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(54) Title: COMBINATION OF PEJVAKIN AND LC3B FOR TREATING HEARING LOSSES

(57) Abstract: The balance between peroxisome biogenesis and degradation is crucial for redox cell homeostasis. The present invention is based on the findings that pejvakine is involved in the early and rapid selective autophagic degradation of peroxisome (pexophagy) in auditory hair cells subjected to sound overstimulation, by interacting with the autophagosome-associated protein MAP1LC3B. It is here demonstrated that it is possible to completely restore sound-induced pexophagy and to prevent oxidative stress in *Pjvk*^{-/-} auditory hair cells by transducing in same the Pejvakine and MAP1LC3B proteins together. Thus, the present invention relates to compositions containing these two proteins, or gene vectors encoding same, as well as their therapeutic use for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, or hereditary hearing loss due to an altered expression level of Pejvakine or to an altered *DFNB59* gene expression, in a subject in need thereof.



COMBINATION OF PEJVAKIN AND LC3B FOR TREATING HEARING LOSSES

Summary

The balance between peroxisome biogenesis and degradation is crucial for redox cell homeostasis. The present invention is based on the findings that pejkakin is involved in the early and rapid selective autophagic degradation of peroxisome (pexophagy) in auditory hair cells subjected to sound overstimulation, by interacting with the autophagosome-associated protein MAP1LC3B. It is here demonstrated that it is possible to completely restore sound-induced pexophagy and to prevent oxidative stress in *Pjvk*^{-/-} auditory hair cells by transducing in same the Pejvakin and MAP1LC3B proteins together. Thus, the present invention relates to compositions containing these two proteins, or gene vectors encoding same, as well as their therapeutic use for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, or hereditary hearing loss due to an altered expression level of Pejvakin or to an altered *DFNB59* gene expression, in a subject in need thereof.

Prior art description

Age-related hearing loss (AHL or presbycusis), which affects more than 30% people above 60 and overall, about 5 million people in France, is surely the result of a combination of factors, genetic and environmental (lifelong exposure to noise and to chemicals). Yet it has been shown in the 1950s that subjects who spend their lives in silent environments do not suffer from any hearing loss even in their 80s. This, and a huge body of evidence collected in subjects occupationally exposed to noise, leads to conclude that noise-induced hearing loss (NIHL) is the dominant cause of hearing impairment in ageing subjects. It is one of the most frequent conditions in workers, and an increasing matter of concern as exposure to loud sound during leisure has increased dramatically, particularly in younger subjects, with the development of inexpensive portable music players. Permanent hearing loss resulting from the loss of auditory hair cells (HCs) and spinal

ganglion neurons (SGNs) is irreversible because the cells are terminally developed and cannot be replaced by mitosis. Although great efforts have been made to regenerate lost HCs and SGNs in mammals, these efforts have been largely unsuccessful so far.

It is well-known that noise overexposure increases reactive oxygen species (ROS) levels, causing oxidative damage to auditory hair cells, resulting in hearing loss (Ohlemiller K.K. et al., 1999). The present inventors have previously demonstrated that an adaptive peroxisome proliferation involving pejvakin, a peroxisome-associated protein from the gasdermin family, buffers this harmful oxidative stress (Delmaghani S. et al, 2015). More precisely, defects of pejvakin underlying an autosomal recessive form of deafness (DFNB59) (Delmahani S. et al, 2006), cause hypervulnerability to sound in humans and mice associated with high oxidative stress in auditory hair cells and neurons (Delmaghani S. et al, 2015; WO 2016/131981).

The precise function of pejvakin in peroxisome biogenesis is still unknown. Yet, if elucidated, it could lead the scientists to identify novel treatments to counteract or alleviate hearing losses.

Peroxisome biogenesis can occur *de novo* or through the proliferation of existing peroxisomes (Smith JJ et al., 2013; Schrader, M. et al 2012). Damaged peroxisomes are degraded by selective autophagy (pexophagy), in yeast and plant vacuoles, and in mammalian lysosomes (Platta, H. W. & Erdmann, R, 2012). The balance between peroxisome biogenesis and degradation is crucial for redox cell homeostasis. To elucidate the mechanisms involved in this balance, the present inventors studied the role of pejvakin in peroxisome dynamics and homeostasis.

Detailed description of the invention

The results below show that pejvakin is involved in the early and rapid selective autophagic degradation of peroxisome (pexophagy) in auditory hair cells subjected to sound overstimulation, by interacting with the autophagosome-associated protein MAP1LC3B.

Moreover, these results show that it is possible to completely restore sound-induced pexophagy and to prevent oxidative stress in *Pjvk*^{-/-} auditory hair cells by transducing in same the Pejvakin and MAP1LC3B proteins together.

These findings have major therapeutic implications, as described below.

- 5 The human gasdermin family has six members (gasdermins A-E and pejvakin), four of which (gasdermins A, B, D, E) trigger pyroptosis, a form of programmed lytic cell death initiated by inflammasome activation (Kovacs SB & Miao E.A., 2017). All 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. Their
- 10 pyroptotic pore-forming activity results from inflammatory caspase cleavage at the linker domain, separating the N-terminal pore-forming from the C-terminal repressor domain (Shi et al, 2015; Kayagaki, N., 2015; Ding et al, 2016; Rogers C. et al, 2017).

- Here, the present Inventors show for the first time that sound overstimulation induces an early and rapid selective autophagic degradation of peroxisome (pexophagy) in auditory
- 15 hair cells from wild-type but not pejvakin-deficient (*Pjvk*^{-/-}) mice. They also show that noise overexposure triggered recruitment of the autophagosome-associated protein MAP1LC3B (LC3B; microtubule-associated protein 1 light chain 3β) to peroxisomes in wild-type but not *Pjvk*^{-/-} mice. Moreover, the inventors demonstrated that pejvakin binds LC3B via an LC3-interacting region (LIR) located in its predicted chaperone domain, this
 - 20 interaction requiring oxidation of the two cysteine residues closest to the C-terminus of pejvakin, C328 and C343. Finally, the inventors herein show that the viral transduction of *Pjvk*^{-/-} auditory hair cells with both *Pjvk* and LC3B cDNAs is required for the complete restoration of sound-induced pexophagy, preventing oxidative stress, and allowing subsequent peroxisome proliferation.

25

Definitions of the proteins of the invention

The term “**gasdermin**” as used herein refers to any member of the gasdermin family of proteins or polypeptides, or any homolog of a member of the gasdermin family of proteins or polypeptides, from humans or non-human mammals such as primates, cats, dogs,

swine, cattle, sheep, goats, horses, rabbits, rats, mice, and the like. This term encompasses for example: gasdermin A, gasdermin B, gasdermin C, gasdermin D, gasdermin E (DFNA5), cyclophilin A, c-dopachrome tautomerase, Mpv17, and DFNB59 (or pejvakin) (Shi et al., 2015; Saeki and Sasaki, 2011). All these proteins have been thoroughly
5 described in WO 2016/131981, which is incorporated herein by reference.

The term “**functional fragment of gasdermin**” designates any fragment of a member of the gasdermin family of proteins or polypeptides, or any fragment of a homolog of a member of the gasdermin family of proteins or polypeptides, wherein said fragment retains at least one biological function of the gasdermin that is of interest in the present
10 context (Shi et al., 2015; Saeki and Sasaki, 2011). Preferably, said fragment contains at least the N-terminal domain of said gasdermin.

Pejvakin or autosomal recessive deafness type 59 protein or PJVK is a protein belonging to the gasdermin family. In human, it has the sequence SEQ ID NO:1 (NCBI Reference Sequence: NP_001036167.1). In mouse, it has the sequence SEQ ID NO:2 (NCBI
15 Reference Sequence: NP_001074180). It is known to be expressed in all the relays of the afferent auditory pathway from the cochlea to the midbrain and is thought to play a critical role in the physiology of auditory neurons (Delmaghani S. et al, 2006). Several impairing mutations have been described (Collin et al., 2007; Ebermann et al., 2007; Hashemzadeh Chaleshtori et al., 2007; Schwander et al., 2007; Borck et al., 2012; Mujtaba et al., 2012;
20 Zhang et al., 2015).

Human pejvakin is encoded by the *DFNB59* gene of SEQ ID NO:3 in human (NCBI Reference Sequence: NM_001042702.3, the coding sequence being comprised between the nucleotides 357 and 1415). The mouse *Dfnb59* gene is of SEQ ID NO:4 (NCBI Reference Sequence: NM_001080711.2, the coding sequence being comprised between
25 the nucleotides 150 and 1208).

In the context of the invention, the term “**pejvakin**” herein designates a polypeptide having the amino acid sequence SEQ ID NO:1 (human PJVK) or SEQ ID NO:2 (mouse PJVK) or an homologous sequence thereof. Said latter **homologous sequence** is for example the PJVK protein of another animal species retaining at least one biological
30 function of human PJVK or mouse PJVK that is of interest in the present context. For

example, this biological function is related to the modulation of the pexophagy and/or the oxidative stress induced by sound stimulation in auditory hair cells. It can be highlighted as proposed by the Inventors in the examples below (see figures 1 and 4), e.g., by restoring the normal level of pexophagy and/or the redox equilibrium in cells that are deficient from Pjvk (*pjvk*^{-/-} cells), after sound exposure. This latter homologous sequence shares preferably at least 50%, preferably at least 60%, more preferably at least 70%, more preferably at least 80%, and even more preferably at least 90% identity with SEQ ID NO:1 or SEQ ID NO:2.

In the context of the invention, the identity percentage between said two homologous sequences is identified by a global alignment of the sequences in their entirety, this alignment being performed by means of an algorithm that is well known by the skilled person, such as the one disclosed in Needleman and Wunsch (1970). Accordingly, sequence comparisons between two amino acid sequences or two nucleotide sequences can be performed for example by using any software known by the skilled person, such as the “needle” software using the “Gap open” parameter of 10, the “Gap extend” parameter of 0.5 and the “Blosum 62” matrix.

In another embodiment, the term “pejvakin” also designates any polypeptide encoded by a *DFNB59* gene. In a preferred embodiment, said *DFNB59* gene is chosen in the group consisting of: SEQ ID NO:3, SEQ ID NO:4, or any homologous gene of another animal species, said homologous gene encoding a protein that shares at least 50% identity with SEQ ID NO:1 or SEQ ID NO:2 and more particularly preferably at least 60%, more preferably at least 70%, more preferably at least 80%, and even more preferably at least 90% identity with SEQ ID NO:1 or SEQ ID NO:2. This polypeptide may contain the N-terminal and C-terminal parts of SEQ ID NO:1 or SEQ ID NO:2, and some amino acids can be substituted, deleted or incorporated between these two essential parts.

The term “**functional fragment of the pejvakin protein**” herein designates any fragment of human PJVK or mouse PJVK or any fragment of a protein or polypeptide having a homologous sequence as defined above, wherein said fragment retains at least one biological function of human PJVK or mouse PJVK that is of interest in the present context. For example, this biological function is related to the modulation of the

pexophagy and/or the oxidative stress induced by sound stimulation in auditory hair cells. It can be highlighted as proposed by the Inventors in the examples below (see figures 1 and 4), e.g., by restoring the normal level of pexophagy and/or the redox equilibrium in cells that are deficient from Pjvk (*pjvk*^{-/-} cells), after sound exposure.

- 5 Autophagy is a major intracellular pathway for the degradation and recycling of long-lived proteins and entire organelles. LC3s (MAP1-LC3A, B and C) are structural proteins of autophagosomal membranes. They have been described for example in Koukourakis MI. et al, PLoS One 2015.

The term “LC3 protein” herein designates the proteins MAP1-LC3A, B and C described
10 in Koukourakis MI. et al, PLoS One 2015.

The LC3B protein, also called “Microtubule Associated Protein 1 Light Chain 3 Beta (MAP1LC3B)” or “ATG8F” is an autophagosome-associated protein that is widely used as biomarker of autophagy.

- In the context of the invention, the term “**LC3B**” designates a polypeptide having the
15 amino acid sequence SEQ ID NO:5 (human LC3B, NP_073729) or SEQ ID NO:6 (mouse LC3B, NP_080436) or an homologous sequence thereof. Said latter **homologous sequence** is for example the LC3B protein of another animal species, retaining at least one biological function of human LC3B or mouse LC3B that is of interest in the present context. For example, this biological function is related to the modulation of the
20 pexophagy and/or the oxidative stress induced by sound stimulation in auditory hair cells. It can be highlighted as proposed by the Inventors in the examples below (see figure 4), e.g., by restoring the normal level of pexophagy and/or the redox equilibrium in cells that are deficient from Pjvk (*pjvk*^{-/-} cells), after sound exposure, when co-injected with a pejvakin protein. This latter homologous sequence shares preferably at least 50%,
25 preferably at least 60%, more preferably at least 70%, more preferably at least 80%, and even more preferably at least 90% identity with SEQ ID NO:5 or SEQ ID NO:6.

