cisplatin injection and were not significantly different from those of WT littermates (p = not significant for all differences; data not shown). Two weeks after a single dose of 0.7 mg/kg cisplatin, TCR-deficient $Csa^{-/-}$ and $Csb^{-/-}$ mice showed significant elevation in hearing thresholds compared with WT controls (Fig. 7*A*). $Csa^{-/-}$ mice exhibited a larger threshold shift compared with WT mice at each frequency measured; however, the shift was more pronounced at 16 kHz ($p = 2.16 \times 10^{-4}$), 24 kHz ($p = 3.96 \times 10^{-6}$), and 32 kHz ($p = 2.48 \times 10^{-6}$) than 8 kHz ($p = 0.0077$) and 12 kHz ($p = 0.0012$). $Csb^{-/-}$ mice were less severely affected than $Csa^{-/-}$ animals but nonetheless showed elevated ABR threshold shifts compared with WT mice at 24 kHz ($p = 0.0028$) and 32 kHz ($p = 0.0077$), which are represented in the base of the cochlea (Müller and Smolders, 2005); thresholds were not significantly elevated at the lower frequencies (p = not significant; Fig. 7*A*).

We assessed cochlear hair cell integrity by performing counts on parvalbumin-stained cochlear surface preparations at 6 weeks of age. Organs of Corti from cisplatin-treated $Csa^{-/-}$ and $Csb^{-/-}$ mice presented severe outer hair cell loss, particularly in the basal and mid-basal regions (Fig. 7*B*,*C*); additionally, a significant number of outer hair cells were missing from the mid-apical region of $Csa^{-/-}$ cochleae in the treated group. The effect on outer hair cell densities in the basal and mid-basal regions of TCR-deficient organs of Corti (Fig. 7*B*,*C*) is consistent with the elevated ABR thresholds at 24–32 kHz (Fig. 7*A*), which correspond tonotopically to locations within ~40% of the base of the cochlea (Müller and Smolders, 2005). In contrast, cisplatin-treated WT littermates showed a normal complement of hair cells (Fig. 7*B*,*C*) throughout the cochlea. No apparent damage was observed in TCR-deficient inner hair cells (Fig. 7*B*,*C*). Together, TCR-deficient CS mice, but not WT littermates, manifested significant hearing threshold elevation and outer hair cell degeneration after cisplatin treatment *in vivo*, with $Csa^{-/-}$ mice exhibiting more profound hearing loss and outer hair cell damage than $Csb^{-/-}$ mice.

At doses >0.7 mg/kg cisplatin, $Csa^{-/-}$ and $Csb^{-/-}$ mice experienced rapid weight loss, dehydration, intestinal hemorrhage, and death (data not shown), which precluded analysis (including potential inner hair cell damage) at higher doses. In contrast, GGR-deficient $Xpc^{-/-}$ mice did not exhibit the systemic sensitivity to higher cisplatin doses observed in TCR-deficient CS mice (data not shown) and presented no significant ABR threshold shift relative to WT littermates at any frequency tested 2 weeks after a single dose of 5 mg/kg cisplatin (p = not significant; Fig.

