

# Pejvakin-mediated pexophagy protects auditory hair cells against noise-induced damage

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**Noise overexposure causes oxidative stress, leading to auditory hair cell damage. Adaptive peroxisome proliferation involving pejvakin, a peroxisome-associated protein from the gasdermin family, has been shown to protect against this harmful oxidative stress. However, the role of pejvakin in peroxisome dynamics and homeostasis remains unclear. Here we show that sound overstimulation induces an early and rapid selective autophagic degradation of peroxisomes (pexophagy) in auditory hair cells from wild-type, but not pejvakin-deficient (*Pjvk*<sup>-/-</sup>), mice. Noise overexposure triggers recruitment of the autophagosome-associated protein MAP1LC3B (LC3B; microtubule-associated protein 1 light chain 3β) to peroxisomes in wild-type, but not *Pjvk*<sup>-/-</sup>, mice. We also show that pejvakin-LC3B binding involves an LC3-interacting region within the predicted chaperone domain of pejvakin. In transfected cells and in vivo transduced auditory hair cells, cysteine mutagenesis experiments demonstrated the requirement for both C328 and C343, the two cysteine residues closest to the C terminus of pejvakin, for reactive oxygen species-induced pejvakin-LC3B interaction and pexophagy. The viral transduction of auditory hair cells from *Pjvk*<sup>-/-</sup> mice in vivo with both *Pjvk* and *Lc3b* cDNAs completely restored sound-induced pexophagy, fully prevented the development of oxidative stress, and resulted in normal levels of peroxisome proliferation, whereas *Pjvk* cDNA alone yielded only a partial correction of the defects. Overall, our results demonstrate that pexophagy plays a key role in noise-induced peroxisome proliferation and identify defective pexophagy as a cause of noise-induced hearing loss. They suggest that pejvakin acts as a redox-activated pexophagy receptor/adaptor, thereby identifying a previously unknown function of gasdermin family proteins.**

pejvakin | noise-induced hearing loss | LC3B | pexophagy | intracochlear viral transduction

Noise overexposure increases reactive oxygen species (ROS) levels, causing oxidative damage to auditory hair cells and resulting in hearing loss (1, 2). Excessive ROS production can be counteracted by the antioxidant activities of ROS-metabolizing organelles, mitochondria, and peroxisomes (3, 4). Diverse metabolic pathways, including the β-oxidation of branched and very-long-chain fatty acids and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) metabolism, occur in peroxisomes. The number, size, shape, and molecular content of these organelles continually adapt to the changing needs of the cell and external conditions (5, 6). The balance between peroxisome biogenesis and degradation is crucial for redox cell homeostasis and may be dysregulated in peroxisomal biogenesis disorders (7). Peroxisome biogenesis can occur de novo or through the proliferation of existing peroxisomes (5, 6), and damaged peroxisomes are degraded by selective autophagy (pexophagy) in yeast and plant vacuoles and in mammalian lysosomes (8).

We recently showed that the physiological response to noise overexposure includes peroxisome proliferation (1). We found that defects of pejvakin, underlying an autosomal recessive form of deafness in humans (DFNB59) (9), cause hypervulnerability to sound in humans and mice (1) associated with high levels of

oxidative stress in auditory hair cells and neurons. Pejvakin is a member of the gasdermin protein family, which has six members in humans (gasdermins A–E and pejvakin), four of which (gasdermins A, B, D, and E) trigger pyroptosis, a form of programmed lytic cell death initiated by inflammasome activation (10). These gasdermins have an autoinhibited two-domain architecture, with an N-terminal pore-forming domain in the plasma membrane and a C-terminal repressor domain, separated by a linker region (11–14). Their pyroptotic pore-forming activity results from inflammatory caspase cleavage at the linker domain, separating the N-terminal pore-forming domain from the C-terminal repressor domain. Pejvakin has a markedly different structure than that of other gasdermins, with a divergent shorter C-terminal domain and no identifiable cleavable linker domain (11, 15).

Pejvakin is associated with peroxisomes and is required for the sound-induced proliferation of these organelles (1). However, its role in peroxisome dynamics, in peroxisome proliferation itself, or at other steps in peroxisome turnover, and the direct or indirect nature of its action, remain unknown. In this study, we addressed these issues. We first studied sound-induced peroxisome proliferation in both wild-type and *Pjvk*<sup>-/-</sup> mice. We then characterized

## Significance

Noise-induced hearing loss is a highly prevalent form of sensorineural hearing impairment affecting individuals of all ages. Noise exposure triggers a rapid increase in reactive oxygen species (ROS) levels, causing oxidative damage to auditory hair cells and resulting in hearing loss. Here we describe a novel mechanism of protection against noise-induced auditory hair cell damage. In response to sound exposure, pejvakin, a peroxisome-associated protein, acts as an ROS sensor and recruits the autophagy machinery to trigger the degradation of peroxisomes (pexophagy) damaged by oxidative stress. We characterized the pejvakin interactions involved in this process. We provide evidence that pejvakin-mediated pexophagy plays a key role in controlling peroxisome proliferation and protecting auditory hair cells against noise-induced damage.

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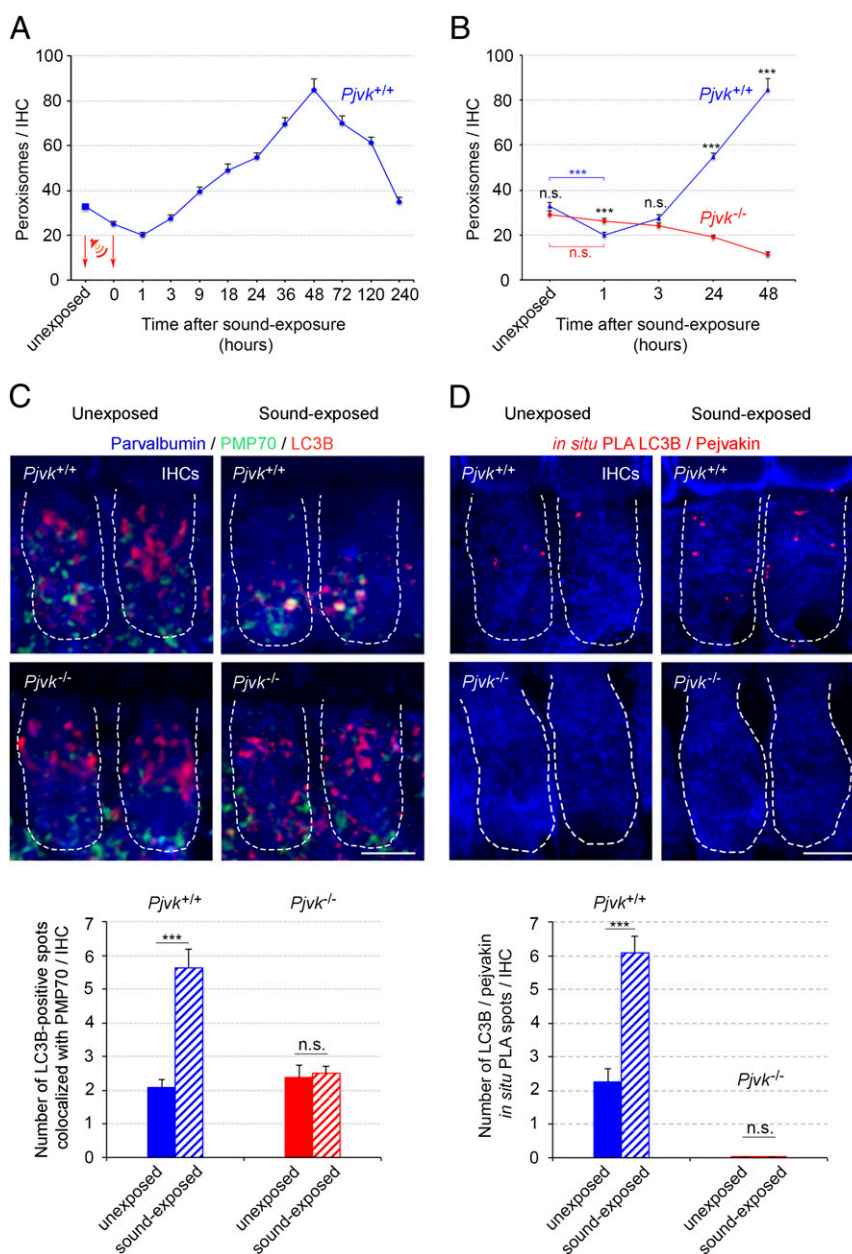
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the role of pejevakin in pexophagy and in protection of the auditory system against noise-induced oxidative stress.