In another embodiment, the term “LC3B” also designates any polypeptide encoded by the *LC3B* gene. In human, said gene has the sequence SEQ ID NO:7 (NCBI NM_022818). In mouse, said gene has the sequence SEQ ID NO:8 (NCBI NM_026160).

The term “**functional fragment of the LC3B protein**” herein designates any fragment of human LC3B or mouse LC3B or any fragment of a polypeptide having a homologous sequence as defined above, wherein said fragment retains at least one biological function of human LC3B or mouse LC3B that is of interest in the present context. For example, this biological function is related to the modulation of the pexophagy and/or the oxidative stress induced by sound stimulation in auditory hair cells. It can be highlighted as proposed by the Inventors in the examples below (see figure 4), e.g., by restoring the normal level of pexophagy and/or the redox equilibrium in cells that are deficient from Pjvk (pjvk^{-/-} cells), after sound exposure, when co-injected with a pejvakin protein.

Gene therapy vectors

The two above-described proteins can be produced *in situ* in the appropriate auditory cells by *in vivo* gene therapy.

Two alternative strategies for gene therapy can be contemplated for treating animal subjects. One strategy is to administer vector(s) encoding the gene(s) of interest directly to the subject. The second is to use cells that have been i) removed from the target subject and ii) treated *ex vivo* with vector(s) expressing the gene(s) of interest; these cells are then re-administered to the same subject.

Different methods for cellular gene therapy are known in the art. These methods include, yet are not limited to, the use of DNA plasmid vectors as well as DNA and RNA viral vectors. In the present invention, such vectors may be used to express the proteins of the invention in cells of the auditory pathway such as cochlear hair cells, afferent auditory neurons and neurons of the auditory brainstem pathway.

The two proteins of the invention can be conveyed into the target cells by either one unique vector (carrying both protein genes) or two separate vectors (each carrying one protein gene). In both cases, the said vector(s) will belong to the “**gene therapy vector system of the invention**”.

More precisely, said system can contain either one gene therapy vector encoding both the gasdermin protein, in particular pejvakin, and the LC3 protein, in particular the LC3B

protein, (or their fragments), or two gene therapy vectors, separately encoding the gasdermin protein and the LC3 protein (or their fragments).

In one particular aspect, the present invention relates to a vector encoding both the gasdermin protein, in particular pejkakin, and the LC3 protein, in particular the LC3B
5 protein, (or their functional fragments described above).

In another particular aspect, the present invention relates to a kit containing at least two distinct vectors, among which one vector encodes the gasdermin protein, in particular pejkakin, or a functional fragment thereof, and one other vector encodes the LC3 protein, in particular the LC3B protein, or a functional fragment thereof.

10 In any case, the vector(s) of the system of the invention are preferably viral vector(s) that is(are) able to transfect the cells of the auditory pathway such as cochlear hair cells, afferent auditory neurons and neurons of the auditory brainstem pathway. These vectors are well-known in the art. They are for example lentiviruses, adenoviruses and Adeno-associated viruses (AAV).

15 In a preferred embodiment, said vector(s) is(are) AAV vector(s).

The AAV vectors display several advantages such as i) a long lasting expression of synthesized genes (Cooper et al, 2006), ii) a low risk for pathogenic reactions (because they are artificially manufactured and not ototoxic), iii) they trigger low immunogenic response, and iv) they do not integrate the human genome (Kaplitt et al., 1994). AAV is
20 therefore preferred to transfer the genes of the proteins of the invention in order to efficiently protect the auditory pathway.

The AAV vectors targeted by the present invention are any adeno-associated virus known in the art including, but not limited to AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, and AAV10.

25 In a more preferred embodiment, the serotype of said vector is AAV8, AAV5, or AAV1.

In order to increase the efficacy of gene expression, and prevent the unintended spread of the virus, genetic modifications of AAV can be performed. These genetic modifications include the deletion of the E1 region, deletion of the E1 region along with deletion of

either the E2 or E4 region, or deletion of the entire adenovirus genome except the cis-acting inverted terminal repeats and a packaging signal. Such vectors are advantageously encompassed by the present invention.

Moreover, genetically modified AAV having a mutated capsid protein may be used so as to direct the gene expression towards a particular tissue type, e.g., to auditory cells. In this aim, modified serotype-2 and -8 AAV vectors in which tyrosine residues in the viral envelope are substituted for alanine residues can be used. In the case of tyrosine mutant serotype-2, tyrosine 444 can be substituted with alanine (AAV2-Y444A). In the case of serotype 8, tyrosine 733 can be substituted with an alanine residue (AAV8-Y733A). Such vectors are advantageously encompassed by the present invention.

Specific AAV vectors that would be able to carry the protein coding genes to auditory cells and methods to administer same are for example disclosed in WO 2011/075838. In the context of the invention, it would be for example possible to use the mutated tyrosine AAVs disclosed in WO 2011/075838 to deliver the coding genes in auditory cells. These mutated vectors avoid degradation by the proteasome, and their transduction efficiency is significantly increased. Mutated tyrosine residues on the outer surface of the capsid proteins include, for example, but are not limited to, mutations of Tyr252 to Phe252 (Y252F), Tyr272 to Phe272 (Y272F), Tyr444 to Phe444 (Y444F), Tyr500 to Phe500 (Y500F), Tyr700 to Phe700 (Y700F), Tyr704 to Phe704 (Y704F), Tyr730 to Phe730 (Y730F) and Tyr733 to Phe733 (Y733F). These modified vectors facilitate penetration of the vector across the round window membranes, which allow for non-invasive delivery of the vectors to the hair cells/spiral ganglion neurons of the cochlea. For example, by using AAV2-Y444A or AAV8-Y733A, it is possible to increase gene transfer by up to 10,000 fold, decreasing the amount of AAV necessary to infect the sensory hair cells of the cochlea.

The skilled person would easily determine if it is required, prior to the administration of the vector of the invention, to enhance the permeability of the round window membrane as proposed in WO 2011/075838, depending on the target cell.

For instance, an appropriate vector in the context of the invention is an AAV8 vector. More particularly, it can be a vector having the nucleotide sequence of an AAV2 genome that is modified so as to encode AAV8 capsid proteins.

5 *Pharmaceutic compositions of the invention*

The gasdermin (e.g., pejevakin) and the LC3 (e.g. LC3B) polypeptides (or their functional fragments) can be administered directly to the subject. In this case, the gasdermin, and the LC3 polypeptides are contained in a pharmaceutical composition further containing a pharmaceutical carrier.

- 10 In another aspect, the present invention relates to a pharmaceutical composition comprising i) a gasdermin protein or a functional fragment thereof, ii) a LC3 protein or a functional fragment thereof (as described above), and iii) a therapeutically acceptable carrier.

- 15 In a particular embodiment, the pharmaceutical composition of the invention contains a gasdermin protein chosen among: gasdermin A, gasdermin B, gasdermin C, gasdermin D, DFNA5 and DFNB59 (or pejevakin).

In a preferred embodiment, the pharmaceutical composition of the invention contains the pejevakin protein, preferably the pejevakin protein of SEQ ID NO:1, or a functional fragment thereof as described above.

- 20 In another preferred embodiment, the pharmaceutical composition of the invention also contains the LC3B protein, preferably the LC3B protein of SEQ ID NO:5, or a functional fragment thereof, and a therapeutically acceptable carrier.

In a most preferred embodiment, the pharmaceutical composition of the invention contains the the pejevakin protein of SEQ ID NO:1 and the LC3B protein of SEQ ID NO:5.

- 25 As explained above, the gasdermin (e.g., pejevakin) and the LC3, in particular the LC3B polypeptides can also be administered via gene therapy vector(s) to the subject. In this case, the vector(s) encoding gasdermin, and the LC3 polypeptides, or functional

fragments thereof, is (are) contained in a pharmaceutical composition further containing a pharmaceutical carrier.

In another aspect, the present invention relates to a pharmaceutical composition comprising a therapeutically acceptable carrier and a gene therapy vector system encoding i) the gasdermin protein (in particular pejpakin) or a functional fragment thereof and ii) the LC3 protein (in particular the LC3B protein), or a functional fragment thereof, as described above.

As used herein, the term "**pharmaceutically acceptable carrier**" or "**pharmaceutical carrier**" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers or pharmaceutical carrier include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it can be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers or pharmaceutical carriers can further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antioxidant compounds or of the pharmaceutical compositions containing same.

The pharmaceutical compositions of the invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The form used depends on the intended mode of administration and therapeutic application. Typical compositions are in the form of injectable or infusible solutions.

Pharmaceutical compositions typically must be sterile and stable under the conditions of manufacture and storage. The pharmaceutical compositions of the invention are preferably formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the polypeptides / vectors of the invention in the required

amount in an appropriate solvent optionally with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the polypeptides / vectors of the invention into a sterile vehicle that contains a basic dispersion medium and optionally other ingredients from those enumerated above, as required. In the case of sterile lyophilized powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and spray-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be achieved by including an agent in the composition that delays absorption, for example, monostearate salts and gelatine.

In certain embodiments, the polypeptides / vectors of the invention may be prepared with a carrier that will protect same against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are generally known to those skilled in the art.

The pharmaceutical compositions of the invention include a "**therapeutically effective amount**" or a "**prophylactically effective amount**" of the polypeptides / vectors of the invention. A "therapeutically effective amount" refers to the amount of polypeptides / vectors of the invention that is effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, in this case for both prophylaxis and treatment of hearing impairment or peroxisomal disorders without unacceptable toxicity or undesirable side effects.

A therapeutically effective amount of the polypeptides / vectors of the invention can vary according to factors such as the disease state, age, sex, and weight of the subject, and the ability of said compound to elicit a desired response in same. A therapeutically effective amount can also be one in which any toxic or detrimental effects of the claimed

compounds are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount of the polypeptides / vectors of the invention that is effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose can be used in subjects prior to
5 or at an earlier stage of disease, the prophylactically effective amount is usually less than the therapeutically effective amount.

Restoration of peroxisome homeostasis and alleviation of ROS-induced damages

In another aspect, the present invention relates to the compositions of the invention for
10 use for restoring peroxisome homeostasis in auditory cells from subjects suffering from peroxisomal disorders.

In another aspect, the present invention relates to the compositions of the invention for use for preventing and/or alleviating ROS-induced cellular damages, especially in cochlear hair cells, afferent auditory neurons and neurons of the auditory brainstem and
15 auditory central pathway, in a subject in need thereof.

In a preferred embodiment, they are used to prevent and/or alleviate ROS-induced cellular damages in Inner Hair Cells (IHC), Outer Hair Cells (OHC), or neurons of the auditory pathway.

In a preferred embodiment, they are used to prevent and/or alleviate ROS-induced cellular
20 damages due to noise exposure or to ototoxic substance exposure.

Said ototoxic substances can be any medication or chemical substances to which a subject has been unfortunately or voluntarily exposed. There are more than 200 known ototoxic medications (prescription and over-the-counter) on the market today. These include medicines used to treat serious infections, cancer, and heart disease. Ototoxic medications
25 known to cause permanent damage include certain aminoglycoside antibiotics, such as gentamicin, and cancer chemotherapy drugs, such as cisplatin and carboplatin. Other medications may reversibly affect hearing. This includes some diuretics, aspirin and NSAIDs, and macrolide antibiotics. On October 18, 2007, the U.S. Food and Drug Administration (FDA) announced that a warning about possible sudden hearing

impairment would be added to drug labels of PDE5 inhibitors, which are used for erectile dysfunction. In addition to medications, hearing impairment including hearing loss and auditory threshold shift may result from specific drugs, metals (such as lead, mercury, trimethyltin), solvents (such as toluene, for example found in crude oil, gasoline and automobile exhaust, styrene, xylene, n-hexane, ethyl benzene, white spirit, carbon disulfide, perchloroethylene, trichloroethylene, or p-xylene), pesticides / herbicides (organophosphates) and asphyxiating agents (carbon monoxide, hydrogen cyanide).

Treatment of auditory diseases

- 10 In another aspect, the present invention relates to the compositions of the invention, containing the polypeptides or the gene therapy vector(s) of the invention, for use for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, in a subject in need thereof.
- 15 In another aspect, the present invention relates to the polypeptides or the gene therapy vector(s) of the invention (as described above), for use for preparing a medicament aiming at preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, in a subject in need thereof.
- 20 As used herein, the term "**treating**" is intended to mean the administration of a therapeutically effective amount of one of the polypeptide combination or the gene vector(s) of the invention to a subject who is suffering from a disease, e.g., a loss or impairment of hearing, in order to minimize, reduce, or completely impair the symptoms of same, e.g., the loss or impairment of hearing. "Treatment" is also intended to designate the complete restoration of hearing function regardless of the cellular mechanisms involved.
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In the context of the present invention, the term "**preventing**" a disease, e.g., presbycusis, herein designates impairing or delaying the development of the symptoms of said disease, e.g., delaying the impairment of hearing sensitivity within the frequency range ranging

from 0.5-6.0 (0.5, 1, 2, 3, 4, and 6) kHz (cf. Dobie, R.A. (2005)), particularly at the high frequency range above 3-4 kHz.