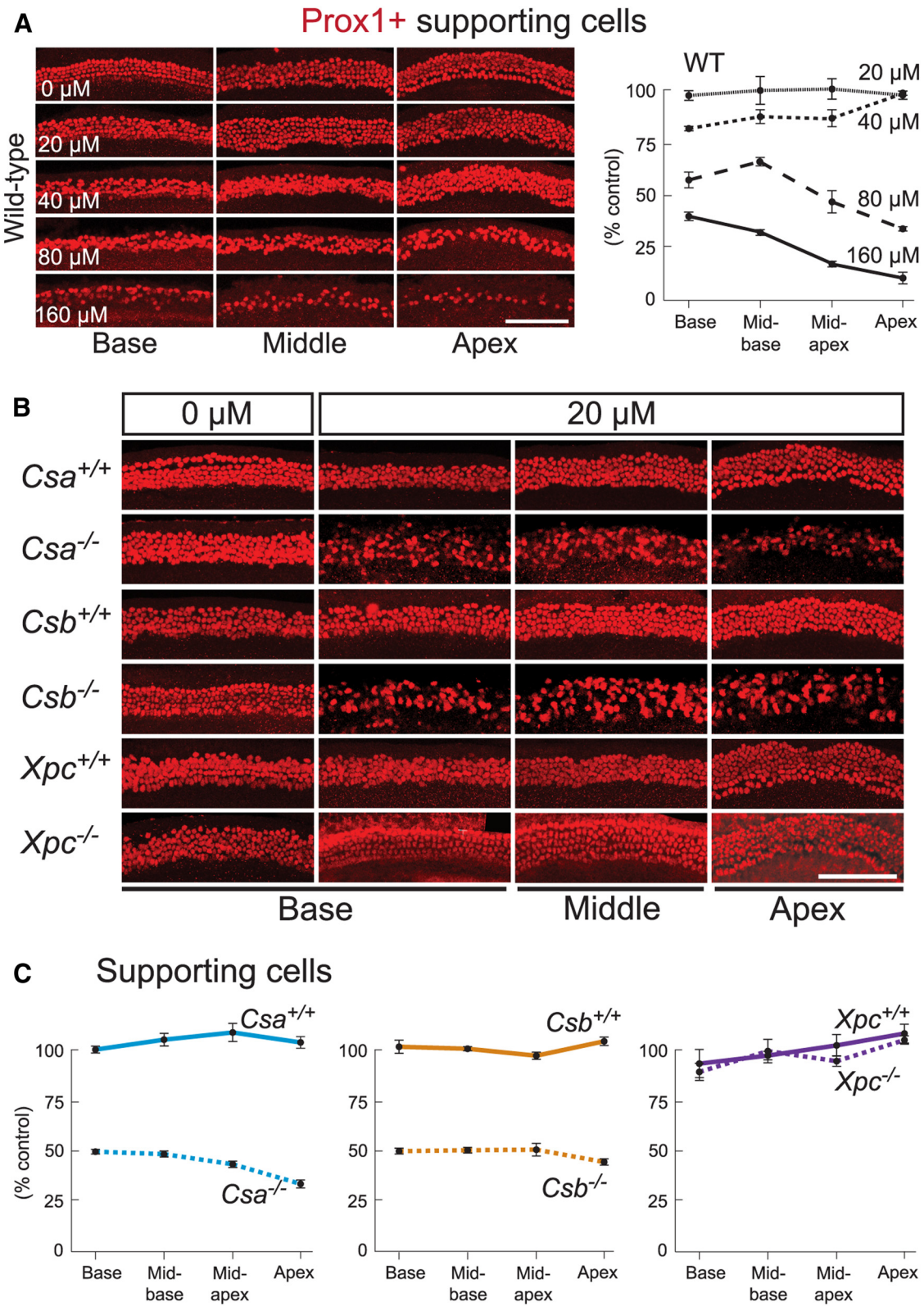

Figure 5. Transcription-coupled repair, but not global genome repair, is crucial for cochlear supporting cell survival in response to cisplatin *in vitro*. ***A***, Supporting cells are lost in cisplatin-exposed WT organ of Corti in a dose-dependent manner. Organ of Corti explants from P1 Math1-GFP $^{+}$ mice were treated with cisplatin at the indicated doses as described in Figure 1 and stained with an antibody against Prox1 (red), which marks supporting cells. Quantification of the change in WT supporting cells along the length of the cochlear explant (base to apex) relative to 48 h untreated control (0 μM cisplatin) is shown on the right. $n = 3$ animals for each dose. ***B***, ***C***, Supporting cells in TCR-deficient $Csa^{-/-}$ and $Csb^{-/-}$ mice, but not in GGR-deficient $Xpc^{-/-}$ mice, are hypersensitive to cisplatin throughout the cochlear turn. ***B***, Organ of Corti explants from P1 mutant mice ($Csa^{-/-}$, $Csb^{-/-}$, $Xpc^{-/-}$) and WT littermates were treated with 20 μM cisplatin as described in Figure 2 and stained with anti-Prox1. ***C***, Supporting cells in ***B*** were quantified as in ***A*** relative to 48 h untreated control of each genotype (0 μM cisplatin in ***B***). $n = 3$ animals per experimental group. Scale bars: ***A***, ***B***, 100 μm. ***A***, ***C***, Error bars indicate SEM.