## Results

**Early Pexophagy Precedes the Proliferation of Peroxisomes Induced by Sound Overexposure.** We studied peroxisome kinetics in response to sound in the inner hair cells (IHCs), the genuine sensory cells of the cochlea, the auditory sensory organ, in wild-type (*Pjvk*<sup>+/+</sup>) and *Pjvk*<sup>-/-</sup> mice. Surprisingly, *Pjvk*<sup>+/+</sup> mice were found to have fewer peroxisomes per IHC than unexposed mice (mean  $\pm$  SEM, 32.8  $\pm$

1.5 peroxisomes per IHC), 24% lower ( $24.8 \pm 1.4$  peroxisomes per IHC;  $P = 0.0002$ ) at the end of the 1 h of exposure to loud broadband sound (5–40 kHz, 105 dB SPL), and 39% lower at 1 h after the end of sound exposure ( $20 \pm 1.2$  peroxisomes per IHC;  $P < 0.0001$ ) (Fig. 1A). Two hours later, the number of peroxisomes had started to increase, peaking at 48 h at a level 4.3 times that at 1 h after the end of the stimulation ( $85.3 \pm 4.2$  peroxisomes per IHC; 2.6 times that before stimulation). It decreased slowly thereafter, reaching baseline values within 10 days (Fig. 1A). In contrast, the number of peroxisomes remained unchanged at 1 h after the



**Fig. 1.** Pejevakin promotes pexophagy in response to sound in auditory hair cells. (A) Changes in peroxisome number in P21 *Pjvk*<sup>+/+</sup> IHCs ( $n = 60$ ; four mice per time point) after sound exposure (5–40 kHz, 105 dB SPL, 1 h). The arrows indicate the sound stimulation period (1 h). (B) Impaired early elimination and proliferation of peroxisomes in *Pjvk*<sup>-/-</sup> IHCs in response to sound ( $n = 60$  IHCs; four mice per time point). (C) LC3B-mediated pexophagy is altered in *Pjvk*<sup>-/-</sup> IHCs. The number of coimmunolabeled LC3B/PMP70 spots increased at 1 h after sound exposure in *Pjvk*<sup>+/+</sup> IHCs, but not in *Pjvk*<sup>-/-</sup> IHCs. (D) The number of LC3B/pejevakin PLA spots (in red) was higher at 1 h after sound exposure in *Pjvk*<sup>+/+</sup> IHCs. No LC3B/pejevakin PLA spots were observed in *Pjvk*<sup>-/-</sup> IHCs. The bar charts quantify LC3B/PMP70 coimmunolocalization in IHCs from *Pjvk*<sup>+/+</sup> and *Pjvk*<sup>-/-</sup> mice (C) and LC3B/pejevakin PLA spots in *Pjvk*<sup>+/+</sup> and *Pjvk*<sup>-/-</sup> IHCs (D) before and at 1 h after sound exposure ( $n = 40$  IHCs; four mice per genotype and condition). Data are mean  $\pm$  SEM. \*\*\* $P < 0.001$ ; n.s., not significant, unpaired Student's *t* test. (Scale bars: 5  $\mu$ m.)

end of sound exposure in *Pjvk*<sup>-/-</sup> IHCs (mean ± SEM, 29.3 ± 1.3 and 26.1 ± 1.2 peroxisomes per IHC before and after sound exposure, respectively; *P* = 0.08) and decreased gradually thereafter, by 56% (mean ± SEM, 12.8 ± 1.8; *P* < 0.0001) over 48 h (Fig. 1B). Therefore, sound-induced peroxisome proliferation is preceded by an early rapid pejkakin-dependent degradation of peroxisomes (pexophagy).

We then investigated the possible involvement of pejkakin in coupling of the autophagosome machinery to peroxisomes, by studying the recruitment of LC3B, an autophagosomal marker, to these organelles (16, 17). At the time of maximal peroxisome degradation (1 h after the end of sound exposure), the number of spots double-immunolabeled for LC3B and peroxisome membrane protein 70 (PMP70) had increased by a factor of 2.7 in *Pjvk*<sup>+/+</sup> IHCs (Fig. 1C) but remained unchanged in *Pjvk*<sup>-/-</sup> IHCs (Fig. 1C). Moreover, at 24 h after sound exposure, peroxisome aggregates, a hallmark of impaired pexophagy (18), were detected in *Pjvk*<sup>-/-</sup> IHCs but not in *Pjvk*<sup>+/+</sup> IHCs (SI Appendix, Fig. S1).

We next investigated the possible recruitment of LC3B by pejkakin in an *in situ* proximity ligation assay (PLA) (19, 20), a highly sensitive method in which each pair of proteins is visualized as a fluorescent spot. The number of LC3B/pejkakin PLA spots had also increased by a factor of 2.7 in *Pjvk*<sup>+/+</sup> IHCs at 1 h after sound exposure (Fig. 1D). The lack of pejkakin in *Pjvk*<sup>-/-</sup> mice did not modify the number of spots double-immunolabeled for LC3B and PMP70 observed under basal conditions, indicating the existence of a pejkakin-independent interaction between peroxisomes and LC3B in unstressed IHCs. Overall, these results suggest that pejkakin triggers the autophagic degradation of noise-induced oxidative stress-damaged peroxisomes by recruiting LC3B.