NIHL (noise-induced hearing loss) encompasses all types of permanent hearing losses resulting from excessive exposure to intense sounds, which induces mechanical deleterious effects (e.g., to stereocilia bundles and to the plasma membrane of auditory hair cells) and metabolic disturbances (e.g., leading to a swelling of the synaptic regions of IHCs and auditory neurons, in relation to the excitotoxicity of the neurotransmitter, glutamate).

Presbycusis, or age-related hearing impairment, affects more than 30% people above 60 and overall, about 5 million people in France. It is the result of a combination of factors, genetic and environmental (lifelong exposure to noise and to chemicals). Yet it has been shown in the 1950s that subjects who spend their lives in silent environments do not suffer from any hearing loss even in their 80s. This, and a huge body of evidence collected in subjects occupationally exposed to noise, leads to conclude that noise-induced hearing loss (NIHL) is the dominant cause of hearing impairment in ageing subjects.

ROS-induced damages may also be due to an **acoustic trauma**, which may occur after a single, short exposure to extremely loud noise (>120 dB SPL). As a matter of fact, it is thought that, after such an acoustic trauma, subjects experience protracted worsening of their hearing lesions in relation to disrupted ROS metabolism and its consequences on cellular homeostasis, even when these subjects have a normal antioxidant equipment. Likely, their antioxidant defences can be easily overwhelmed by the after-effects of the acoustic trauma. The compositions of the invention could improve the way that these patients heal and recover hearing after a damaging exposure.

In addition to direct effects of noise exposure, other factors also involve ROS metabolism and increased oxidative stress, notably chemical substances known for their **ototoxicity**. A targeted application of the compositions, combination products or vectors of the invention would alleviate these side-effects, even when the initial insult that triggers ROS production is not mechanical, but chemical.

Said ototoxic substances can be any medication or chemical substances described above.

As used herein, the term “**hearing impairment**” refers to a hearing defect that can either be congenital or not.

As used herein, the term “**hearing loss**” refers to a hearing defect that develops in
5 previously normal hearing individual. It can appear at any age.

As used herein the term “**auditory damages**” is intended to mean any reduction in a subject's ability to detect sound. Auditory damages are for example a 10 decibel (dB) standard threshold shift or greater in hearing sensitivity for two of 6 frequencies ranging from 0.5-6.0 (0.5, 1, 2, 3, 4, and 6) kHz (cited in Dobie, R.A. (2005)). Auditory damages
10 can also occur only on high frequency, for example as a 5 dB auditory threshold shift at two adjacent high frequencies (2-6 kHz), or 10dB at any frequency above 2kHz.

In another aspect, the present invention relates to the compositions of the invention, for use for treating subjects suffering from hereditary hearing loss due to an altered expression level of Pejvakin in their auditory cells or to an altered *DFNB59* gene
15 expression in said cells.

In another aspect, the present invention relates to the polypeptides or the gene therapy vector(s) of the invention (as described above), for use for preparing a medicament for treating subjects suffering from hereditary hearing loss due to an altered expression level of Pejvakin in their auditory cells or to an altered *DFNB59* gene expression in said cells.
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“**Congenital / hereditary hearing impairment due to altered *DFNB59* gene expression or deficiency**” herein designates the so-called “DFNB59 patients” described in the art. These patients exhibit an endogenous PJKV that is either truncated or mutated,
25 and consequently not functional. These mutations are for example p.T54I, p.R183W, p.C343S, p.K41SfsX18, p.R167X and p.V330LfsX7 (Collin et al., 2007; Ebermann et al., 2007; Hashemzadeh Chaleshtori et al., 2007; Schwander et al., 2007; Borck et al., 2012; Mujtaba et al., 2012; Zhang et al., 2015).

Kits of parts

In another aspect, the present invention relates to a combination product comprising a gasdermin protein and a LC3 protein, in particular the LC3B protein, or functional fragments thereof (as described above), for use for simultaneous, separated or staggered use for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, or hereditary hearing loss due to an altered expression level of Pejvakin in auditory cells or to an altered *DFNB59* gene expression in said cells, in a subject in need thereof. Preferably, said gasdermin protein is pejvakin.

10 Said combination product can contain the proteins themselves, or the gene therapy vectors described above.

The present invention therefore also relates to a combination product comprising a gene therapy vector system encoding a gasdermin, and a LC3 protein, in particular the LC3B protein, or functional fragments thereof, for use for simultaneous, separated or staggered use for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, or hereditary hearing loss due to an altered expression level of Pejvakin in auditory cells or to an altered *DFNB59* gene expression in said cells, in a subject in need thereof. Preferably, said gasdermin protein is pejvakin.

20 In the context of the invention, the typical mode of administration of the two components of these combination products is intratympanic (in the middle ear), intracochlear, or parenteral (e.g., intravenous, subcutaneous, intraperitoneal, intramuscular, intrathecal). In one example, the combination products of the invention can be administered by intravenous infusion or injection. In another example, the combination products of the invention can be administered by intramuscular or subcutaneous injection. In another example, the combination products of the invention can be administered perorally. In yet another example, the combination products of the invention can be delivered to a specific location using stereostatic delivery, particularly through the tympanic membrane or mastoid into the middle ear.

Dosage regimens can be adjusted to provide the optimum desired response (e.g., a therapeutic or prophylactic response). For example, a single bolus can be administered, several divided doses can be administered over time or the dose can be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It can be especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of the polypeptides / vectors of the invention calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms can be dictated by and directly dependent on (a) the unique characteristics of the polypeptides / vectors and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of formulating such polypeptides / vectors for treating or preventing hearing impairment or peroxisomal disorders in a subject.

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Methods of treatment

In another aspect, the present invention relates to methods for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, or hereditary hearing loss due to an altered expression level of Pejvakin in auditory cells or to an altered *DFNB59* gene expression in said cells, in a subject in need thereof, comprising the steps of administering to said subject an effective amount of:

- The gasdermin protein and the LC3 protein, in particular the LC3B protein, or functional fragments thereof (as described above);
- Two vectors encoding the gasdermin protein and the LC3 protein, in particular the LC3B protein, or functional fragments thereof (as described above); or
- One vector encoding the gasdermin protein and the LC3 protein, in particular the LC3B protein, or functional fragments thereof.

The administration of the two proteins / two vectors can be simultaneous, separated or staggered in time. Preferably, said gasdermin protein is pejvakin.

In another aspect, the present invention relates to a method for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, or hereditary hearing loss due to an altered expression level of Pejvakin in auditory cells or to an altered *DFNB59* gene expression in said cells, in a subject in need thereof, comprising the step of
 5 administering to said subject an effective amount of a vector encoding the gasdermin protein and the LC3 protein, in particular the LC3B protein, or functional fragments thereof (as described above). Preferably, said gasdermin protein is pejvakin.

All the embodiments concerning the compositions of the invention, vectors of the
 10 invention, and combination products of the invention do apply mutatis mutandis for these treatment methods.

As used herein, the term "**subjects**" is intended to designate humans and non-human mammals such as primates, cats, dogs, swine, cattle, sheep, goats, horses, rabbits, rats, mice and the like. In a preferred embodiment, said subjects are human subjects.

Figure legends

Figure 1 – Pejvakin promotes pexophagy in response to sound in auditory hair cells.

a, Changes in peroxisome number in P21 (Post natal 21) *Pjvk*^{+/+} IHCs (*n* = 60; 4 mice per time point) after sound exposure (5-40 kHz, 105 dB SPL, 1 hour). The arrows indicate
 20 the sound stimulation period (1 hour). **b**, Impaired early elimination and proliferation of peroxisomes in *Pjvk*^{-/-} IHCs in response to sound (*n* = 60 IHCs; 4 mice per time point). **c**, LC3B-mediated pexophagy is altered in *Pjvk*^{-/-} IHCs. The number of co-immunolabeled LC3B/PMP70 spots increased one hour after sound exposure in *Pjvk*^{+/+} IHCs, but not *Pjvk*^{-/-} IHCs. **d**, The number of LC3B/pejvakin PLA spots was higher one hour after sound
 25 exposure in *Pjvk*^{+/+} IHCs. No LC3B/pejvakin PLA spots were observed in *Pjvk*^{-/-} IHCs. Bar charts quantifying LC3B/PMP70 co-immunolocalization in IHCs from *Pjvk*^{+/+} and *Pjvk*^{-/-} mice (**c**) and LC3B/pejvakin PLA spots in *Pjvk*^{+/+} IHCs (**d**) before and one hour after sound exposure (*n* = 40 IHCs; 4 mice per genotype and condition). Scale bars, 5 μm. Mean ± s.e.m.; ****P* < 0.001, n.s. not significant (unpaired Student's *t*-test).

Figure 2 – Pejvakin recruits LC3B directly via its LC3-interacting region (LIR), promoting autophagy in response to oxidative stress. **a**, Co-immunolabeling of pejvakin and LC3B in untreated and H₂O₂-treated HepG2 cells. The number of LC3B-positive spots colocalizing with PMP70 labeling increased after oxidative stress (bar charts, $n = 20$ cells). **b**, Interactions between LC3B and pejvakin, detected as *in situ* PLA spots, in untreated and H₂O₂-treated HepG2 cells. Bar charts showing an increase in the number of LC3B/pejvakin PLA spots after oxidative stress, but no change in the number of p62/pejvakin or NBR1/pejvakin PLA spots ($n = 20$ cells per condition). **c**, Schematic representation of the putative LIR motif in the predicted pejvakin chaperone domain. **d**, **e**, Interactions between pejvakin and LC3B in transfected HeLa cells producing EGFP alone or together with murine pejvakin or pejvakin with mutated core residues, p.F215A and p.L218A, detected by double-immunolabeling (**d**) and *in situ* PLA (**e**) for pejvakin and LC3B, before and after oxidative stress. The bar charts in **d** and **e** demonstrate that H₂O₂ treatment increases LC3B recruitment to pejvakin in transfected HeLa cells producing EGFP and murine pejvakin, but not in cells producing mutated pejvakin or untransfected cells ($n = 20$ cells per condition). Scale bars, 5 μ m. Mean \pm s.e.m.; ** $P < 0.01$, *** $P < 0.001$, n.s. not significant (unpaired Student's *t*-test).

Figure 3 – Stress-induced pejvakin binding to LC3B is regulated by pejvakin C328 and C343 residue oxidation. **a**, Schematic representation of cysteines N- or C-terminal positions to the predicted chaperone domain of pejvakin. **b**, **c**, Interactions between pejvakin and LC3B in transfected HeLa cells producing EGFP together with murine pejvakin or the mutated forms of pejvakin, C328S and C343S, detected by double-immunolabeling (**b**) and *in situ* PLA (**c**) for pejvakin and LC3B, before and after oxidative stress. The bar charts in **b** and **c** show that H₂O₂ treatment increases LC3B recruitment by pejvakin in transfected HeLa cells producing EGFP and murine pejvakin, but not in cells producing mutated pejvakin ($n = 20$ cells per condition). Scale bars, 5 μ m. Mean \pm s.e.m.; ** $P < 0.01$, *** $P < 0.001$, n.s. not significant (unpaired Student's *t*-test).

Figure 4 – Transfer of Pjvk and Lc3b into *Pjvk*^{-/-} IHCs restores pexophagy and abolishes lipid peroxidation after sound exposure. **a**, The number of LC3B-positive spots colocalizing with PMP70 labeling was higher one hour after sound exposure in *Pjvk*^{-/-} IHCs cotransduced with AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b. Inset:

LC3B recruitment to peroxisomes. **b**, The number of LC3B/pejvakin *in situ* PLA spots increased one hour after sound exposure (5-40 kHz, 105 dB SPL, 1 hour) in *Pjvk*^{-/-} IHCs cotransduced with AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b. **c**, The number of LC3B/pejvakin *in situ* PLA spots had increased slightly one hour after sound exposure in *Pjvk*^{-/-} IHCs transduced with AAV2/8-Pjvk-IRES-EGFP (lower panel). The bar charts in **(a)**, **(b)**, and **(c)** quantify LC3B-positive spots colocalizing with PMP70 labeling **(a)**, and LC3B/pejvakin *in situ* PLA spots **(b)** and **(c)** in treated IHCs before and after sound exposure ($n = 40$ IHCs; 4 mice per condition). **d**, Peroxisome degradation (one hour after sound exposure) and proliferation (48 hours after sound exposure) were fully restored in *Pjvk*^{-/-} IHCs cotransduced with AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b. AAV2/8-Pjvk-IRES-EGFP transduction of *Pjvk*^{-/-} IHCs partially rescued early pexophagy and proliferation, whereas the transduction of *Pjvk*^{-/-} IHCs with AAV2/8-Flag-Lc3b had no effect ($n = 40$ IHCs from 4 mice). **e**, Lipid peroxidation levels, as assessed by 4-HNE immunolabeling, were higher in *Pjvk*^{-/-} IHCs than in *Pjvk*^{+/+} IHCs, 3 hours after sound exposure. Lipid peroxidation was abolished in *Pjvk*^{-/-} IHCs cotransduced with AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b, but had significantly increased in both *Pjvk*^{-/-} IHCs and AAV2/8-Flag-Lc3b *Pjvk*^{-/-} IHCs. 4-HNE immunoreactivity was weaker in AAV2/8-Pjvk-IRES-EGFP *Pjvk*^{-/-} IHCs than in *Pjvk*^{-/-} IHCs ($n = 50$ cells; 4 mice per condition). Scale bars, 5 μ m. Mean \pm s.e.m.; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s. not significant (unpaired Student's *t*-test).