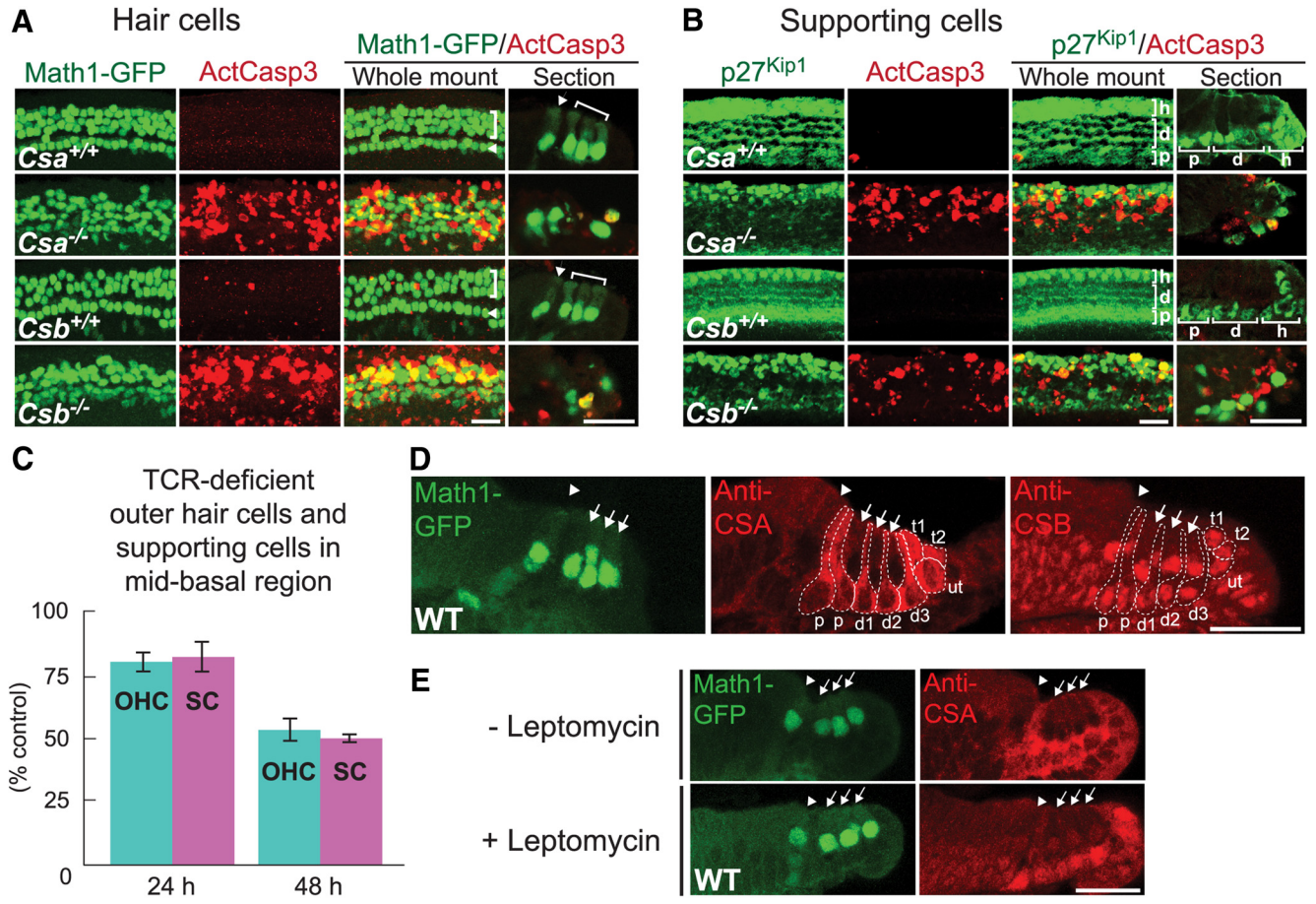

Figure 6. Cisplatin-exposed transcription-coupled repair-deficient cochlear hair cells and supporting cells are lost concomitantly through apoptosis *in vitro*, and the transcription-coupled repair factor CSA is differentially expressed in WT hair cells and supporting cells. ***A***, ***B***, Organ of Corti explants from P1 mice defective in TCR ($Csa^{-/-}$, $Csb^{-/-}$) and WT littermates were treated with 20 μM cisplatin as described in Figure 2. Whole-mount preparations and cross sections of 48 h post-treated explants were double-labeled with an antibody against ActCasp3 (red), a marker of apoptosis, and Math1-GFP (green) or $p27^{Kip1}$ (green) to visualize hair cells (***A***) or supporting cells (***B***), respectively. Representative pictures from the basal/mid-basal region of each explant are shown. Brackets indicate outer hair cells. Arrowheads indicate inner hair cells. Supporting cells are indicated as follows: p, Pillar cells; d, Deiters' cells; h, Hensen's cells. ***C***, Parvalbumin$^+$ outer hair cells (OHC) and Prox1$^+$ supporting cells (SC) within the mid-basal region of 20 μM cisplatin-exposed $Csb^{-/-}$ explants were quantified after incubation in cisplatin-free medium for the indicated times relative to 24 or 48 h