**Pejkakin Promotes Stress-Induced Pexophagy Through the Direct Recruitment of LC3B.** Most selective organelle autophagy pathways in mammalian cells require specific receptors to target organelles for autophagic degradation. The key players and regulators of pexophagy in vertebrates are largely unknown. However, the autophagic receptor/adaptor complex, composed of p62/SQSTM1 (hereinafter referred to as p62) (21) and NBR1 (22), has been shown to induce pexophagy in mammals by binding the targeted peroxisome to a phagophore, particularly in conditions of oxidative stress. Both p62 and NBR1 bind LC3B via their LC3-interacting region (LIR) motifs and ubiquitinated lysine residues of peroxisomal membrane proteins via their ubiquitin-associated (UBA) domains (3). Therefore, we investigated whether the binding of LC3B to pejkakin induced by oxidative stress also involves a direct interaction of pejkakin with p62 or NBR1. HepG2 human hepatoblastoma cells, which are enriched in peroxisomes and strongly express pejkakin, displayed an increase in the numbers of both LC3B/pejkakin double-immunolabeled spots and LC3B/pejkakin PLA spots in response to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress (Fig. 2A and B). In contrast, the number of p62/pejkakin and NBR1/pejkakin PLA spots was unaffected by H<sub>2</sub>O<sub>2</sub> treatment (Fig. 2A and B). Thus, there was no evidence for a direct interaction of pejkakin with p62/NBR1 in the ROS-induced recruitment of LC3B.

An analysis of the murine pejkakin sequence with the Phyre2 web portal (23) found no predicted UBA domain. A predicted chaperone domain was detected between amino acids 154 and 227 (76% confidence), with a putative LIR motif, FIYL, between amino acids 215 and 218 (Fig. 2C). The LIR motif contains a core consensus motif, [W/F/Y]X<sub>1</sub>X<sub>2</sub>[L/I/V] (24, 25), either containing or adjacent to (X<sub>1</sub>, X<sub>2</sub>) negatively charged residues. In pejkakin, this motif is flanked by the charged acidic residues E213 and D219, positioned N- and C-terminal to the aromatic residue F215, and I216 is one of only five amino acids acceptable in position X<sub>1</sub> relative to F215 in F-type LIRs (24, 25). This putative LIR motif has been strongly conserved throughout evolution (SI Appendix, Fig. S2). We assessed its functional relevance by

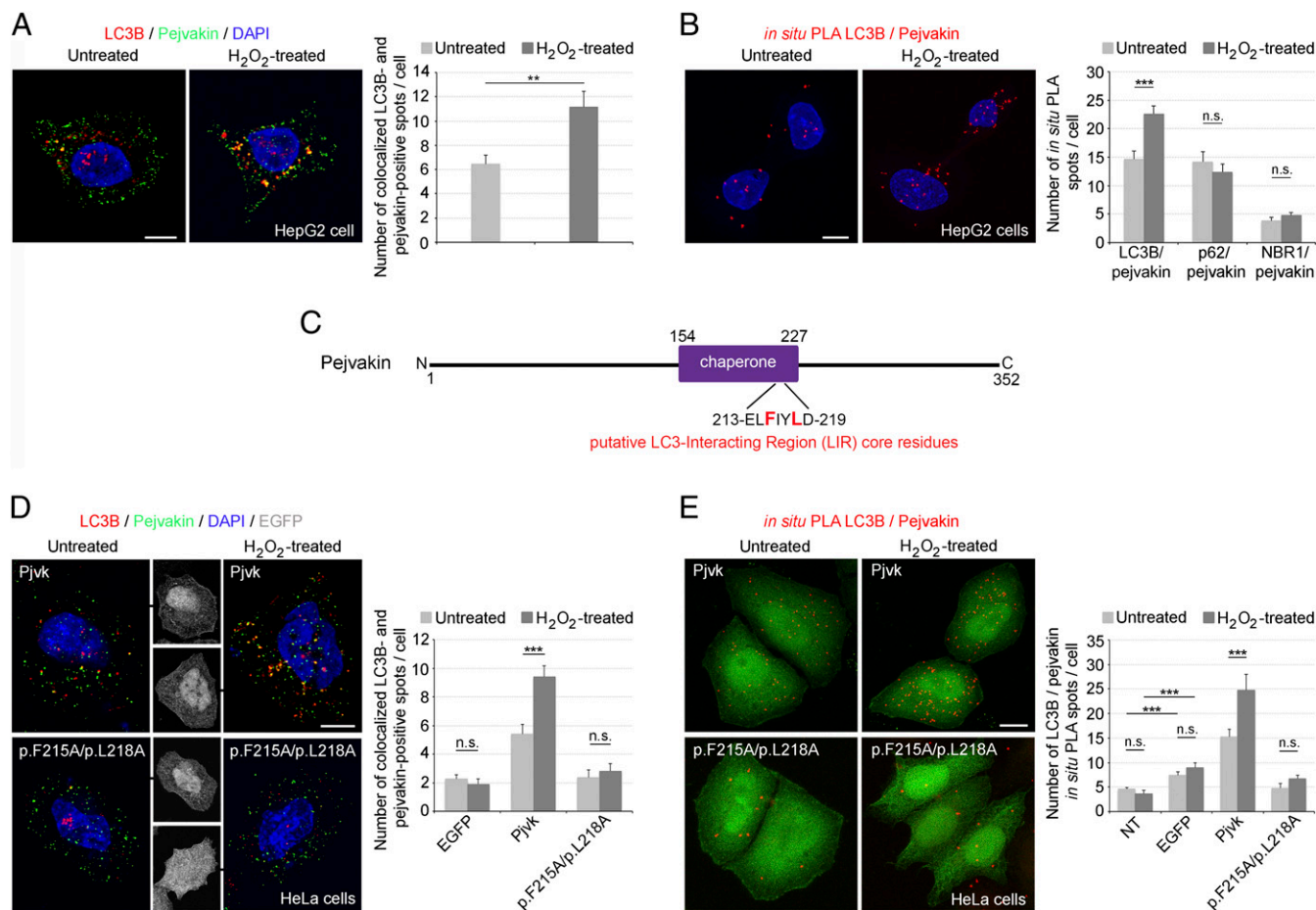
investigating whether mutations of its key residues, F215 and L218, affected pejkakin binding to LC3B under H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. After H<sub>2</sub>O<sub>2</sub> treatment, the numbers of LC3B/pejkakin-coimmunolabeled spots and LC3B/pejkakin PLA spots increased in transfected HeLa cells expressing EGFP and normal murine pejkakin (Pjvk-IRES-EGFP), but not in transfected cells expressing EGFP and a mutated form of pejkakin (mutPjvk-IRES-EGFP) carrying the p.F215A and/or p.L218A amino acid substitutions or expressing EGFP alone (Fig. 2D and E). However, in basal conditions, the number of LC3B/pejkakin PLA spots was larger in transfected cells producing EGFP alone than in untransfected cells, suggesting that transfection induced a stress that triggered the recruitment of LC3B by endogenous pejkakin (Fig. 2E). Five other putative LIR motifs were identified outside the predicted chaperone domain of pejkakin. Site-direct mutagenesis, performed as above, showed that none of these motifs was involved in LC3B recruitment by pejkakin in response to oxidative stress (SI Appendix, Fig. S3). Overall, these findings suggest that oxidative stress-induced pexophagy is dependent on the direct interaction of the defined LIR motif of pejkakin with LC3B.