Figure 5 - Transfer of the p.C328S / p.C343S mutated form of pejvakin into *Pjvk*^{-/-} IHCs does not restore neither early pexophagy nor peroxisome proliferation after sound exposure. (A) The number of LC3B/pejvakin *in situ* PLA spots does not increase one hour after sound exposure (5-40 kHz, 105 dB SPL, 1 hour) in *Pjvk*^{-/-} IHCs transduced with AAV2/8-Pjvk p.C328S/p.C343S-IRES-EGFP ($n = 40$ IHCs; 4 mice per condition). (B) Peroxisome degradation (one hour after sound exposure) and proliferation (48 hours after sound exposure) are not restored in *Pjvk*^{-/-} IHCs transduced with AAV2/8-Pjvk p.C328S/p.C343S-IRES-EGFP ($n = 60$ IHCs; 4 mice per condition). Scale bar, 5 μ m. Mean \pm s.e.m.; *** $P < 0.001$, n.s. not significant (unpaired Student's *t*-test).

Examples

The experiments described below show that pexophagy plays a key role in controlling noise-induced peroxisome proliferation. The present results reveal the role of pejvakin as a redox-activated pexophagy receptor, and suggest a new role for gasdermins in the triggering of selective organelle autophagy.

MATERIAL AND METHODS

Animals

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*^{-/-} mice were produced in a C57BL/6-129/Sv mixed genetic background (Delmaghani, S. *et al.*2015).

Acoustic overexposure

Three-week-old animals were exposed, for one hour, to broad-band 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 (The Mathworks), and was delivered by an amplifier to a set of four Ultrasonic Vifa speakers (Avisoft Bioacoustics) (Delmaghani, S. *et al.*2015).

Treatment of HepG2 and HeLa cells with H₂O₂

The cells were treated with 0.5 mM H₂O₂ at 37°C for 6 hours, under normoxic conditions (95% air) and were then fixed in 4% paraformaldehyde (PFA).

Plasmids and DNA transfection

The full-length pejvakin cDNA was obtained by RT-PCR, as previously described (Delmaghani, S. *et al.*2015). The mutant pejvakin clones were prepared from the wild-type pejvakin clone with the QuikChange™ Site-Directed 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 hour 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 F7425) in 1% bovine serum albumin (BSA) in PBS. Anti-parvalbumin (1:200, Abcam Ab32895) and anti-myosin VI (1:100, Santa Cruz sc-23568) antibodies were used to delimit the contours of IHCs and OHCs. Anti-PMP70 (1:100, Sigma 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 minutes, washed in PBS, and incubated in 50 mM NH₄Cl, 0.2% Triton X-100 for 15 minutes at room temperature. The cells were washed and incubated in 20% normal goat serum in PBS for 1 hour. Cells were incubated with the primary antibody in 1% BSA in PBS for 1 hour. Antibodies against peroxisome membrane protein 70 (PMP70, 1:100, Sigma SAB4200181) and LC3B (1:100, Abcam ab51520) were used to label peroxisomes and autophagosomes, respectively. The mouse monoclonal antibody against pejvakin (Pjvk-G21)¹ was used at a concentration of 100 µg/ml. Cells were washed in PBS and incubated with the appropriate secondary antibody for 1 hour at room temperature.

For immunofluorescence studies, Atto-647-conjugated goat anti-rabbit IgG (1:500, Sigma 18772, 40839), Atto-550-conjugated goat anti-mouse IgG (1:500, Sigma 43394), and Alexa-Fluor-488-conjugated goat anti-chicken IgG (1:500, Invitrogen A11039) as secondary antibodies were used. DAPI (1:7500, Sigma D9542) was used to label cell nuclei. Images were acquired with a Zeiss LSM700 Meta confocal microscope (Carl Zeiss MicroImaging, Inc.). For whole-mount cochlea experiments, the auditory hair cells from the middle part of the cochlea were analyzed.

***In situ* proximity ligation assay**

- The Duolink *in situ* proximity ligation assay was performed according to the manufacturer's protocol (www.olink.com/products-services/duolink), to detect the interaction between pejvakin and Lc3b, NBR1 or p62 in HepG2, HeLa, and cochlear hair cells. Cells were immunolabeled with a mouse monoclonal antibody against pejvakin (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, goat Santa Cruz 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-Pjvk-IRES-EGFP and AAV2/8-Flag-Lc3b were obtained by inserting the murine pejvakin and Lc3b cDNAs flanked by an IRES-EGFP or Flag reporter gene into the multiple cloning site of the pENN.AAV.CB7.CI.RBG vector (PennVector P1044, 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 (Post natal 3) *Pjvk*^{-/-} mice, as previously described (Delmaghani, S. *et al.* 2015). A fixed volume (2μl) of a solution containing AAV2/8-Pjvk-IRES-EGFP, AAV2/8-Flag-Lc3b, or both (10¹³ viral genomes/ml) 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 immunohistofluorescence with an antibody against 4-hydroxy-2-nonenal (4-HNE) (1:200, Abcam ab48506). Images were acquired with a Zeiss LSM700 Meta confocal microscope (Carl Zeiss MicroImaging,

Inc.) under identical settings, to allow comparison. The mean pixel intensity of 4-HNE immunolabeling in cochlear hair cells was quantified with ImageJ software (NIH Image).

Prediction tools

The Phyre2 web portal was used to predict and analyze pejvakin structure and function (Kelley et al, 2015). Fpocket, an open-source platform, was used to detect the ligand-binding pocket (Le Guilloux V. et al, 2009). The pejvakin protein sequences were multiply aligned using MAFFT web server.

Statistical analyses

Quantitative data are presented as the mean \pm s.e.m. and statistical analyses were performed with GraphPad. Data were analysed in unpaired Student's *t*-tests. The differences between groups were considered significant if $p < 0.05$.

DISCUSSION

Peroxisome kinetics were observed 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*^{+/+}) and *Pjvk*^{-/-} mice. Surprisingly, the number of peroxisomes per IHC in *Pjvk*^{+/+} mice had decreased relative to baseline values in unexposed mice (32.8 ± 1.5 peroxisomes per IHC, mean \pm s.e.m.), by 24% (24.8 ± 1.4 peroxisomes per IHC; $p = 0.0002$) at the end of one hour of exposure to loud broad-band sound (5-40 kHz, 105 dB SPL), and by 39% one hour later (20 ± 1.2 peroxisomes per IHC; $p < 0.0001$) (**Fig. 1a**). The number of peroxisomes then began to increase, peaking at a level 4.3 times that one hour after stimulation (2.6 times that pre-stimulation), at 48 hours (85.3 ± 4.2 peroxisomes per IHC), slowly decreasing thereafter, to reach baseline values within 10 days (**Fig. 1a**). By contrast, the number of peroxisomes remained unchanged one hour after the end of sound exposure in *Pjvk*^{-/-} IHCs (29.3 ± 1.3 and 26.1 ± 1.2 peroxisomes per IHC before and after sound exposure, respectively, mean \pm s.e.m.; $p = 0.08$), gradually decreasing thereafter, by 56% (12.8 ± 1.8 , mean \pm s.e.m.; $p < 0.0001$) over 48 hours (**Fig. 1b**). Sound-induced peroxisome proliferation is, therefore, preceded by an early, rapid pejvakin-dependent degradation of peroxisomes. It was then investigated whether pejvakin coupled the autophagosome machinery to peroxisomes through recruitment of LC3B to these

organelles (Kabeva Y et al, 2000; Tanida I et al, 2008). At the time of maximal peroxisome degradation (1 hour after 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*^{+/+} IHCs (**Fig. 1c**), but remained unchanged in *Pjvk*^{-/-} IHCs (**Fig. 1c**). Moreover, 24 hours after sound exposure, peroxisome aggregates, a hallmark of impaired pexophagy (Shibata M et al, 2013), were detected in *Pjvk*^{-/-} but not *Pjvk*^{+/+} IHCs (not shown). LC3B recruitment by pejvakin was further investigated by using *in situ* proximity ligation assay (PLA) (Gauthier T et al, 2015; Soderberg O et al, 2006), a highly sensitive method in which each pair of proteins is visualized as a fluorescent spot. The number of LC3B/pejvakin PLA spots had also increased by a factor of 2.7 in *Pjvk*^{+/+} IHCs, one hour after sound exposure (**Fig. 1d**). Thus, pejvakin promotes the autophagic degradation of noise-induced oxidative stress-damaged peroxisomes by recruiting 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, p62 (Kim PK et al, 2008) and NBR1 (Deosaran, E. et al 2013) have been identified as receptors for mammalian pexophagy. They bind to LC3B via their LC3-interacting region (LIR) motif, and ubiquitinated lysine residues of peroxisomal membrane proteins via their ubiquitin-associated (UBA) domain. It was investigated whether LC3B binding to pejvakin in conditions of oxidative stress was direct or required p62 or NBR1. Human hepatoblastoma HepG2 cells, which are enriched in peroxisomes and express pejvakin strongly, displayed both an increase in the number of LC3B/pejvakin double-immunolabeled spots and LC3B/pejvakin PLA spots in response to H₂O₂-induced oxidative stress (**Fig. 2a,b**). By contrast, the number of p62/pejvakin and NBR1/pejvakin PLA spots was unaffected by H₂O₂ treatment (**Fig. 2a,b**), suggesting that pejvakin recruits LC3B directly.

From murine pejvakin sequence analysis with the Phyre2 web portal (Kelley L.A. et al, 2015), no predicted UBA domain was identified. 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]X₁X₂[L/I/V]) (Alemu E.A. et al, 2012; Birgisdottir, A. B. et al.

2013) with negatively charged residues within (X_1 , X_2) or adjacent. In pejvakin, it is flanked by the charged acidic residues, E213 and D219, N- and C-terminal, respectively, to the aromatic residue (F215) and contains I216 as one of only five amino acids acceptable in position X_1 relative to F215 in F-type LIRs (Alemu E.A. et al, 2012; 5 Birgisdottir, A. B. et al. 2013). This putative LIR motif is highly conserved throughout evolution (not shown). The functional relevance of this findings was assessed by determining whether mutations of its key residues, F215 and L218, affected pejvakin binding to LC3B under H_2O_2 -induced oxidative stress. After H_2O_2 treatment, the numbers of LC3B/pejvakin co-immunolabeled spots and LC3B/pejvakin PLA spots both increased 10 in transfected HeLa cells expressing EGFP and normal murine pejvakin (Pjvk-IRES-EGFP), but not in cells expressing EGFP and a mutated pejvakin (mutPjvk-IRES-EGFP) carrying the p.F215A and/or p.L218A mutations (**Fig. 2d,e**). Five other putative LIR motifs were identified outside the predicted chaperone domain of pejvakin. Site-direct mutagenesis as above, showed that none of them was implicated in LC3B recruitment by 15 pejvakin in response to oxidative stress (not shown). Thus, oxidative stress-induced pexophagy is dependent on the direct interaction between the pejvakin LIR motif and LC3B.

Pejvakin-mediated pexophagy in response to oxidative stress raises the possibility of redox-regulation of pejvakin activity. The functional LIR motif of pejvakin is part of the 20 largest putative protein-binding pocket detected by Fpocket tool (Le Guilloux V. et al, 2009) within the predicted chaperone domain (not shown) and chaperone protein activity is often regulated by the redox state of the cell. Cysteine oxidation releases the compact conformation of the protein, exposing the high-affinity binding site. The C-terminus of pejvakin contains a cysteine-rich region (residues 309-343). In HeLa cells transfected 25 with mutated forms of pejvakin carrying cysteine-to-serine substitutions, the pejvakin-LC3B binding elicited by H_2O_2 was assessed by using co-immunolabeling and *in situ* PLA. Mutations of the two cysteine residues closest to the C-terminus, p.C328S and p.C343S (a deafness-causing missense mutation in humans (Mujtaba G et al, 2012), affected this interaction (**Fig. 3**). No mutation of the other C-terminal (p.C309S, p.C312S, 30 and p.C325S), or N-terminal (p.C43S and p.C49S) cysteine residues (not shown) had this effect.