untreated control. $n = 3$ animals at each time point. Error bars indicate SEM. ***D***, Adjacent sections through P1 Math1-GFP$^+$ organ of Corti were visualized using Math1-GFP or stained with an antibody against CSA or CSB. High-resolution image shows the overlap of CSA with supporting cells (outlined in dotted white). p, Pillar cells; d1–d3, Deiters' cells. Tectal cells (t1–t2) and undertectal cells (ut) are a subset of Hensen's cells. Arrowheads indicate inner hair cells. Arrows indicate outer hair cells. ***E***, P1 Math1-GFP$^+$ explants were cultured in the presence of 20 ng/ml protein export inhibitor Leptomycin B (+Leptomycin) or its solvent methanol (−Leptomycin) for 16 h and visualized using Math1-GFP or an antibody against CSA. Scale bars: ***A***, ***B***, ***D***, ***E***, 25 μm.

7*D*), a dose previously reported as non-ototoxic in guinea pigs (Hellberg et al., 2009). Cisplatin-treated $Xpc^{-/-}$ mice and WT littermates showed no abnormalities in the numbers or arrangement of hair cells (Fig. 7*E*,*F*). The data suggest that TCR, but not GGR, is crucial for protection against cisplatin-induced hearing loss and cochlear hair cell damage *in vivo*, and indicate that the differential sensitivity of TCR versus GGR observed *in vitro* translates to *in vivo* models.

