Autophagy is essential for hearing in mice

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Hearing loss is the most frequent sensory disorder in humans. Auditory hair cells (HCs) are postmitotic at late-embryonic differentiation and postnatal stages, and their damage is the major cause of hearing loss. There is no measurable HC regeneration in the mammalian cochlea, and the maintenance of cell function is crucial for preservation of hearing. Here we generated mice deficient in autophagy-related 5 (Atg5), a gene essential for autophagy, in the HCs to investigate the effect of basal autophagy on hearing acuity. Deletion of Atg5 resulted in HC degeneration and profound congenital hearing loss. In autophagy-deficient HCs, polyubiquitinated proteins and p62/SQSTM1, an autophagy substrate, accumulated as inclusion bodies during the first postnatal week, and these aggregates increased in number. These findings revealed that basal autophagy has an important role in maintenance of HC morphology and hearing acuity.

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Autophagy is an intracellular system where cytoplasmic components are delivered to and degraded in the lysosome. Macroautophagy is one type of autophagy that is initiated by the formation of a sequestering structure, called the phagophore or isolation membrane. The phagophore matures into a closed double-membrane-bound structure, termed the autophagosome, which then fuses with a lysosome. The inner membrane of the autophagosome and the materials contained within are then degraded by lysosomal hydrolases. Autophagy is typically induced by starvation as well as other stresses, and basal autophagy is also important for quality control of cytoplasmic components and homeostasis of various postmitotic cells, such as neurons, hepatocytes, and cardiomyocytes.

As the auditory HCs are postmitotic and long lived, we hypothesized that basal autophagy in these cells may have an important role in the acquisition and/or preservation of hearing function in mammals. Here we report that loss of autophagy in the auditory HCs leads to damage to these cells. Mice deficient in autophagy-related 5 (Atg5), a gene essential for autophagy, in the auditory HCs develop profound congenital hearing loss. Mice deficient in autophagy-related 5 (Atg5), a gene essential for autophagy, in the auditory HCs develop profound congenital hearing loss and degeneration of the HCs. We revealed that intracellular quality control by basal autophagy in the HCs has an important role in hearing acuity and maintenance of cell morphology.

Results

Basal autophagy flux detected in auditory HCs. First, we aimed to examine basal autophagy flux in the auditory HCs. We monitored green fluorescence protein (GFP)-labeled microtubule-associated protein 1 light chain 3b (LC3) puncta, which represent autophagy-related structures, in the HC cytoplasm in a cochlear explant culture established from postnatal day 5 (P5) GFP-LC3 transgenic mice (Figure 1a). GFP-LC3 mice express a fusion cDNA encoding enhanced GFP jointed at its C-terminus to rat LC3 under the control of the CAG promoter. The occurrence of autophagy in mouse tissues can be monitored by the GFP. In both the inner and outer HCs, only a small number of GFP-LC3 puncta were observed. However, the number of GFP-LC3 puncta increased after a 4-h treatment with the lysosomal protease inhibitors E64d and pepstatin A (Figures 1b and c). These data suggest that autophagosomes are continuously turned over dependently on lysosomal function in both the inner and outer HCs (Figure 1).

Generation of Atg5<sup>fl</sup><sub>flow</sub>/Pou4f3-Cre mice. Next, we generated mice deficient in Atg5 in auditory HCs to...
investigate the physiological function of basal autophagy in these cells. Mice bearing an \( \text{Atg}^{5\text{flo}} \) allele\(^{12} \) were crossed with POU domain, class 4, transcription factor 3 (\( \text{Pou4f3} \))-\( \text{Cre} \) transgenic mice.\(^{13} \) To confirm the inhibition of autophagy in the HCs, we crossed these mice with GFP-LC3 transgenic mice.\(^{11} \) In the control auditory HCs from P5 \( \text{Atg}^{5\text{flo}}/+;\text{Pou4f3-Cre} \) mice, a number of GFP-LC3 puncta were observed (Figure 2), suggesting that autophagy is constitutively active. Conversely, P5 \( \text{Atg}^{5\text{flo}}/\text{flo};\text{Pou4f3-Cre} \) mice showed almost no GFP-LC3 puncta in the HCs. These results suggest that autophagosome formation is suppressed in the \( \text{Atg}^{5\text{def}} \) HCs.