To confirm the essential role of these two cysteine residues, a rescue experiment was performed in *Pjvk*^{-/-} mice, using adeno-associated virus 2/8 (AAV2/8) vector-mediated transfer of the p.C328S / p.C343S mutated form of pejvakin into *Pjvk*^{-/-} IHCs. The interaction between LC3B and pejvakin was not increased in transduced *Pjvk*^{-/-} IHCs 1 hour after sound exposure (**Fig. 5A**). Moreover, neither early peroxisome degradation nor peroxisome proliferation was restored in response to sound (**Fig. 5B**).

The pejvakin C328 and C343 residues play a key role in oxidative stress-induced pexophagy, and pejvakin, which displays redox-regulated binding to LC3B, likely acts as a ROS sensor.

Finally, the role of pexophagy was investigated in redox homeostasis of auditory hair cells and peroxisome proliferation elicited by sound overexposure. The effects of pejvakin and LC3B were assessed, separately and together, on pexophagy and peroxisome proliferation, by injecting AAV2/8-Pjvk-IRES-EGFP, AAV2/8-Flag-LC3B, or both, into *Pjvk*^{-/-} cochleas. One hour after sound exposure, Lc3b targeting to peroxisomes in *Pjvk*^{-/-} IHCs overexpressing both Pjvk and LC3B cDNAs, as detected by PLA and co-immunolabeling, was similar to that observed in wild-type mice (5.25 ± 0.85 LC3B/pejvakin PLA spots per IHC vs. 6.08 ± 0.5 in *Pjvk*^{+/+}, mean \pm s.e.m.; $p = 0.40$) (4.88 ± 0.85 LC3B/pejvakin co-immunolabeled spots per IHC vs. 5.63 ± 0.55 in *Pjvk*^{+/+}, mean \pm s.e.m.; $p = 0.40$), whereas the re-expression of Pjvk alone increased pexophagy only slightly (2.73 ± 0.4 LC3B/pejvakin PLA spots per IHC vs. 6.08 ± 0.5 in *Pjvk*^{+/+}, mean \pm s.e.m.; $p < 0.0001$) (**Fig. 4a-c**). Forty-eight hours later, peroxisome numbers had increased by a factor of 2.2 with respect to their prestimulation number in *Pjvk*^{-/-} IHCs transduced with both AAV2/8-Pjvk-IRES-EGFP and AAV2/8-Flag-LC3B, and by a factor of 2.4 in non-injected *Pjvk*^{+/+} IHCs, the difference between these two values being non-significant (**Fig. 4d**), whereas the transduction of *Pjvk*^{-/-} IHCs with Pjvk cDNA alone increased peroxisome numbers by a factor of only 1.4 (**Fig. 4d**). The injection of LC3B cDNA alone did not affect early pexophagy or peroxisome proliferation in *Pjvk*^{-/-} mice (**Fig. 4d**). The lipid peroxidation induced by oxidative stress-damaged peroxisomes was also determined in auditory hair cells by assessing immunoreactivity for a by-product of this process, 4-hydroxy-2-nonenal (4-HNE). The levels of 4-HNE also varied with deficient pexophagy (**Fig. 4e**), providing evidence for a major role of pejvakin-mediated

pexophagy in redox homeostasis and protection of auditory hair cells against noise-induced damage. This is reminiscent of the link between oxidative stress-induced pexophagy and redox homeostasis observed in cell lines (Zhang et al, 2015). Moreover, the parallel changes in the magnitude of these pexophagic and peroxisome proliferation responses to sound, with the strongest effects observed after the co-expression of pejvakin and LC3B, show that noise-induced peroxisome proliferation is controlled by pejvakin-mediated pexophagy.

The present findings demonstrate that pejvakin acts as a redox-regulated pexophagy receptor and protects cells through its key role in oxidative stress-induced pexophagy.

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Claims

1. A pharmaceutical composition comprising i) a gasdermin protein or a functional fragment thereof, ii) a LC3 protein, or a functional fragment thereof, and iii) a therapeutically acceptable carrier.
- 5 2. A pharmaceutical composition comprising a therapeutically acceptable carrier and a gene therapy vector system encoding i) a gasdermin protein or a functional fragment thereof and ii) a LC3 protein, or a functional fragment thereof.
3. The composition of claim 1 or 2, wherein said gasdermin fragment contains at least the N-terminal domain of a gasdermin protein.
- 10 4. The composition of claim 1 or 2, wherein said LC3 protein is a protein chosen in the group consisting of: LC3A, LC3B and LC3C.
5. The composition of any of claims 1 to 4, wherein said gasdermin protein is the pejevakin protein..
6. The composition of any of claims 1 to 5, wherein said gasdermin protein is the pejevakin
15 protein of SEQ ID NO:1.
7. The composition of any of claims 1 to 6, wherein said LC3 protein is the LC3B protein.
8. The composition of any of claims 1 to 7, wherein said LC3 protein is the LC3B protein of SEQ ID NO:5.
9. The composition of any of claims 1 to 8, wherein said gasdermin protein is the pejevakin
20 protein and said LC3 protein is the LC3B protein.
10. The composition of any of claims 1 to 9, wherein said gasdermin protein shares at least 70% identity with SEQ ID NO:1 and wherein said LC3 protein shares at least 70% identity with SEQ ID NO:5.
11. The composition of any of claims 1 to 10, wherein said gasdermin protein is of SEQ ID
25 NO:1 and said LC3 protein is of SEQ ID NO:5.

12. The composition of any one of claims 2 to 11, comprising one gene therapy vector encoding both the gasdermin protein and the LC3 protein, or functional fragments thereof, or two gene therapy vectors, separately encoding the gasdermin protein and the LC3 protein, or functional fragments thereof.
- 5 13. The composition of any one of claims 2 to 12, wherein said gene therapy vector(s) is (are) viral vector(s), preferably AAV vector(s).
14. The composition of claims 1 to 13, for use for restoring peroxisome homeostasis in auditory cells in a subject in need thereof.
- 10 15. The composition of claims 1 to 13, for use for preventing and/or alleviating ROS-induced cellular damages, preferably in cochlear cells, afferent auditory neurons or neurons of the auditory central pathway, said damages being more preferably due to noise exposure or to ototoxic substance exposure, in a subject in need thereof.
- 15 16. The composition of claims 1-13, for use for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, in a subject in need thereof.
17. The composition of claims 1-13, for use for treating subjects suffering from hereditary hearing loss due to an altered expression level of *Pejvakin* in their auditory cells or to an altered *DFNB59* gene expression in said cells.
- 20 18. A combination product comprising i) a gasdermin protein or a functional fragment thereof, and ii) a LC3 protein, or a functional fragment thereof, for simultaneous, separated or staggered use for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, or hereditary hearing loss due to an altered expression level of *Pejvakin* in auditory cells or to an altered *DFNB59* gene expression
25 in said cells, in a subject in need thereof.
19. A combination product comprising i) a gasdermin protein or a functional fragment thereof, and ii) a LC3 protein, or a functional fragment thereof, for simultaneous, separated or staggered use for restoring peroxisome homeostasis in auditory cells in a subject in need thereof.

20. A combination product comprising i) a gasdermin protein or a functional fragment thereof, and ii) a LC3 protein, or a functional fragment thereof, for simultaneous, separated or staggered use for preventing and/or alleviating ROS-induced cellular damages, preferably in cochlear cells, afferent auditory neurons or neurons of the auditory central pathway, said damages being more preferably due to noise exposure or to ototoxic substance exposure, in a subject in need thereof.
21. A combination product comprising a gene therapy vector system encoding i) a gasdermin or a functional fragment thereof, and ii) a LC3 protein, or a functional fragment thereof, for simultaneous, separated or staggered use for preventing and/or treating presbycusis, noise-induced hearing-loss or sudden sensorineural hearing loss or auditory damages induced by acoustic trauma or ototoxic substances, or hereditary hearing loss due to an altered expression level of Pejvakin in auditory cells or to an altered *DFNB59* gene expression in said cells, in a subject in need thereof.
22. A combination product comprising a gene therapy vector system encoding i) a gasdermin or a functional fragment thereof, and ii) a LC3 protein, or a functional fragment thereof, for simultaneous, separated or staggered use for restoring peroxisome homeostasis in auditory cells in a subject in need thereof.
23. A combination product comprising a gene therapy vector system encoding i) a gasdermin or a functional fragment thereof, and ii) a LC3 protein, or a functional fragment thereof, for simultaneous, separated or staggered use for preventing and/or alleviating ROS-induced cellular damages, preferably in cochlear cells, afferent auditory neurons or neurons of the auditory central pathway, said damages being more preferably due to noise exposure or to ototoxic substance exposure, in a subject in need thereof.
24. The combination product for use according to claim 18 or 23, wherein said gasdermin protein is the pejvakin protein, preferably the pejvakin protein of SEQ ID NO:1, and wherein said LC3 protein is the LC3B protein, preferably the LC3B protein of SEQ ID NO:5.
25. A vector encoding a gasdermin protein and a LC3 protein, or functional fragments thereof.

26. The vector of claim 25, wherein said gasdermin protein is pejvakin, preferably the pejvakin protein of SEQ ID NO:1 and wherein said LC3 protein is the LC3B protein, preferably the LC3B protein of SEQ ID NO:5.

27. A kit containing at least two distinct vectors, among which one vector encodes the gasdermin protein or a functional fragment thereof, and one other vector encodes the LC3 protein or a functional fragment thereof.

28. The kit of claim 27, wherein said gasdermin protein is pejvakin, preferably the pejvakin protein of SEQ ID NO:1 and wherein said LC3 protein is the LC3B protein, preferably the LC3B protein of SEQ ID NO:5.

Figure 1

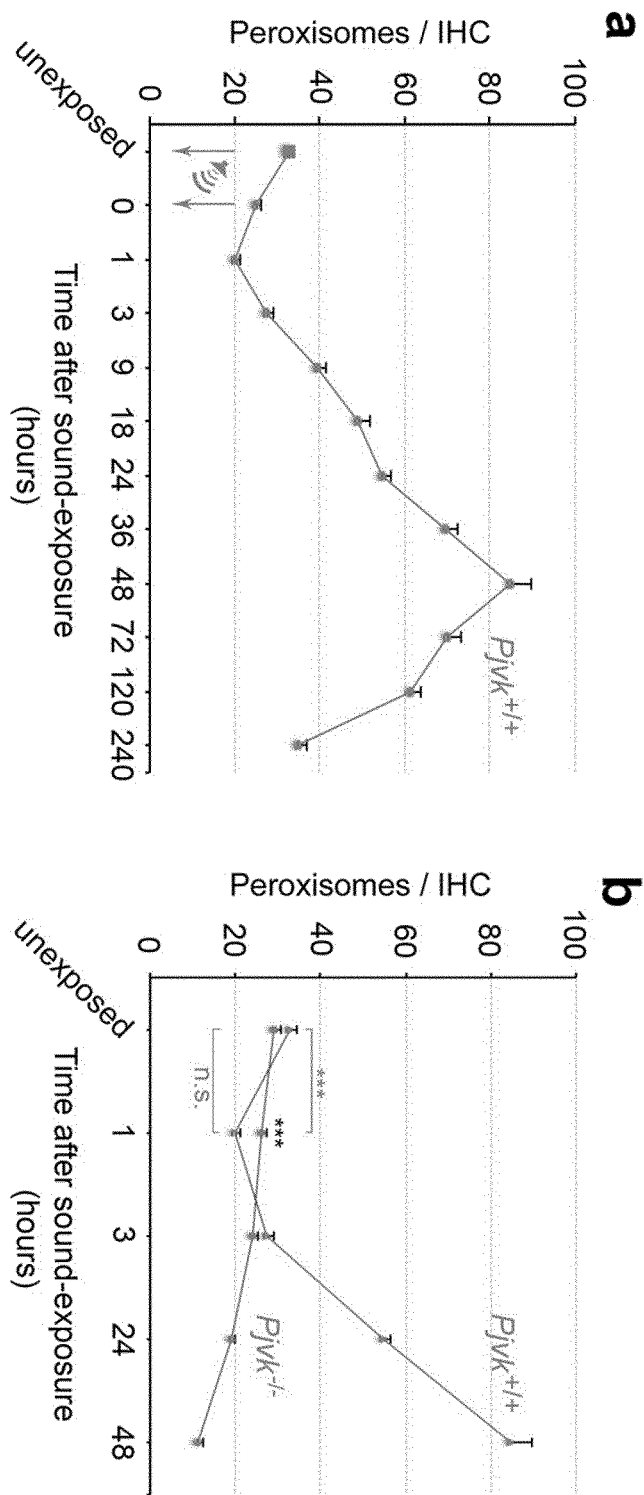
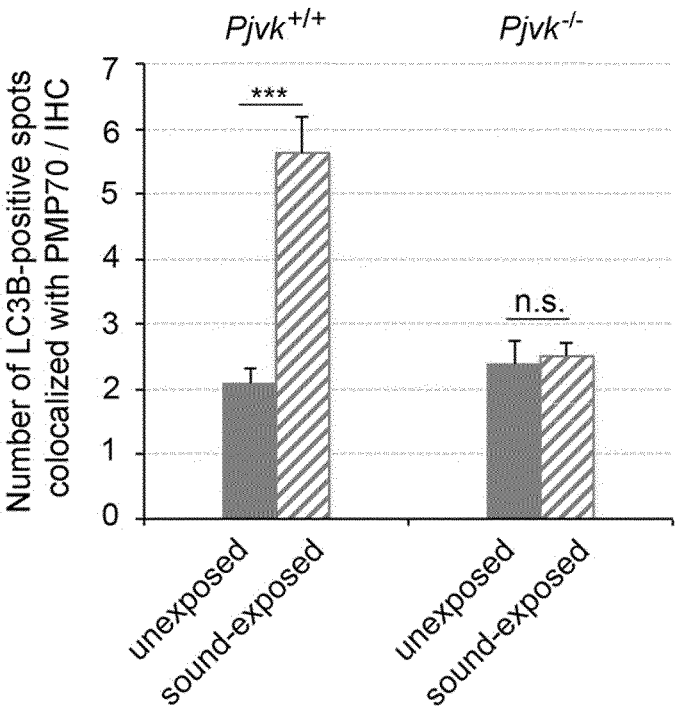