**The Two Most C-Terminal Cysteine Residues of Pejkakin Regulate Stress-Induced Binding to LC3B.** The pejkakin-mediated pexophagy elicited by oxidative stress raised the possibility of redox-regulated LC3B recruitment by pejkakin. The functional LIR motif of pejkakin is part of the largest putative protein-binding pocket detected by the F-pocket tool (26) within the predicted chaperone domain (SI Appendix, Fig. S4), and chaperone protein activity is often regulated by the redox state of the cell (27). Cysteine oxidation releases the compact conformation of the protein, exposing high-affinity binding sites (27–29). The C terminus of pejkakin contains a cysteine-rich region (residues 309–343). We assessed the pejkakin-LC3B binding elicited by H<sub>2</sub>O<sub>2</sub> in HeLa cells transfected with mutated forms of pejkakin carrying cysteine-to-serine substitutions by coimmunolabeling and *in situ* PLA. Mutations of either of the two cysteine residues closest to the C terminus, p.C328S and p.C343S (a deafness-causing missense mutation in humans) (30), abolished this interaction (Fig. 3). No mutation of the other C-terminal (p.C309S, p.C312S, and p.C325S) or N-terminal (p.C43S and p.C49S) cysteine residues had such an effect (SI Appendix, Fig. S5). Thus, the C328 and C343 residues are essential for ROS-induced pejkakin-LC3B interaction.

We then investigated the role of these two cysteine residues *in vivo* in sound-induced LC3B recruitment by pejkakin and pexophagy. We performed a rescue experiment in *Pjvk*<sup>-/-</sup> mice, involving the adeno-associated virus 2/8 (AAV2/8) vector-mediated transfer of the p.C328S/p.C343S mutated form of pejkakin [AAV2/8-Pjvk(p.C328S;C343S)-IRES-EGFP] into the cochlea of *Pjvk*<sup>-/-</sup> mice. Before sound stimulation, the basal interaction between pejkakin and LC3B detected in the form of LC3B/pejkakin PLA spots (Fig. 4A) was similar in transduced *Pjvk*<sup>-/-</sup> and control *Pjvk*<sup>+/+</sup> IHCs. In contrast, at 1 h after the end of sound exposure, no increase in LC3B-mutated pejkakin interaction could be detected in transduced *Pjvk*<sup>-/-</sup> IHCs, whereas these interactions had increased by a factor of three in *Pjvk*<sup>+/+</sup> IHCs (Fig. 4A). Moreover, neither pexophagy nor peroxisome proliferation following sound exposure was restored in *Pjvk*<sup>-/-</sup> IHCs transduced with the vector expressing the mutated form of pejkakin (Fig. 4B). Thus, the pejkakin C328 and C343 residues play a key role in noise-induced pexophagy, and pejkakin, which displays redox-regulated binding to LC3B, likely acts as a ROS sensor.

**Transfer of the *Pjvk* and *Lc3b* Genes Together Restores Pexophagy and Peroxisome Proliferation in *Pjvk*<sup>-/-</sup> IHCs.** The foregoing experiments provide strong evidence for a role of pejkakin in pexophagy and the involvement of the pexophagy in peroxisome proliferation. However, we thought that additional *in vivo* evidence correlating pexophagy, changes in ROS damage to peroxisomes, and peroxisome





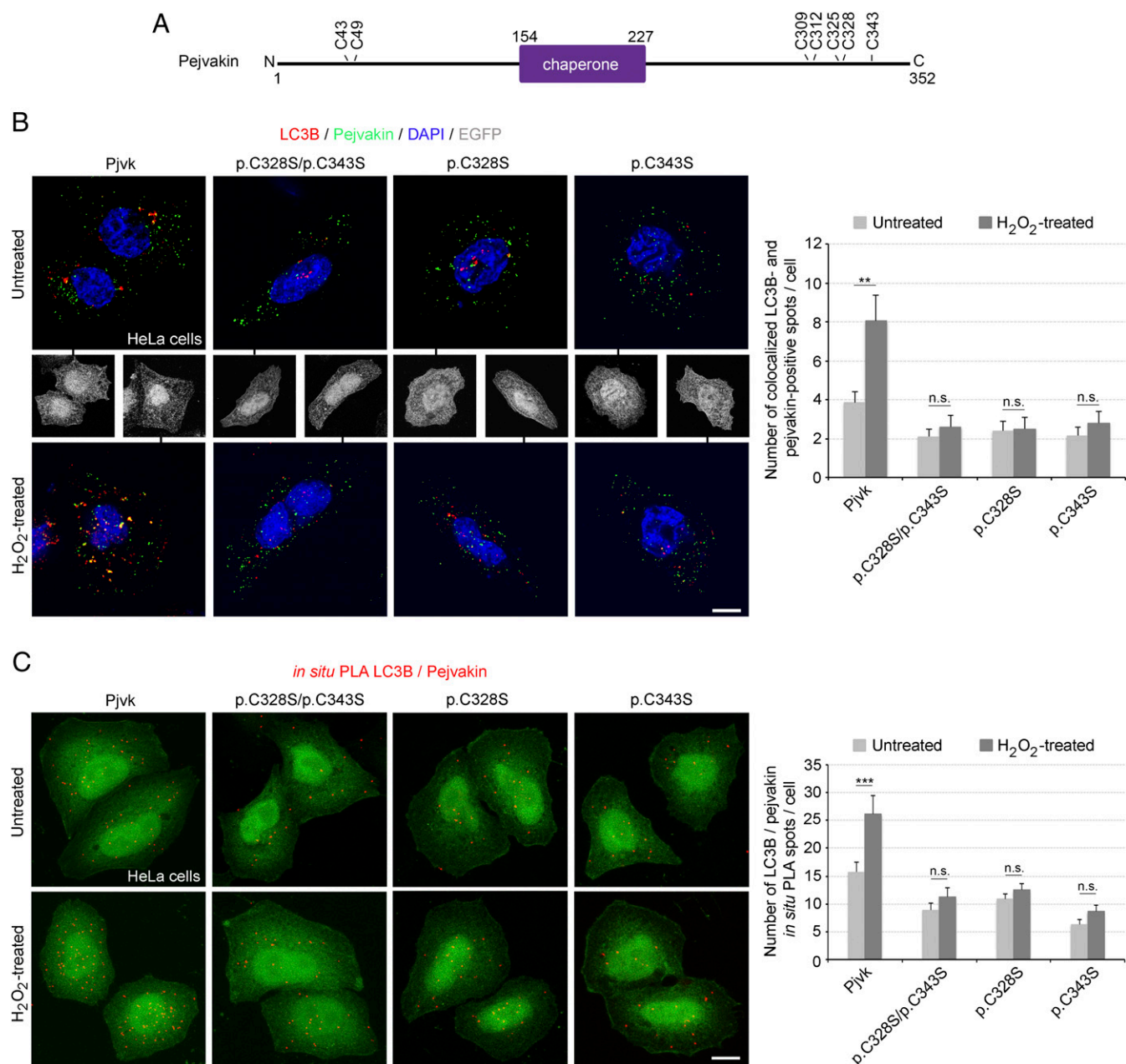
**Fig. 2.** Pejvakín recruits LC3B directly via its LIR, promoting autophagy in response to oxidative stress. (A) Coimmunolabeling of pejvakín and LC3B in untreated and H<sub>2</sub>O<sub>2</sub>-treated HepG2 cells. The number of LC3B-positive spots colocalizing with PMP70 labeling increased after oxidative stress (bar charts;  $n = 20$  cells per condition). (B) Interactions between LC3B and pejvakín, detected as *in situ* PLA spots, in untreated and H<sub>2</sub>O<sub>2</sub>-treated HepG2 cells. The bar charts show an increase in the number of LC3B/pejvakín PLA spots after oxidative stress but no change in the number of p62/pejvakín or NBR1/pejvakín PLA spots ( $n = 20$  cells per condition). (C) Schematic representation of the putative LIR motif (core residues in red) in the predicted pejvakín chaperone domain. (D and E) Interactions between pejvakín and LC3B in untransfected HeLa cells (NT) and transfected HeLa cells producing EGFP alone, EGFP and wild-type pejvakín (Pjvk), or EGFP and pejvakín with mutated core residues. p.F215A and p.L218A were detected by double-immunolabeling (D) and *in situ* PLA (E) for pejvakín and LC3B before and after oxidative stress. The bar charts in D and E show that H<sub>2</sub>O<sub>2</sub> treatment increases LC3B recruitment to pejvakín in transfected HeLa cells producing EGFP and wild-type pejvakín but not in cells producing mutated pejvakín or untransfected cells ( $n = 20$  cells per condition). Data are mean  $\pm$  SEM.  $**P < 0.01$ ;  $***P < 0.001$ ; n.s., not significant, unpaired Student's *t* test. (Scale bars: 5  $\mu$ m.)