**Aggregates containing ubiquitin and p62 observed in \( \text{Atg}^{5\text{def}} \) HCs.** Because autophagy has an important role in intracellular quality control, dysfunction of autophagy gives rise to the accumulation of abnormal ubiquitinated proteins and organelles.\(^8 \) Therefore, we examined whether polyubiquitinated proteins and p62/SQSTM1, which is an autophagy-specific substrate and accumulates under autophagy-deficient conditions,\(^{14,15} \) were accumulated in the \( \text{Atg}^{5\text{def}} \) HCs. In the control HCs from P5 \( \text{Atg}^{5\text{flo}}/+;\text{Pou4f3-Cre} \) mice, punctate signals positive for ubiquitin and/or p62 were rarely observed (Figures 3a and b). However, in the HCs from P5 \( \text{Atg}^{5\text{flo}}/\text{flo};\text{Pou4f3-Cre} \) mice, aggregates containing ubiquitin and p62 were observed (Figures 3a and b), suggesting that the failure of intracellular quality control by the dysfunction of basal autophagy started before the maturation of the organ of Corti. At P14, these aggregates increased in number in the \( \text{Atg}^{5\text{def}} \) HCs (Figures 3a and b). These aggregates were rarely generated in the HCs from \( \text{Atg}^{5\text{flo}}/+;\text{Pou4f3-Cre} \) mice at P14 (Figures 3a and b). Quantitative analysis showed that the number of aggregates containing ubiquitin and p62 in the

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**Figure 1** Basal autophagy flux was detected in auditory HCs. (a) Experimental design after the administration of lysosomal protease inhibitors, E64d and pepstatin A, to cochlear explant cultures established from P5 GFP-LC3 transgenic mice. Fixation was performed 4 h after the administration of lysosomal protease inhibitors. (b) Basal autophagy flux in the auditory HCs. GFP-LC3 puncta in the HC cytoplasm in a cochlear explant culture established from P5 GFP-LC3 transgenic mice were monitored. The accumulation of GFP-LC3 puncta was increased in cultures treated with E64d and pepstatin A for 4 h in comparison with cultures without these inhibitors. MYO7A was used as a marker of the HC cytoplasm. Magnified images of GFP-LC3 staining are shown in the rightmost panels. IHC, inner HC; OHC, outer HC. Scale bars: 10 μm. (c) Quantitative analysis of the accumulation of GFP-LC3 puncta. The average number of GFP-LC3 puncta was significantly increased in cultures treated with E64d and pepstatin A (\( n=5 \)) in comparison with cultures without these inhibitors (\( n=5 \)). Error bars: S.E. **\( p<0.01 \), ***\( p<0.001 \)
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HCs of P14 Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice was significantly greater than those in P14 Atg5\textsuperscript{flox/flox};Pou4f3-Cre and P5 Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice (Figure 3b). The progressive accumulation of aggregates in the Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice was confirmed both in the inner and outer HCs (Figures 3c and d) and observed in every cochlear turn (Supplementary Figure S1). These results suggest that basal autophagy in the auditory HCs is important for preventing the accumulation of abnormal aggregates before the onset of hearing.

Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice developed congenital severe hearing loss and HC damage. To assess the effect of basal autophagy on hearing acuity, we recorded auditory brainstem responses (ABRs) in Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice. The ABR threshold of Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice was extremely high and significantly elevated compared with that of Atg5\textsuperscript{flox/+};Pou4f3-Cre mice at every tested frequency at P14, 4 weeks, and 8 weeks of age (Figures 4a and b). These results suggest that Atg5 deficiency leads to congenital profound hearing loss and that Atg5 in the cochlear HCs is required for gaining normal hearing acuity.

We then investigated the effect of Atg5 on the morphogenesis of the auditory HCs. The HCs in Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice had normal morphogenesis and no reduction in the number of HCs at P5 (Figures 5a and b). At P14, however, many stereocilia labeled with fluorescent phalloidin were damaged in both inner and outer HCs, and the body of the outer HCs was destroyed to some extent (Figure 5a). At 8 weeks of age, almost all the outer HC bodies were destroyed and most stereocilia and many cell bodies in the inner HCs were damaged. These findings suggest that the damage to stereocilia and cell bodies of the HCs in Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice was progressively developed with age (Figure 5a).

The process of mechanotransduction in the auditory HCs is initiated by the opening of cation channels located at the tips of the stereocilia, which can be assessed visually by uptake of the fluorescent styryl dye FM1-43.\textsuperscript{16,17} To evaluate the function of morphologically normal neonatal HCs in Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice before morphological degeneration, we performed an FM1-43 uptake assay using P5 cochlear explants (Figure 7a). FM1-43 uptake into the HCs was not affected in P5 Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice (Figures 7b and c), indicating that the HCs of the Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice develop normally and have functioning mechanotransduction channels at least until P5. These results suggest that the cause of hearing loss in Atg5\textsuperscript{flox/flox};Pou4f3-Cre mice is degeneration, rather than maldevelopment, of auditory HCs and that Atg5 is required for maintenance of morphology of these cells (Figure 8).