Figure 1 (continuation)

C



d

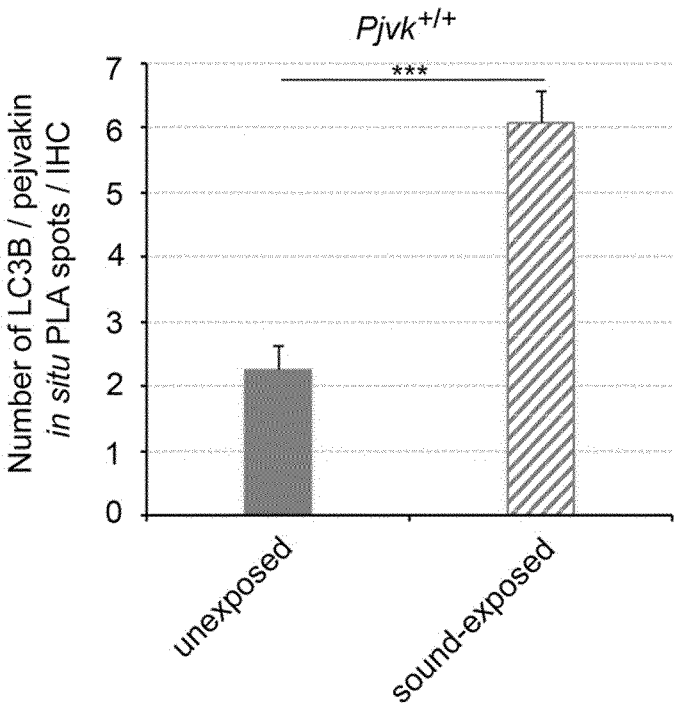
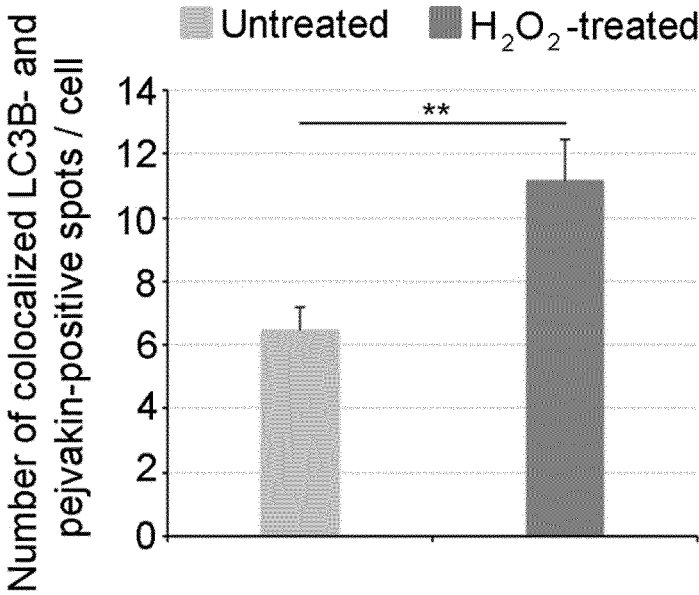


Figure 2

a



b

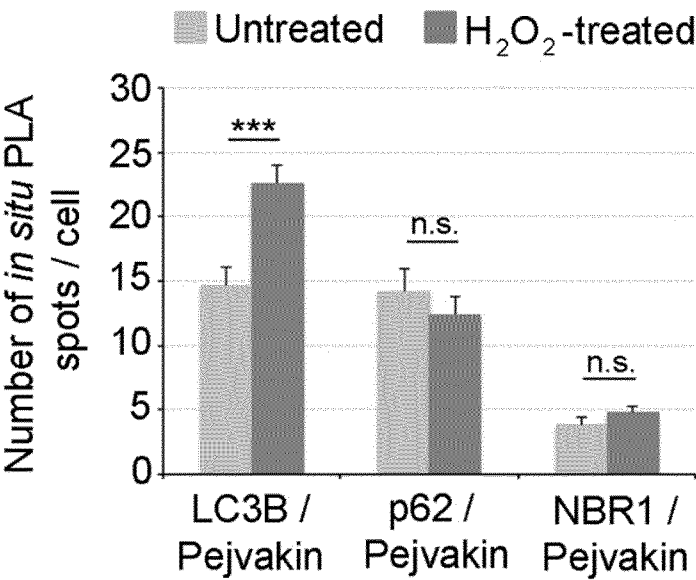
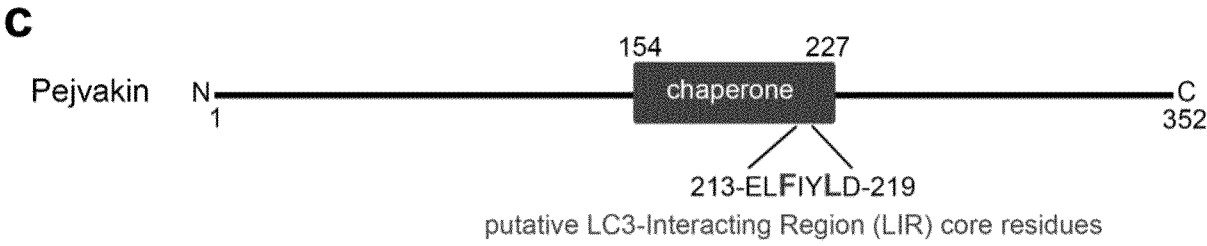


Figure 2 (continuation)



d

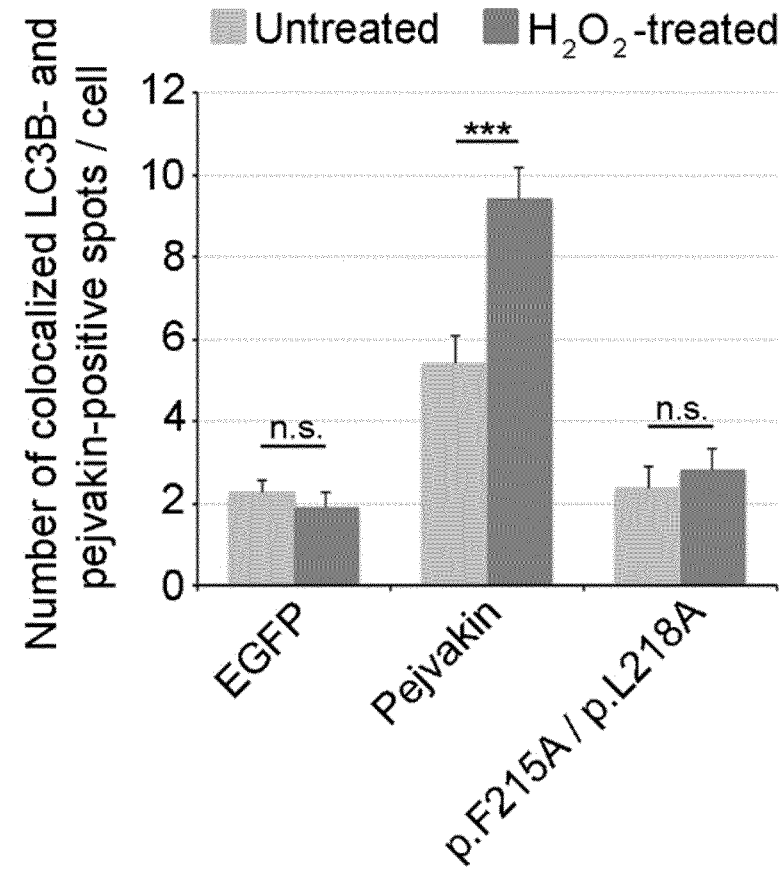


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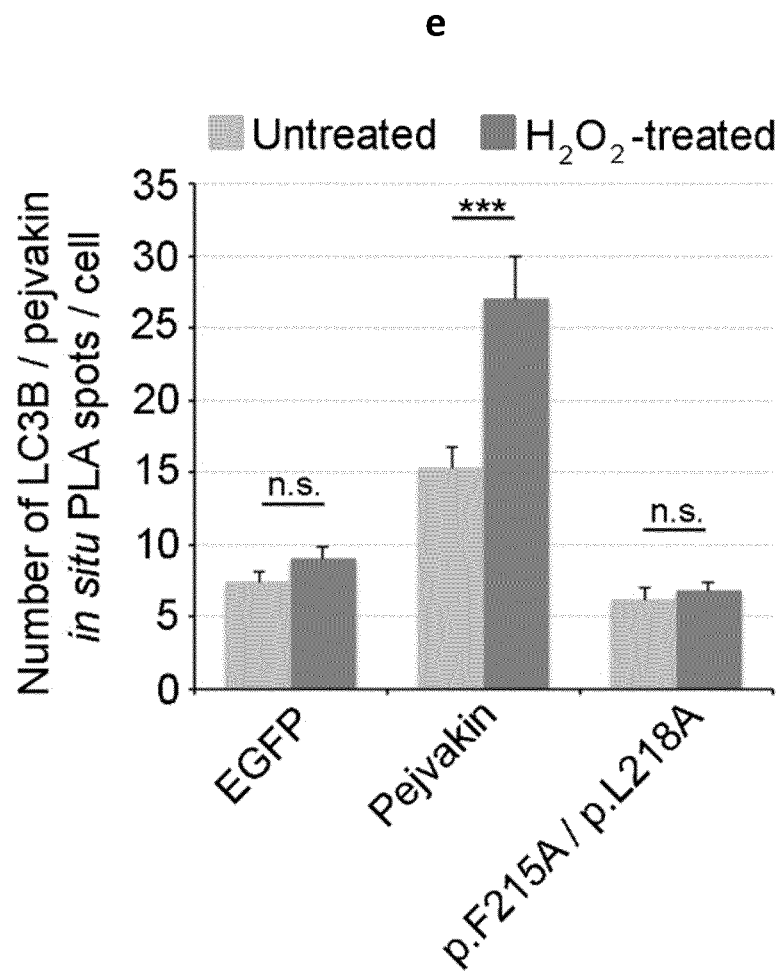


Figure 3

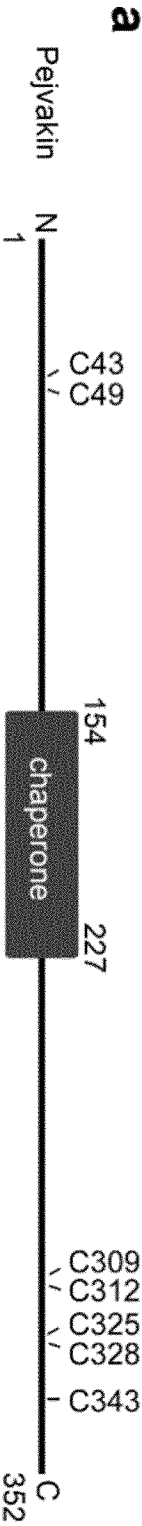


Figure 3 (continuation)