proliferation might accrue from rescue experiments in *Pjvk*<sup>-/-</sup> mice. Therefore, we assessed the effects of pejvakín and LC3B, separately and together, on these processes by injecting AAV2/8-Pjvk-IRES-EGFP, AAV2/8-Flag-Lc3b, or both into *Pjvk*<sup>-/-</sup> cochleas. At 1 h after the end of sound exposure, LC3B targeting to peroxisomes in *Pjvk*<sup>-/-</sup> IHCs overexpressing both Pjvk and Lc3b cDNAs, as detected by PLA and coimmunolabeling, was similar to that observed in wild-type mice (mean  $\pm$  SEM,  $5.25 \pm 0.85$  LC3B/pejvakín PLA spots per transduced IHC vs.  $6.08 \pm 0.5$  spots per *Pjvk*<sup>+/+</sup> IHC;  $P = 0.40$  and  $4.88 \pm 0.85$  LC3B/pejvakín-coimmunolabeled spots per transduced IHC vs.  $5.63 \pm 0.55$  spots per *Pjvk*<sup>+/+</sup> IHC;  $P = 0.40$ ), whereas the reexpression of Pjvk alone led to a smaller increase in the number of these spots (mean  $\pm$  SEM,  $2.73 \pm 0.4$  LC3B/pejvakín PLA spots per transduced IHC vs.  $6.08 \pm 0.5$  spots per *Pjvk*<sup>+/+</sup> IHC;  $P < 0.0001$ ) (Fig. 5A–C).

Forty-eight hours later, peroxisome numbers had increased by a factor of 2.2 with respect to their prestimulation number in *Pjvk*<sup>-/-</sup> IHCs transduced with both AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b and by a factor of 2.4 in *Pjvk*<sup>+/+</sup> IHCs, the difference between these two values being nonsignificant (Fig. 5D). In contrast, the transduction of *Pjvk*<sup>-/-</sup> IHCs with Pjvk

cDNA alone increased peroxisome numbers by a factor of 1.4 (Fig. 5D). The injection of Lc3b cDNA alone neither increased early LC3B-pejvakín interactions nor elicited peroxisome proliferation in *Pjvk*<sup>-/-</sup> mice (Fig. 5D).

We then investigated lipid peroxidation in auditory hair cells (as a marker of ROS damage) by assessing immunoreactivity for a byproduct of this process, 4-hydroxy-2-nonenal (4-HNE). The inverse correlation between the level of pexophagy (Figs. 1C and D and 5B and C) and that of 4-HNE (Fig. 5E) under the various experimental conditions, with the maximal effect obtained following cotransduction with Pjvk and Lc3b cDNAs, provided evidence for a major role of pejvakín-mediated pexophagy in redox homeostasis and the protection of auditory hair cells against noise-induced damage. This situation is reminiscent of the link between oxidative stress-induced pexophagy and redox homeostasis observed in cell lines (31). Moreover, the parallel changes in the magnitude of pexophagic and peroxisome proliferation responses to sound under the various experimental conditions, together with the observation of the strongest effects when LC3B was coexpressed with pejvakín (Fig. 5), suggested



**Fig. 3.** Stress-induced pejkvkin binding to LC3B is regulated by pejkvkin C28 and C343 residue oxidation. (A) Schematic representation of cysteines N- or C-terminal positions to the predicted chaperone domain of pejkvkin. (B and C) Interactions between pejkvkin and LC3B in transfected HeLa cells producing EGFP together with wild-type pejkvkin (Pjvk) or the mutated forms of pejkvkin, C328S and C343S, detected by double-immunolabeling (B) and *in situ* PLA (C) for pejkvkin and LC3B before and after oxidative stress. The bar charts in B and C show that H<sub>2</sub>O<sub>2</sub> treatment increases LC3B recruitment by pejkvkin in transfected HeLa cells producing EGFP and wild-type pejkvkin but not in cells producing mutated pejkvkin ( $n = 20$  cells per condition). Data are mean  $\pm$  SEM. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., not significant, unpaired Student's  $t$  test. (Scale bars: 5  $\mu$ m.)