Discussion

Sensory hair and supporting cells depend on *Csa* and *Csb* to survive cisplatin insult and remove cisplatin-DNA adducts

It was recently reported that TCR-deficient $Csa^{-/-}$ and $Csb^{-/-}$ mice manifest progressive hearing loss in the high frequencies during normal development (Nagtegaal et al., 2015). This correlates with outer hair cell degeneration that presents in a basal-to-apical gradient in the organ of Corti of mutant mice by 16 weeks of age. Inner hair cell and supporting cell loss was relatively less affected in these mutants. Here we tested the response to cisplatin in mice carrying mutations in one of the DNA repair genes *Csa*, *Csb*, or *Xpc* both *in vitro* and *in vivo*. We report that TCR-deficient $Csa^{-/-}$ and $Csb^{-/-}$ mice are hypersensitive to cisplatin, in contrast to GGR-deficient $Xpc^{-/-}$ mice. TCR-deficient explants manifested hypersensitivity to cisplatin in both hair cells and supporting cells (Figs. 2, 5, 6). We used a cisplatin adduct-specific antibody to track removal of cisplatin-DNA adducts after sublethal treatment with cisplatin *in vitro*. We observed higher levels of unresolved cisplatin-DNA adducts in mutant $Csa^{-/-}$ and $Csb^{-/-}$ hair cells compared with baseline controls (Fig. 3*B*), indicating a failure to remove adducts. Unrepaired cisplatin-DNA lesions could be the underlying cause of cell death observed in TCR-deficient explants (Figs. 2, 5). Notably, $Csa^{-/-}$ hair cells accumulated more cisplatin-DNA adducts compared with $Csb^{-/-}$ hair cells (Fig. 3*B*), indicating a slower overall cisplatin adduct removal rate in $Csa^{-/-}$ cells. This may explain our observation that $Csa^{-/-}$ mice are more susceptible to cisplatin treatment than $Csb^{-/-}$ mice *in vivo* (Fig. 7*A*–*C*).

TCR-deficient mice presented a basal-to-apical gradient of cell death following cisplatin treatment *in vivo*, with outer hair cells exhibiting differential sensitivity compared with inner hair cells (Fig. 7*A*–*C*), consistent with previous findings in un-