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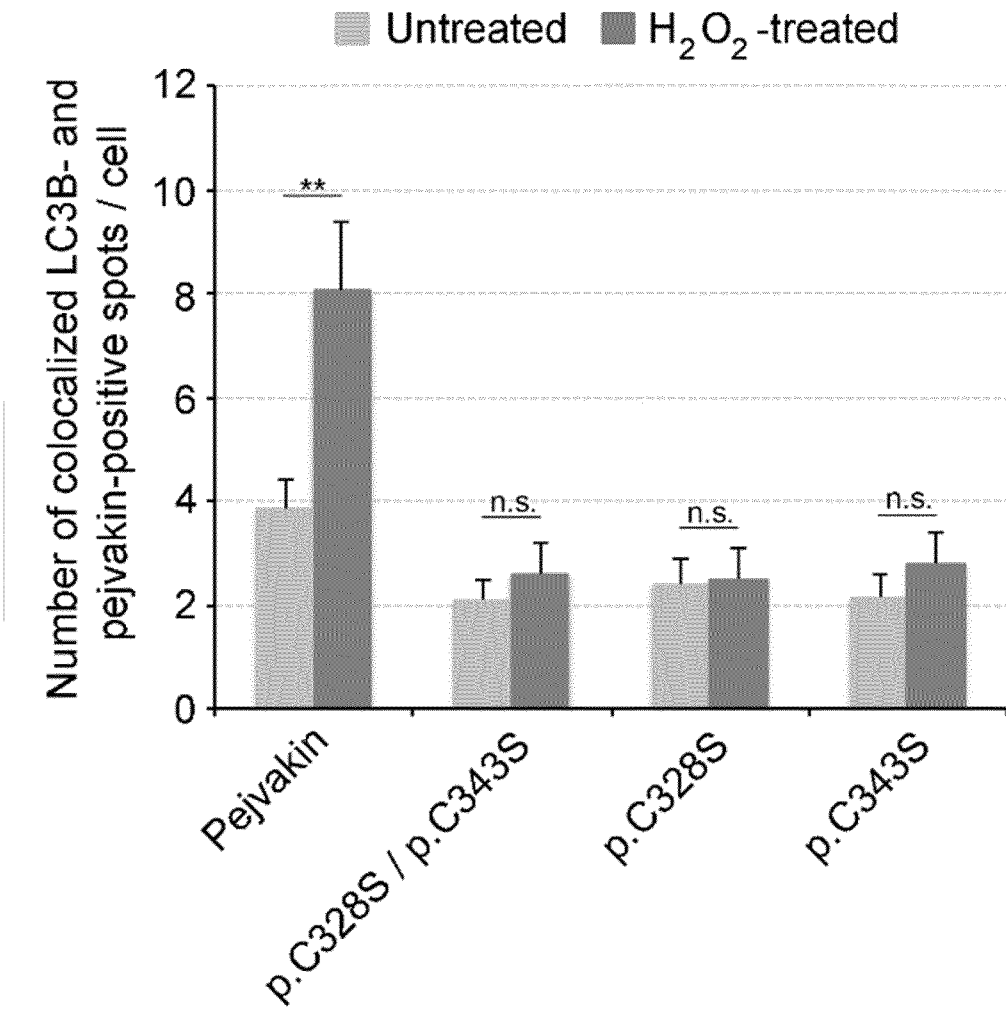


Figure 3 (continuation)

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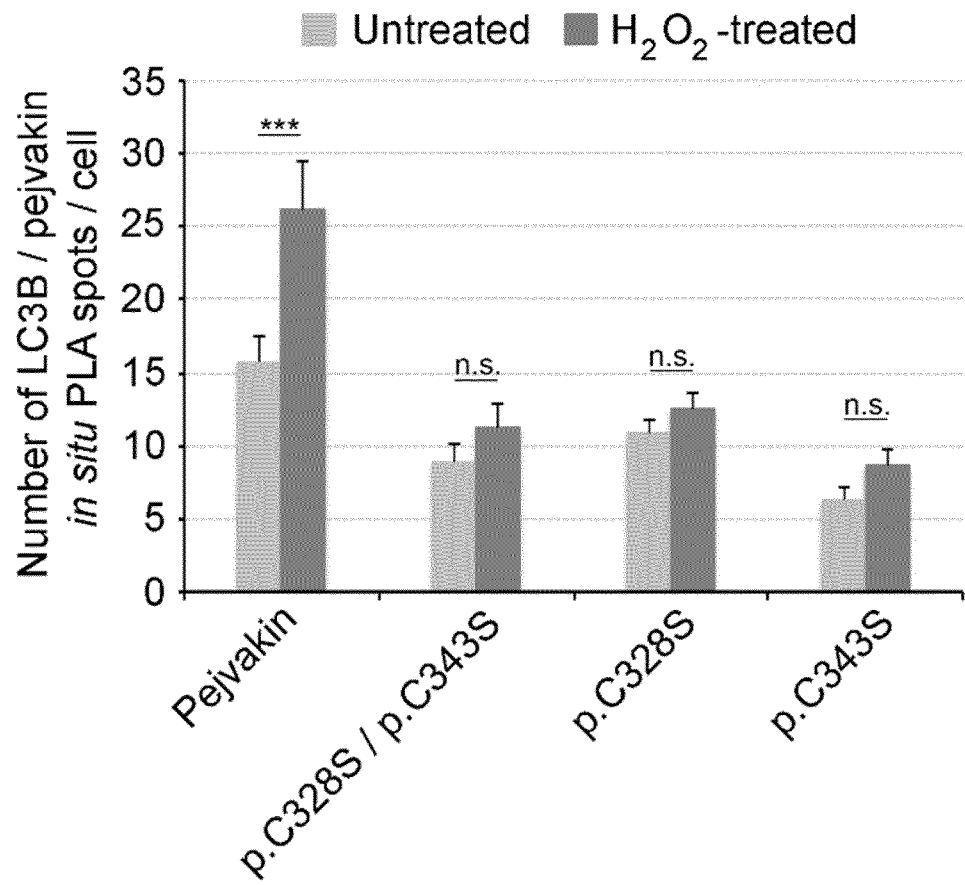
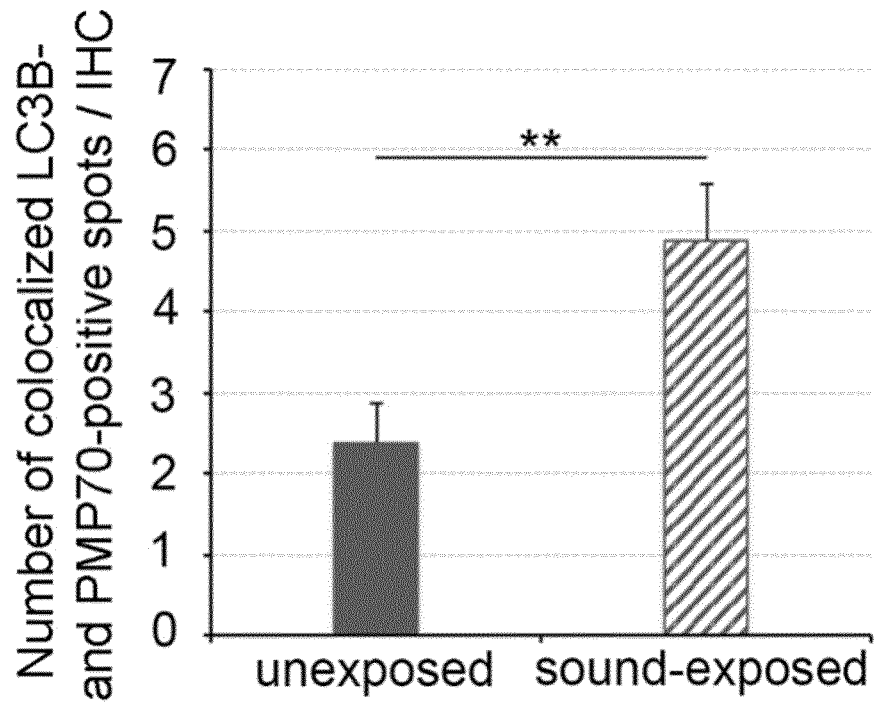


Figure 4

a



b

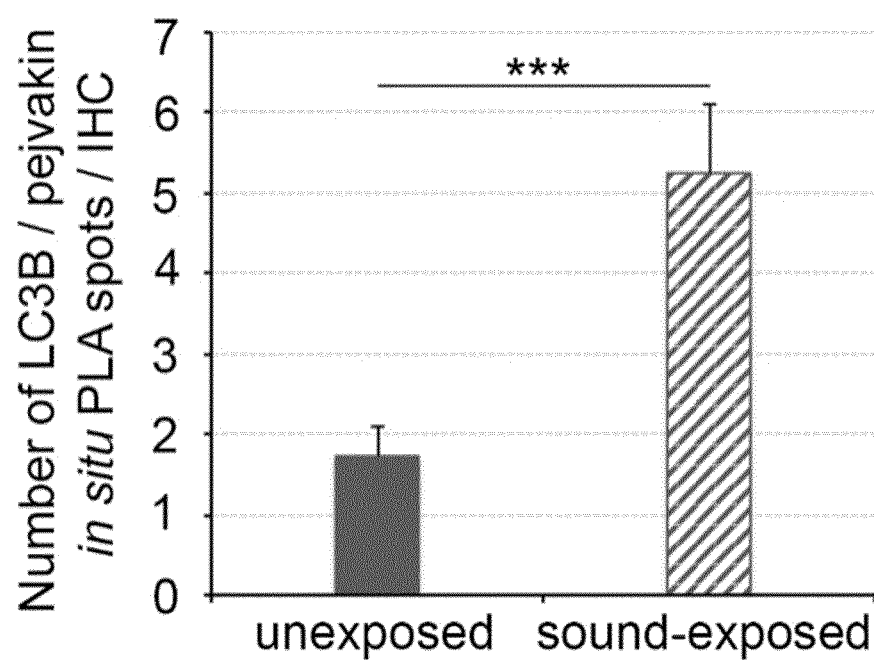


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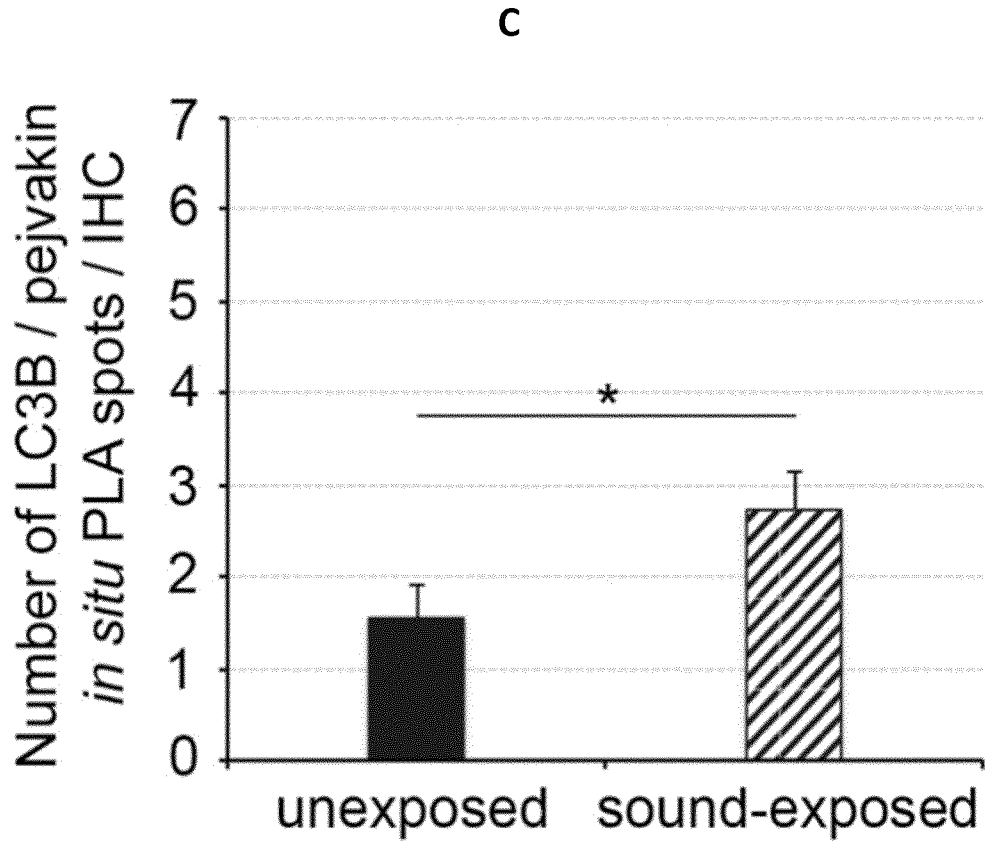