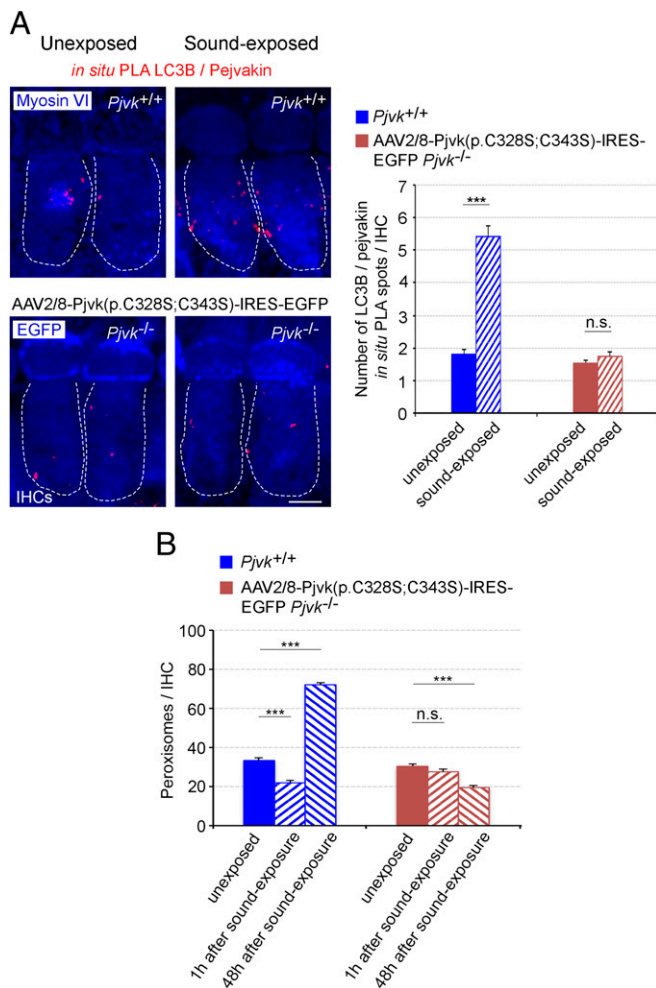
that noise-induced peroxisome proliferation is dependent on pejkvkin-mediated pexophagy.

### Discussion

Noise overexposure increases ROS levels, causing oxidative damage to auditory hair cells and neurons, resulting in hearing loss (1, 2). The peroxisome is a major source of H<sub>2</sub>O<sub>2</sub>. Its production and processing of H<sub>2</sub>O<sub>2</sub> enables it to maintain its own redox balance, but this balance can be overwhelmed, leading to ROS-mediated damage to peroxisomal proteins and membrane lipids and a disturbance in the redox homeostasis of the cell (5). Peroxisomes adapt to the metabolic state of the cell by varying

their proliferation and degradation and modifying their enzymatic content and shape (5, 6). We previously showed that peroxisome proliferation occurs at 48 h after noise overexposure, and that this proliferation is impaired in a mouse model of human deafness (DFNB59) lacking pejkvkin and characterized by hypervulnerability to sound (1).

We show here that peroxisome proliferation in response to sound overstimulation is preceded by an early autophagic degradation of peroxisomes (pexophagy). The molecular mechanisms of pexophagy have been deciphered in detail in yeast and plants, but they remain poorly understood in mammals (32). Mammalian pexophagy, the principal mechanisms by which damaged or



**Fig. 4.** Transfer of the p.C328S/p.C343S mutated form of pejkakin into *Pjvk*<sup>-/-</sup> IHCs does not restore neither early pexophagy nor peroxisome proliferation after sound exposure. (A) The number of LC3B/pejkakin in situ PLA spots does not increase at 1 h after sound exposure (5–40 kHz, 105 dB SPL, 1 h) in *Pjvk*<sup>-/-</sup> IHCs transduced with AAV2/8-*Pjvk*(p.C328S;C343S)-IRES-EGFP ( $n = 40$  IHCs; four mice per condition). (B) Peroxisome degradation (at 1 h after sound exposure) and proliferation (at 48 h after sound exposure) are not restored in *Pjvk*<sup>-/-</sup> IHCs transduced with AAV2/8-*Pjvk*(p.C328S;C343S)-IRES-EGFP ( $n = 60$  IHCs; four mice per condition). Data are mean  $\pm$  SEM. \*\*\* $P < 0.001$ ; n.s., not significant, unpaired Student's *t* test. (Scale bar: 5  $\mu$ m.)

excess peroxisomes are degraded, involves the sequestration of these organelles in autophagosomes and their transfer to lysosomes. Three ubiquitin-dependent pexophagy pathways have been described (3), including one triggered by high levels of ROS. In this recently deciphered pathway, the ataxia telangiectasia mutated kinase (ATM), activated by ROS via the formation of a disulfide cross-linked dimer, is localized to peroxisomes (31) by the peroxisomal import receptor, PEX5 (33, 34). In response to ROS, ATM phosphorylates PEX5, promoting its monoubiquitination. Ubiquitinated PEX5 is recognized by the autophagy receptor/adaptor protein p62, directing the autophagosome to peroxisomes to induce selective pexophagy (31). NBR1 is also involved in this process (22). The p62 and NBR1 proteins have similar domain organizations; p62 has one LIR, and NBR1 has two LIRs, which bind to the LC3 associated with the nascent autophagosome.

We show here that pejkakin plays an essential role in sound-induced pexophagy. Unlike p62 and NBR1, pejkakin, which also contains an LIR domain, has no UBA domain. The lack of detection of a direct interaction of pejkakin with p62 and NBR1 by

PLA under oxidative stress conditions does not exclude the involvement of pejkakin in this pathway. However, the absence of a UBA domain in pejkakin suggests that this protein may mediate an ROS-dependent and ubiquitination-independent pexophagy pathway. This situation is reminiscent of the pexophagy induced by nutrient starvation in mammalian cells, which has been shown to involve activation of the peroxisomal membrane protein, PEX14, which binds directly to LC3B (35). In vitro and in vivo experiments identified two cysteine residues of pejkakin, C328 and C343, as playing crucial roles in ROS-induced pejkakin-LC3B interaction and the pexophagy pathway. It remains to be clarified how these cysteine residues regulate pejkakin activity according to their redox state, and whether they are involved in intramolecular or intermolecular disulfide bond formation.

Our results reveal that pejkakin-mediated pexophagy precedes the proliferation of peroxisomes and protects the auditory hair cells against oxidative damage. Rescue experiments in *Pjvk*<sup>-/-</sup> mice stimulated with sound revealed a strong correlation between levels of pexophagy induction and the restoration of peroxisome proliferation. From the finding that adding LC3B to pejkakin increased not only pexophagy, but also peroxisome proliferation, we infer that ROS-induced pexophagy is involved in controlling peroxisome proliferation. The role of pejkakin in pexophagy can itself account for the reported lack of peroxisome proliferation in *Pjvk*<sup>-/-</sup> mice (1), but we cannot formally exclude the possibility of an additional direct role of pejkakin in peroxisome proliferation.

Overall, our findings reveal the key role of pexophagy in maintaining redox homeostasis and protecting the auditory system against noise-induced hearing loss. Peroxisomes and mitochondria are closely related organelles with similar clearance mechanisms (32, 36). Therefore, it would be interesting to investigate whether mitochondrial autophagy (mitophagy) also protects the hearing system against noise-induced damage.

Finally, our results establish that the DFNB59 form of deafness is a pexophagy disorder. Several peroxisomal membrane proteins have been implicated in pexophagy, and dysfunctions of pexophagy are being increasingly reported in peroxisomal disorders (37). The role of pejkakin in oxidative stress-induced pexophagy reported here demonstrates another function for gasdermin family proteins, which for most of them triggers pyroptosis (11–14).