Discussion

We revealed that mice deficient in Atg5 in auditory HCs showed profound congenital hearing loss and progressive degeneration of these cells. Polyubiquitinated proteins and p62 accumulated in autophagy-deficient HCs as inclusion bodies during the first postnatal week and these aggregates increased in number. Auditory HCs are postmitotic and do not regenerate after their loss in mammals. The effect of the loss of basal autophagy, which is important for homeostasis of postmitotic cells, on hearing acuity has been unknown, although autophagy-related 4b (Atg4b), a gene essential for autophagy, is necessary for the development of otoconia in the murine vestibular system.\textsuperscript{18} Our study first revealed the contribution of basal autophagy to intracellular quality control in HCs and hearing acuity in mammals.

Recently, several studies showed that autophagy is involved in the prevention of auditory damage.\textsuperscript{19–21} According to an in vitro study using a murine auditory cell line, the House Ear Institute-Organ of Corti 1 cell line, suppression of autophagy by Atg7 (autophagy-related 7) knockdown decreased cell...
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viability in H₂O₂-induced cell death, whereas activation of autophagy by rapamycin protected against H₂O₂-induced cell death.²⁰ A previous study in rats revealed that treatment with rapamycin, which is an autophagy activator, significantly attenuated cisplatin-induced hearing loss, decreased the level of malondialdehyde (an oxidative stress marker), and alleviated HC damage.¹⁹ Another study revealed that treatment of mice with rapamycin significantly increased LC3B expression, decreased the levels of the oxidative stress markers 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT), reduced noise-induced cell loss in the outer HCs, and reduced the amount of hearing loss.²¹ Conversely, treatment with 3-methyladenine, an autophagy inhibitor, or LC3B siRNA reduced LC3B expression, increased 4-HNE and 3-NT levels, and exacerbated temporary-to-permanent threshold shift.

Hearing loss has recently been reported in an autophagy-related disease, Vici syndrome.²²–²⁴ This syndrome, which is due to recessive mutations in the EPG5 gene encoding
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Figure 4 Atg5flox/flox;Pou4f3-Cre mice developed congenital severe hearing loss. (a) ABR waveform recordings over 10 ms to 8-kHz tone-burst stimuli from representative Atg5flox/flox;Pou4f3-Cre mice and Atg5flox/-;Pou4f3-Cre mice at P14. Atg5flox/flox;Pou4f3-Cre mice showed reproducible waveforms and Atg5flox/flox;Pou4f3-Cre mice showed no waveforms at 95 dB sound pressure level (dBSPL). (b) A comparison of the ABR threshold between Atg5flox/flox;Pou4f3-Cre and Atg5flox/-;Pou4f3-Cre mice was performed by Student’s t-test at P14, 4 weeks of age, and 8 weeks of age. When no response was obtained, we used 100 dBdBSPL as the threshold volume for calculations. Atg5flox/flox;Pou4f3-Cre mice showed profound congenital hearing loss already at P14 and thereafter and had significantly elevated hearing thresholds compared with Atg5flox/-;Pou4f3-Cre mice. The following genotypes were tested at P14: Atg5flox/flox;Pou4f3-Cre (n = 6) and Atg5flox/-;Pou4f3-Cre (n = 10); at 1 month: Atg5flox/flox;Pou4f3-Cre (n = 6) and Atg5flox/-;Pou4f3-Cre (n = 6); and at 2 months: Atg5flox/flox;Pou4f3-Cre (n = 6) and Atg5flox/-;Pou4f3-Cre (n = 8). Error bars: S.E. *P < 0.01 for the study at P14; **P < 0.001 for the study at 4 weeks of age; ***P < 0.001 for the study at 8 weeks of age.

ectopic P granules protein 5 (EPGS), a key autophagy regulator, is a congenital multisystem disorder characterized by corpus callosal agenesis, cataracts, cardiomyopathy, oculocutaneous hypopigmentation, and immunodeficiency. Life expectancy of patients in Vici syndrome is severely reduced. Sensorineural hearing loss has subsequently been reported in several cases of Vici syndrome with or without confirmed EPGS mutations, although the detailed mechanisms underlying the sensorineural hearing loss have not been elucidated.