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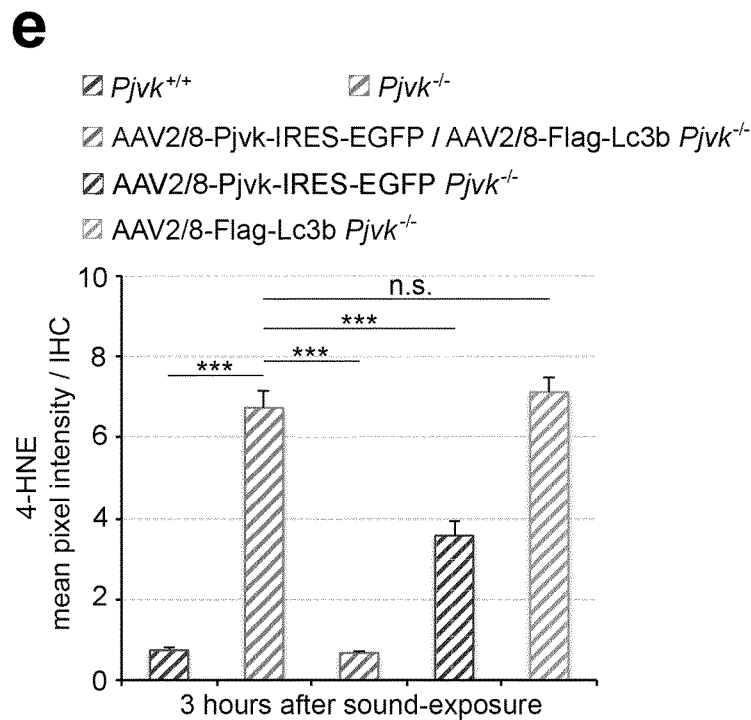
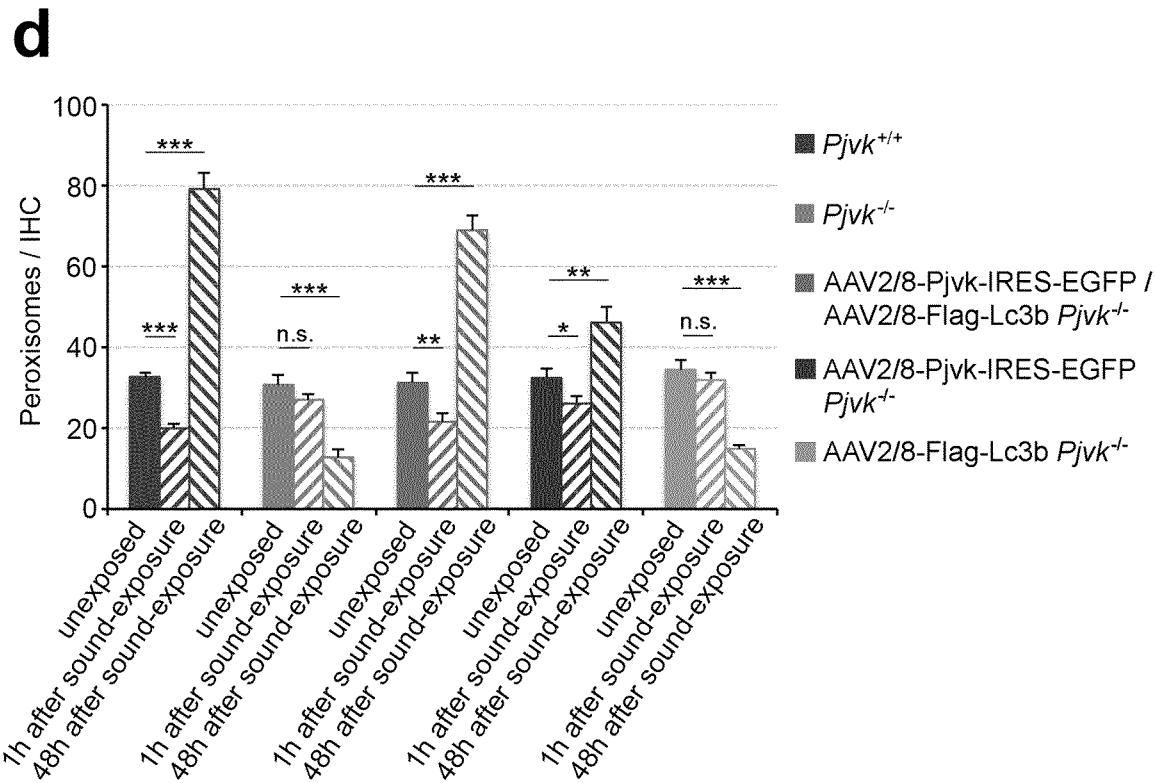


Figure 5

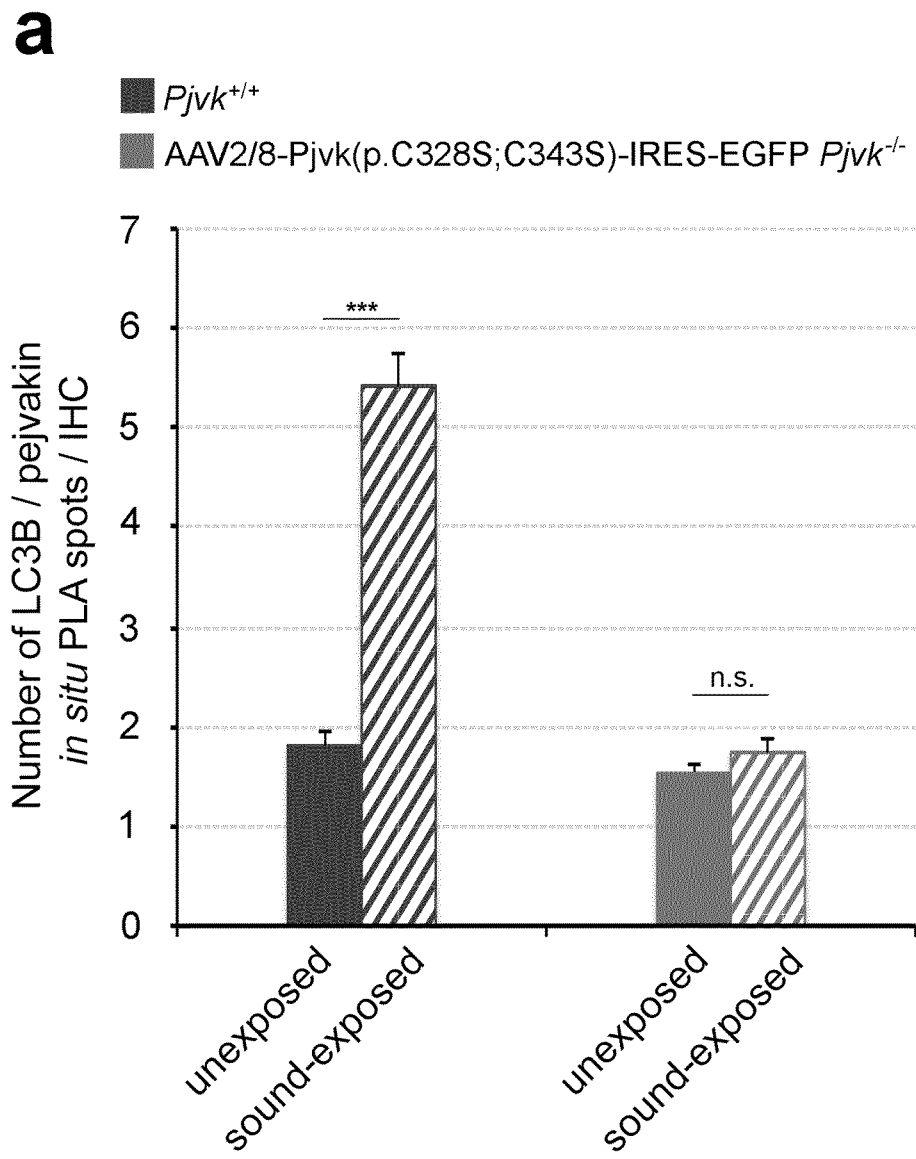
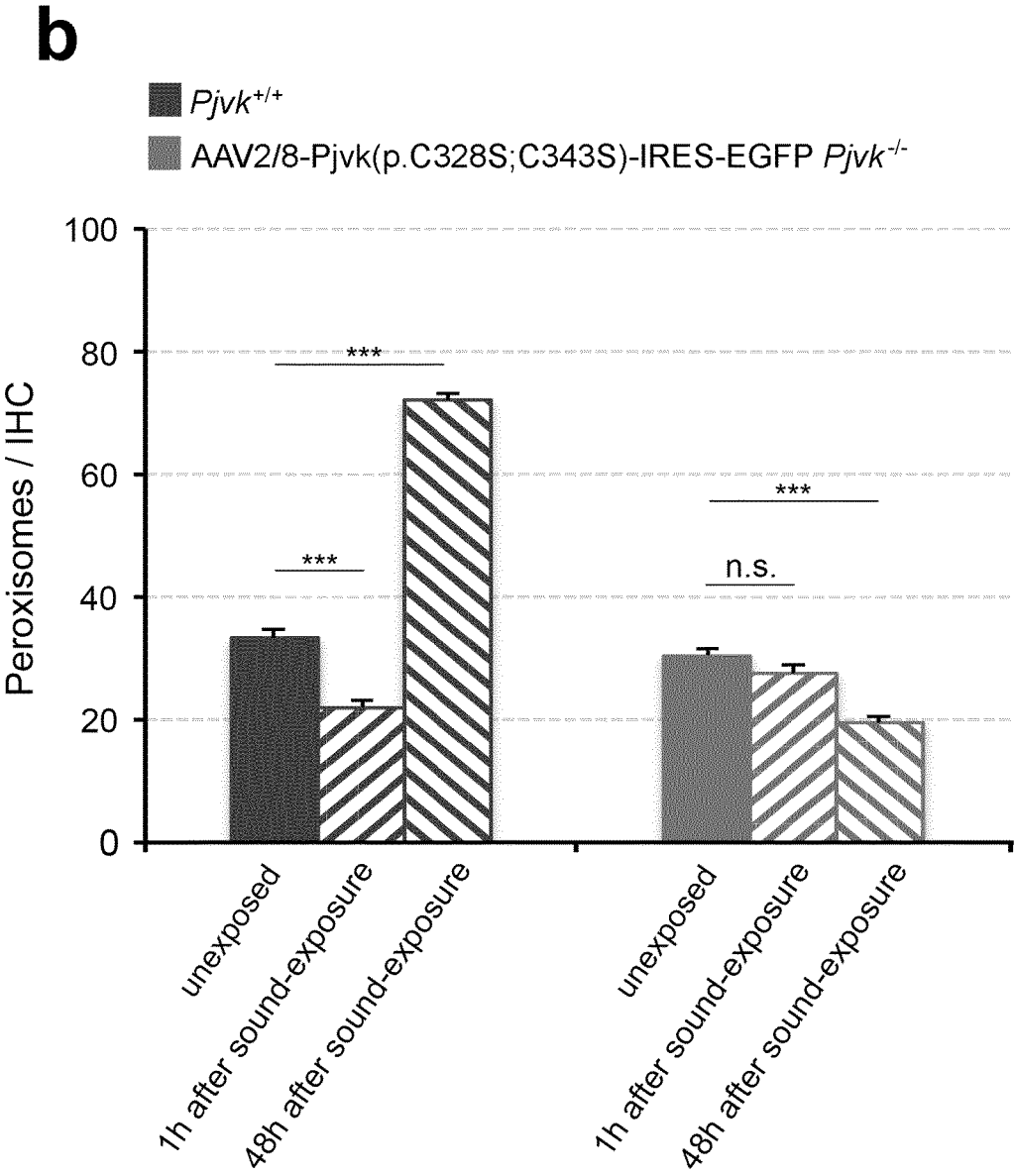


Figure 5 (continuation)



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/081674

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17 A61K48/00 A61P27/16
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>W0 2016/131981 A1 (PASTEUR INSTITUT [FR]) 25 August 2016 (2016-08-25) cited in the application abstract page 1, line 1 - page 83, line 17 claims; figures; examples sequence 1</p> <p>----- -/--</p>	<p>1-23,25, 27</p>

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

17 February 2020

Date of mailing of the international search report

28/02/2020

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/081674

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>JEAN DEFOURNY ET AL: "Pejvakin-mediated pexophagy protects auditory hair cells against noise-induced damage", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 116, no. 16, 1 April 2019 (2019-04-01), pages 8010-8017, XP055585472, US</p> <p>ISSN: 0027-8424, DOI: 10.1073/pnas.1821844116</p> <p>abstract</p> <p>page 8010, left-hand column, paragraph 1 -</p> <p>page 8017, right-hand column, paragraph 5</p> <p>-----</p>	1-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/081674

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 2016131981 A1	25-08-2016	EP 3258955 A1	27-12-2017
		US 2018055908 A1	01-03-2018
		W0 2016131981 A1	25-08-2016