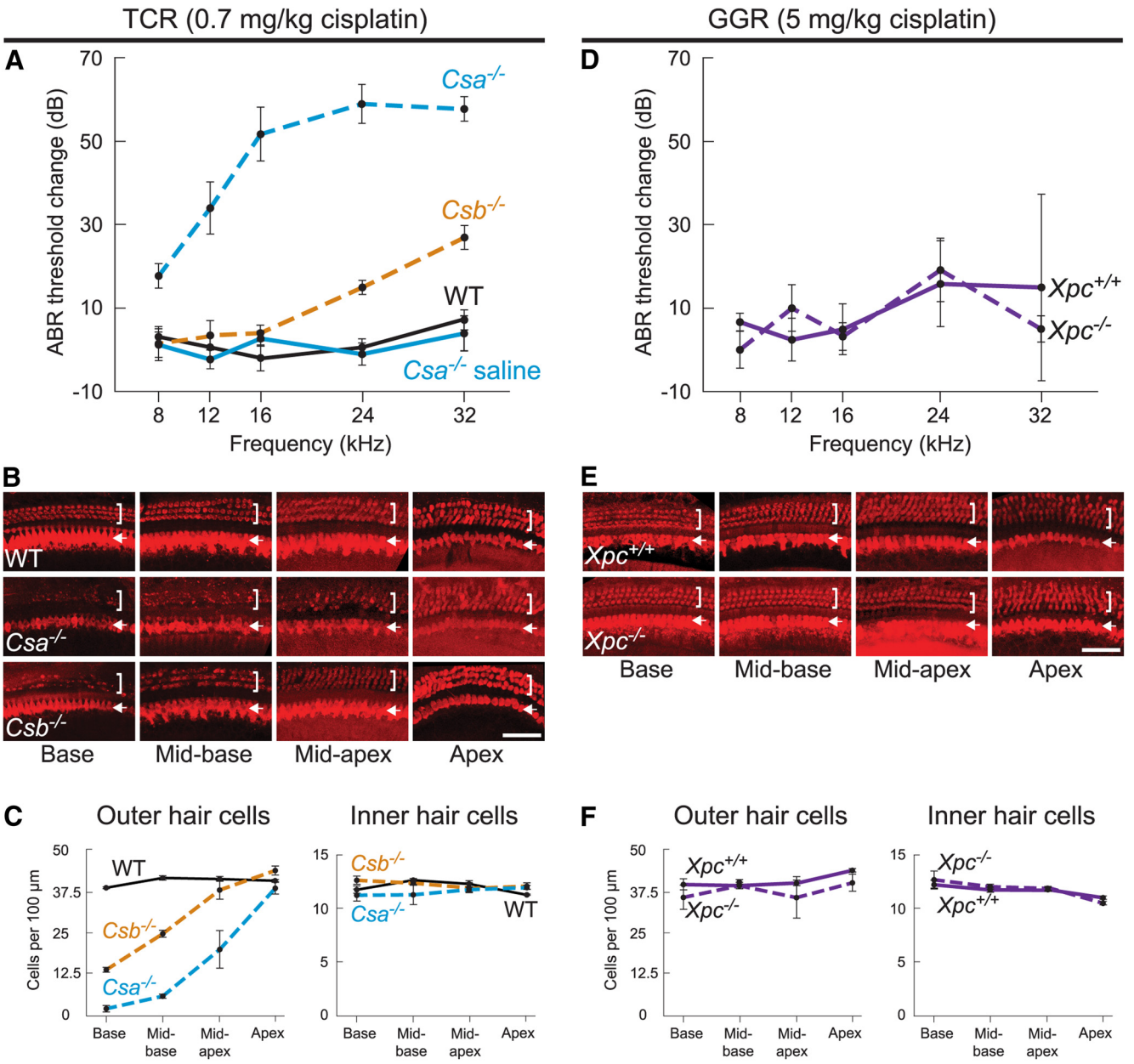

Figure 7. Transcription-coupled repair-deficient mice, but not global genome repair-deficient mice, challenged with systemic cisplatin injection present elevated ABR threshold shifts in the high frequencies and outer hair cell loss in a basal-to-apical gradient. ***A***, ***D***, Change in ABR thresholds 2 weeks after 0.7 mg cisplatin/kg body weight treatment in mice defective in TCR ($Csa^{-/-}$, $Csb^{-/-}$) and WT littermates (***A***) or after 5 mg cisplatin/kg body weight treatment in mice defective in GGR ($Xpc^{-/-}$) and WT littermates (***D***). WT littermates of *Csa* and *Csb* mutant mice were combined. ***B***, ***E***, Whole-mount preparations of the organ of Corti 2 week postcisplatin intraperitoneal injection from ***A***, ***D*** were stained with the hair cell marker parvalbumin (red). Representative pictures from each cochlear position are shown. Brackets indicate outer hair cells. Arrows indicate inner hair cells. Scale bars, 50 μm. ***C***, ***F***, Quantification of the change in outer hair cells and inner hair cells in ***B***, ***E*** along the length of the cochlear duct (base to apex) is presented as average number of cells per 100 μm in the different cochlear regions. $n \geq 3$ animals in each experimental group. Error bars indicate SEM.

treated CS mice at a much later age (Nagtegaal et al., 2015). The basal-to-apical gradient of sensitivity is recapitulated *in vitro* in WT explant cultures (Fig. 1*B*,*C*) but is absent from the TCR-deficient organs *in vitro*, with outer hair cells showing similar levels of hypersensitivity throughout the entire cochlear turn (Fig. 2). Moreover, in explants from $Csa^{-/-}$ mutants (but not $Csb^{-/-}$ mutants), inner hair cells are similar to outer hair cells in their sensitivity to cisplatin. Prolonged incubation at lower concentrations of cisplatin (0.2 μM), where hair cell loss is minimal, failed to reveal a cell death gradient in TCR-deficient explants (data not shown). The reason(s) for these differences in regional sensitivity between *in vivo* and *in vitro* are unclear, but the maintenance of the gradients of sensitivity in WT explants *in vitro* seems to rule out a simple model of differing access to cisplatin between the different cell types or between model systems.