## Materials and Methods

**Animals.** All animal experiments were performed in accordance with French and European legislation on the care and protection of laboratory animals (EC Directive 2010/63, French Law 2013-118, February 6, 2013) and the regulations of the Institut Pasteur Animal Care Committee. *Pjvk*<sup>-/-</sup> mice (approved allele designation *Pjvk*<sup>tm2.1Ugd</sup>; MGI accession ID 5908028) were produced in a C57BL/6-129/Sv mixed genetic background (1).

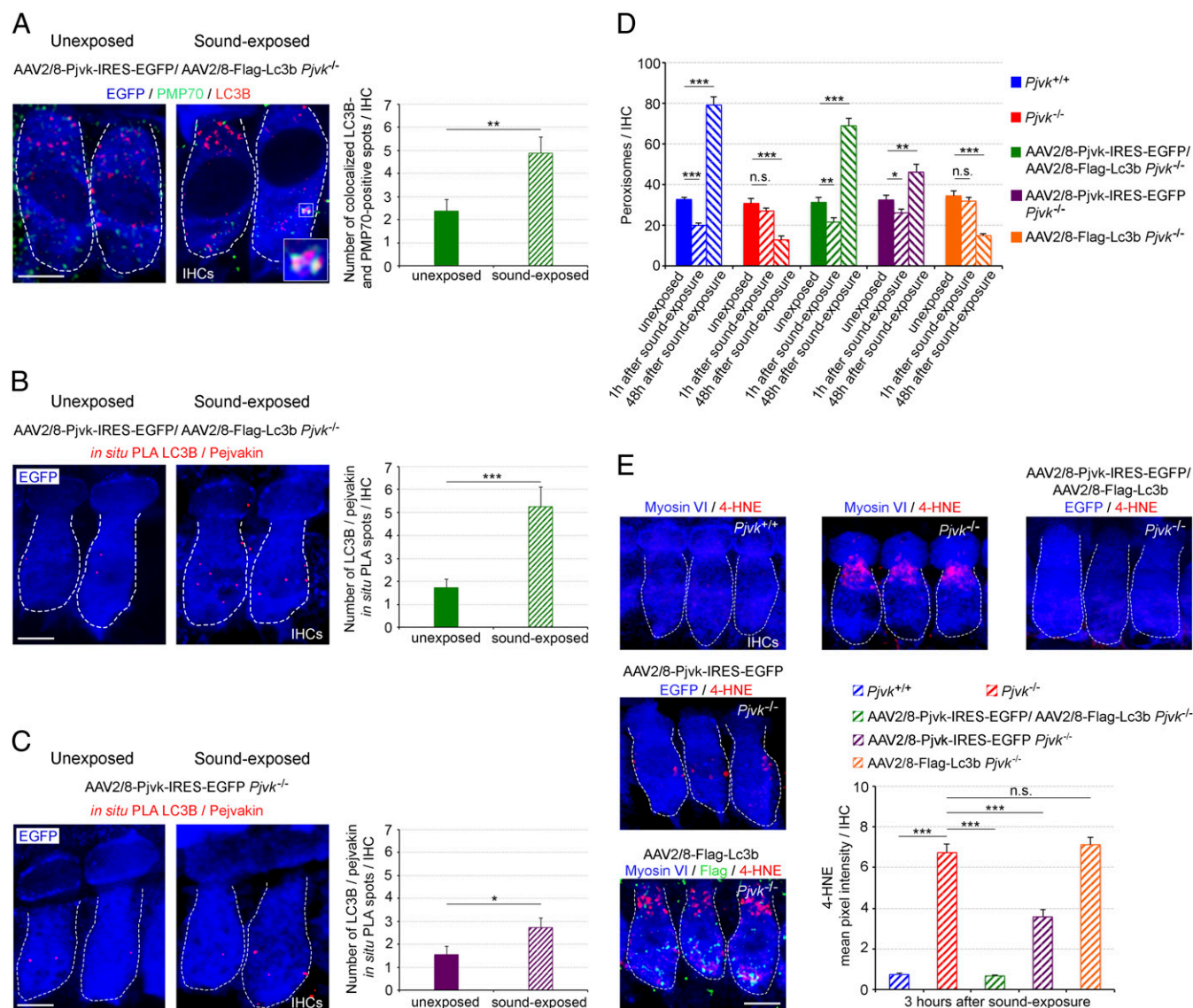
**Housing of Mice in an Acoustically Quiet Environment.** Because *Pjvk*<sup>-/-</sup> mice are hypervulnerable to the natural acoustic environment (1), we split pups from the same litter and placed in isolated boxes. The pups were separated before P10, corresponding to several days before onset of hearing in mice. The boxes were kept in quiet booths, shielded from the sounds emanating from other cages. Each cage contained four mice and a foster mother.

**Acoustic Overexposure.** Three-week-old animals were exposed for 1 h to broadband white noise subjected to bandpass filtering over the 5–40 kHz frequency interval and applied at an intensity of 105 dB SPL. The white noise signal was generated with in-house MATLAB software (MathWorks) and was delivered by an amplifier to a set of four Ultrasonic Vifa speakers (Avisoft Bioacoustics).

**Treatment of HepG2 and HeLa Cells with H<sub>2</sub>O<sub>2</sub>.** The cells were treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> at 37 °C for 6 h under normoxic conditions (95% air) and then fixed in 4% paraformaldehyde (PFA).

**Plasmids and DNA Transfection.** The full-length pejkakin cDNA was obtained by RT-PCR, as described previously (1). The mutant pejkakin clones were prepared from the wild-type pejkakin clone with the QuikChange Site-Directed





**Fig. 5.** Transfer of Pjvk and Lc3b into *Pjvk*<sup>-/-</sup> IHCs restores pexophagy and abolishes lipid peroxidation after sound exposure. (A) The number of LC3B-positive spots colocalizing with PMP70 labeling was higher at 1 h after sound exposure (5–40 kHz, 105 dB SPL, 1 h) in *Pjvk*<sup>-/-</sup> IHCs cotransduced with AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b. (Inset) LC3B recruitment to peroxisomes. (B) The number of LC3B/pejavkin in situ PLA spots was increased at 1 h after sound exposure in *Pjvk*<sup>-/-</sup> IHCs cotransduced with AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b. (C) The number of LC3B/pejavkin in situ PLA spots had increased slightly at 1 h after sound exposure in *Pjvk*<sup>-/-</sup> IHCs transduced with AAV2/8-Pjvk-IRES-EGFP. The bar charts in A–C quantify LC3B-positive spots colocalizing with PMP70 labeling (A) and LC3B/pejavkin in situ PLA spots (B and C) in treated IHCs before and after sound exposure ( $n = 40$  IHCs; four mice per condition). (D) Peroxisome degradation (at 1 h after sound exposure) and proliferation (at 48 h after sound exposure) were fully restored in *Pjvk*<sup>-/-</sup> IHCs cotransduced with AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b. AAV2/8-Pjvk-IRES-EGFP transduction of *Pjvk*<sup>-/-</sup> IHCs partially rescued early pexophagy and proliferation, whereas the transduction of *Pjvk*<sup>-/-</sup> IHCs with AAV2/8-Flag-Lc3b had no effect ( $n = 40$  IHCs from four mice). (E) Lipid peroxidation levels, as assessed by 4-HNE immunolabeling, were higher in *Pjvk*<sup>-/-</sup> IHCs than in *Pjvk*<sup>+/+</sup> IHCs at 3 h after sound exposure. Lipid peroxidation was abolished in *Pjvk*<sup>-/-</sup> IHCs cotransduced with AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b but was significantly increased in both *Pjvk*<sup>-/-</sup> IHCs and AAV2/8-Flag-Lc3b *Pjvk*<sup>-/-</sup> IHCs. 4-HNE immunoreactivity was weaker in AAV2/8-Pjvk-IRES-EGFP *Pjvk*<sup>-/-</sup> IHCs than in *Pjvk*<sup>-/-</sup> IHCs ( $n = 50$  cells; four mice per condition). Data are mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., not significant, unpaired Student's  $t$  test. (Scale bars: 5  $\mu$ m.)

Mutagenesis kit (Stratagene). HeLa cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

**Immunofluorescence Studies.** For whole-mount immunolabeling analyses, inner ears were fixed in 4% PFA in PBS, and the cochlear sensory areas (organ of Corti) were microdissected. The tissues were rinsed twice in PBS, then permeabilized and blocked by incubation in 20% normal goat serum and 0.3% Triton X-100 in PBS for 1 h at room temperature. For GFP or Flag detection, whole-mount cochleas were incubated with a chicken anti-GFP antibody (1:100; Abcam ab13970) or a rabbit anti-Flag antibody (1:100; Sigma-Aldrich F7425) in 1% BSA in PBS. Anti-parvalbumin (1:200; Abcam Ab32895)

and anti-myosin VI (1:100; Santa Cruz Biotechnology sc-23568) antibodies were used to delimit the contours of IHCs and outer hair cells (OHCs). Anti-PMP70 (1:100; Sigma-Aldrich SAB4200181) and anti-LC3B (1:100; Abcam ab51520) antibodies were used to label peroxisomes and autophagosomes, respectively.

For immunocytofluorescence analyses, HeLa and HepG2 cells were fixed by incubation in 4% PFA in PBS for 15 min, washed in PBS, and incubated in 50 mM  $\text{NH}_4\text{Cl}$  and 0.2% Triton X-100 for 15 min at room temperature. The cells were then washed and incubated in 20% normal goat serum in PBS for 1 h, then incubated with the primary antibody in 1% BSA in PBS for 1 h. Antibodies against peroxisome membrane protein 70 (PMP70, 1:100; Sigma-Aldrich SAB4200181) and LC3B (1:100; Abcam ab51520) were used to label

peroxisomes and autophagosomes, respectively. The mouse monoclonal antibody against pejkakin (Pjvk-G21) (1) was used at a concentration of 100  $\mu\text{g}/\text{mL}$ . Cells were washed in PBS and incubated with the appropriate secondary antibody for 1 h at room temperature.

For immunofluorescence studies, we used Atto-647-conjugated goat anti-rabbit IgG (1:500; Sigma-Aldrich 40839), Atto-550-conjugated goat anti-mouse IgG (1:500, Sigma-Aldrich 43394), and Alexa Fluor 488-conjugated goat anti-chicken IgG (1:500; Invitrogen A11039) as secondary antibodies. DAPI (1:7,500, Sigma-Aldrich D9542) was used to label cell nuclei. Images were acquired with a Zeiss LSM700 Meta confocal microscope (Carl Zeiss MicroImaging). For whole-mount cochlea experiments, we analyzed the auditory hair cells from the middle part of the cochlea.

**In Situ PLA.** The Duolink in situ PLA was performed according to the manufacturer's protocol (<https://www.sigmaldrich.com/technical-documents/protocols/biology/duolink-fluorescence-user-manual.html>) to detect the interaction between pejkakin and Lc3b, NBR1, or p62 in HepG2, HeLa, and cochlear hair cells. Cells were immunolabeled with a mouse monoclonal antibody against pejkakin (Pjvk-G21; 1:50) and rabbit polyclonal antibodies against LC3B (1:100; Abcam ab51520), NBR1 (1:100; Abcam ab219862), and p62 (1:100; Abcam ab91526). Anti-mouse and anti-rabbit secondary antibodies with PLA probes were supplied in the Duolink kit (Olink; DUO92101-1KT). DAPI was used to label cell nuclei.

For GFP detection, whole-mount cochleas were incubated with a chicken anti-GFP antibody (1:100; Abcam ab13970). Goat anti-myosin VI antibody (1:100; Santa Cruz Biotechnology sc-23568) was used to delimit the contours of IHCs and OHCs. Cells were observed by confocal microscopy, and interactions were detected as a fluorescent signal (red spot).

**AAV-Pjvk and AAV-Lc3b Viral Constructs and Intracochlear Transduction.** AAV2/8 expressing either wild-type pejkakin or a mutated form of pejkakin (p.C328S;C343S), and AAV2/8-Flag-Lc3b were obtained by inserting the murine wild-type or mutated pejkakin cDNAs flanked by an IRES-EGFP reporter gene and Lc3b cDNA flanked by a Flag reporter gene into the multiple cloning site

of the pENN.AAV.CB7.CI.RBG vector (PennVector PL-C-PV1044; Penn Medicine Vector Core, University of Pennsylvania School of Medicine). The viruses were produced and titrated by Penn Medicine Vector Core.

Intracochlear viral transduction was performed in P3 *Pjvk*<sup>-/-</sup> mice as described previously (1). A fixed volume (2  $\mu\text{L}$ ) of a solution containing AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b alone or together ( $10^{13}$  viral genomes/mL), or AAV2/8-Pjvk(p.C328S;C343S)-IRES-EGFP alone, was carefully injected into the perilymphatic compartment of the cochlea through the round window.

**Quantification of Lipid Peroxidation.** Lipid peroxidation in the cochlea was detected by immunohistochemistry with an antibody against 4-hydroxy-2-nonenal (4-HNE) (1:200; Abcam ab48506). Images were acquired with a Zeiss LSM700 Meta confocal microscope under identical settings to allow comparisons. The mean pixel intensity of 4-HNE immunolabeling in cochlear hair cells was quantified with ImageJ software.

**Prediction Tools.** The Phyre2 web portal was used to predict and analyze pejkakin structure and function (23). Fpocket, an open-source platform, was used to detect the ligand-binding pocket (26). The pejkakin protein sequences were multiply aligned using the MAFFT web server.

**Statistical Analyses.** Quantitative data are presented as mean  $\pm$  SEM, and statistical analyses were performed with GraphPad Prism. Data were analyzed using the unpaired Student *t* test and, for multiple comparisons, by the *t* test with Bonferroni correction. The differences between groups were considered significant at  $P < 0.05$ .

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