The human spiral ganglion neuron shares the same sensitivity as the organ of Corti to cisplatin-induced cell loss, with the destruction of cells progressing in a basal-to-apical gradient (Strauss et al., 1983; Hoistad et al., 1998; Cheng et al., 2001). Notably, it has been reported that, in rat spiral ganglion neurons, the cytoplasmic-to-nuclear translocation of NER factors is less efficient at the base relative to the apex in response to cisplatin (Guthrie et al., 2008).

Do TCR mutations cause hearing loss or susceptibility to hearing loss?

Our finding that systemic cisplatin injection triggers accelerated hearing loss and hair cell degeneration in CS mice indicates that the loss of *Csa* or *Csb* predisposes the mutant mice to hair cell loss due to accumulation of unrepaired DNA lesions. Previously, we observed that untreated *Csa* and *Csb* mutants showed progressive hearing loss and sensory hair cell degeneration, but that this only occurred starting after 6 weeks of age (Nagtegaal et al., 2015). On the basis of the delayed onset of hearing loss, we suggested that the loss of TCR may represent a susceptibility to environmental stress, as opposed to being a direct cause of hair cell death, and that the untreated mice in our previous study may nonetheless be experiencing low-level environmental insult in the normal course of animal maintenance. While now demonstrating a clear susceptibility to cisplatin-induced hearing loss associated with the loss of *Csa* or *Csb*, this does not necessarily confirm our previous hypothesis. Given the accumulating data indicating that CSA and CSB function in transcriptional processes beyond NER (Vélez-Cruz and Egly, 2013), we cannot rule out a direct effect on the transcriptional machinery contributing to hair cell death, either in the untreated state after 6 weeks of age (Nagtegaal et al., 2015) or in synergy with the increased susceptibility to environmental insult demonstrated here. The question of whether *Csa* or *Csb* mutations directly affect hair cell survival, or are limited to affecting susceptibility to environmental stress, requires further investigation.

TCR mutants suggest a new mechanism underlying hair cell sensitivity to DNA-damaging agents

In dividing cells, cisplatin is believed to induce replication-related DNA damage and thus apoptosis (Kartalou and Essigmann, 2001; Damsma et al., 2007). Because hair cells are a nondividing population, it is counterintuitive that they should be hypersensitive to platinating agents designed to kill dividing cells. We speculate that hair cell sensitivity to cisplatin is caused by blockade of transcribed genes secondary to DNA damage, rather than primarily by ROS generation as is commonly believed (Schacht et al., 2012). From this perspective, it is interesting that the *Csa* and *Csb* mutations do not cause an increase in sensitivity to gentamicin, an agent that is thought to cause hair cell death through an increase in ROS (Fig. 3*A*,*B*) (Schacht et al., 2012). Indeed, Thomas et al. (2006) reported no additional formation of 8-oxoG adducts, an early response to ROS, up to 48 h after cisplatin treatment, and suggested that the generation of ROS may be a secondary event in cisplatin ototoxicity. Similarly, the well-known phenomena of "chemo-brain" associated with cisplatin chemotherapy (Dietrich et al., 2006; Gong et al., 2011; Andres et al., 2014) may be the result of loss of postmitotic neurons, as well as a depletion of dividing progenitors.

Although it is unclear why TCR-deficient hair cells display higher rates of survival at the higher (0.1 mM) dose of gentamicin (Fig. 3*A*,*C*), we speculate that it may indicate a role of CSA and CSB as damage sensors, in which case mutating CS factors could lead to delayed death signals. Alternatively, this observation may be related to the well-documented increase in resistance to noise following a gradual preconditioning (Ohlemiller et al., 2011), and thus be associated with an adjusted threshold for apoptosis caused by low-level genotoxic stress secondary to the mutation.

Relative contribution of NER and non-NER activities to protect against cisplatin-induced hearing loss and cochlear cell damage

CSA and CSB proteins have been shown to possess functions outside of TC-NER, including non-NER TCR, which has been proposed to repair transcription-blocking oxidative DNA lesions via the recruitment of alternative DNA repair pathways, such as base excision repair (Stevnsner et al., 2008; Aamann et al., 2014; Marteijn et al., 2014), in addition to being required for transcription itself (Vélez-Cruz and Egly, 2013). This makes it difficult to determine to what extent TC-NER, non-NER TCR, and general transcriptional regulation affects cisplatin-induced hearing loss and cochlear damage. Notably, $Ercc1^{\delta/-}$ mice with a hypomorphic mutation in the downstream NER function, that functions as a common pathway for TC-NER and GGR, display a progressive and accelerated loss of hearing, especially at higher frequencies (Spoor et al., 2012). This observation is consistent with the possibility that, in CS mice, the loss of TC-NER plays a major role in contributing to the sensitivity to cisplatin. Our findings that $Csa^{-/-}$ and $Csb^{-/-}$ mice are not equally sensitive to cisplatin treatment both *in vitro* and *in vivo* (Figs. 2, 7) suggest that non-NER TCR activities of CSA and CSB might contribute to the observed phenotypic differences. This may stem from a possible involvement in mitochondrial deficiency because CSA and CSB proteins are implicated in base excision repair, which is the predominant DNA repair pathway in mitochondria (Scheibye-Knudsen et al., 2013). More studies are needed to determine to what extent, if any, general transcriptional deficits contribute to cisplatin-induced cochlear damage in CS mice.

Neurons and other permanently postmitotic cells downregulate GGR, with transcribed genes still being efficiently repaired by TC-NER/TCR (Nouspikel, 2007). The lack of effect of the *Xpc* mutant suggests that repair activities in postmitotic hair cells and supporting cells behave consistently with this observation. However, others have suggested that there is a general reliance for cisplatin adduct removal on TC-NER, to the exclusion of GGR (Furuta et al., 2002), offering an alternative explanation. In this context, transcriptional analysis of TCR and GGR pathways in hair cells relative to the rest of the organ of Corti suggests that, rather than downregulation, GGR is expressed at the same level in hair cells and the surrounding cell types, whereas CSA and CSB are expressed at significantly higher levels (Tao and Segil, 2015). This does not rule out the possibility of post-transcriptional control of relative abundance; and indeed, our immunostaining results indicate the TCR factor CSA is more highly expressed in supporting cells than in hair cells (Fig. 6*C*,*D*). This observation may be relevant to the observed hypersensitivity of hair cells to cisplatin, relative to supporting cells (Anniko and Sobin, 1986; van Ruijven et al., 2005). Given that TCR requires both CSA and CSB proteins, the relative abundance of CSA alone could possibly influence its rate-limiting step. The question of whether supporting cells possess more robust TCR compared with hair cells, and whether this underlies the difference in cell-type sensitivity to DNA-damaging agents, bears further investigation.

Approximately two-thirds of children receiving platinum-based chemotherapy experience hearing loss (Orgel et al., 2012). Our study strongly suggests that individuals with CS may be at increased risk for cisplatin-induced hearing loss. As noted previously (Rogers et al., 2000; Arlett et al., 2006; Wei et al., 2010); and in light of our data, guidelines on the use of radiotherapy or chemotherapy in CS and XP patients need to be carefully considered. In conclusion, our work provides new insight for understanding the mechanisms underlying hair cell sensitivity to

DNA-damaging agents. Whether a normal variation in TCR is among the susceptibility factors in ototoxin-induced hearing loss now warrants further study.

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