In mice, cochlear HC stereocilia continue to develop in a well-organized process during the early postnatal weeks. The auditory ribbon synapses also form and mature after birth. The external auditory canal remains closed until P12, and mice do not respond to air-conducted sound until then. Therefore, it has been considered that the onset of hearing occurs around P12. In fact, ABRs cannot be reliably recorded before P12–P14. In the present study, the morphology and mechanotransduction of Atg5-deficient auditory HCs were both normal in P5, although polyubiquitinated proteins and p62 were already accumulated. At P14, however, mice deficient in Atg5 in auditory HCs displayed progressive accumulation of polyubiquitinated protein aggregates in the HCs, as well as HC degeneration and profound hearing loss. These results strongly suggest the importance of basal autophagy maintaining the morphology of auditory HCs during the early postnatal stage until the maturation of the auditory system.

Pou4f3 is also expressed in the retinal ganglion cells (RGCs), and Atg5 might be deficient in RGCs in Atg5flox/flox;Pou4f3-Cre mice. We have not analyzed the role of Atg5 on RGCs using these mice. However, a previously study examined the effects of downregulation of Atg5 expression in RGCs by RGC-specific transduction using adenovirus serotype 2 (AAV2) and demonstrated that the downregulation of Atg5 in RGCs made RGCs more vulnerable to optic nerve axotomy. Fewer surviving RGCs were observed postaxotomy in Atg5flox/flox mice injected with AAV2-GFP-Cre in comparison with those injected with AAV2-GFP control vector.

The relationship between p62 accumulation and pathogenesis of disease has previously been well discussed. p62 has been identified as a common component of ubiquitin-containing protein aggregates, known as inclusion bodies in alcoholic hepatitis and steatohepatitis. The inclusion bodies have also been found in neurodegenerative diseases, such as Parkinson’s disease and amyotrophic lateral sclerosis. Reduction of autophagic activity might contribute to the generation of inclusion bodies in these diseases. The hepatic damage in Atg7 knockout mice was ameliorated by simultaneous genetic deletion of p62. However, the effect of the simultaneous deletion of p62 on neurodegeneration was very small, indicating that the contribution of p62 accumulation associated with autophagy suppression on the pathogenesis of disease is cell-type specific.

In the present study, both cell damage and auditory dysfunction in mice deficient in Atg5 in auditory HCs increased with the exacerbation of p62 accumulation. p62 accumulation, therefore, might have an important role in the progression of damage in autophagy-deficient HCs.

Materials and Methods

Animals. Experimental procedures to produce Atg5flox/flox;Pou4f3-Cre transgenic, and GFP-LC3 transgenic mice have been described previously. Genotyping for Atg5flox/flox;Cre, and GFP-LC3 was conducted as described. Wild-type C57BL/6 mice were obtained from Japan CLEA (Tokyo, Japan). All mice were given free access to food. All animal experiments were performed in accordance with the institutional guidelines of the Animal Care and Use Committees of the University of Tokyo (no. P11-100, P12-77, P13-086) and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Antibodies and fluorescent probes. We purchased the following primary antibodies: chicken polyclonal antibodies against GFP (Abcam, Cambridge, MA, USA), rabbit polyclonal antibodies against MYO7A (Proteus Biosciences, Ramona,
CA, USA), mouse monoclonal antibodies against ubiquitin 1B3 (MBL, Nagoya, Japan), and guinea pig polyclonal antibody against p62 (PROGEN, Heidelberg, Germany). We also purchased Alexa 488-phalloidin and the following secondary antibodies from Molecular Probes (Eugene, OR, USA): Alexa 488-conjugated goat anti-chicken IgG, Alexa 488-conjugated goat anti-mouse IgG, Alexa 568-conjugated goat anti-rabbit IgG, Alexa 568-conjugated goat anti-guinea pig IgG, and Alexa 680-conjugated goat anti-rabbit IgG.

Immunohistochemical analyses of cochlear explant cultures. P5 GFP-LC3 transgenic mice were anesthetized and decapitated. The temporal bones were dissected, and the cochleae were freed from the surrounding tissue. After removing the lateral wall of the cochlea, the organ of Corti was dissected from the cochlear modiolus. Tissue samples were then placed on glass-mesh inserts (Falcon, Franklin Lakes, NJ, USA) and cultured in serum-free modified Eagle’s medium (MEM; Invitrogen, Waltham, MA, USA), supplemented with 3 g/l glucose (Wako Pure Chemicals, Osaka, Japan) and 0.3 g/l penicillin G (Wako Pure Chemicals), at 37 °C for 4 h in a humidified incubator with 95% air and 5% atmospheric carbon dioxide. For tissue samples with lysosomal protease inhibitors, E64d (10 μg/ml) and pepstatin A (10 μg/ml) were added to the above-mentioned medium. After the culture was finished, the specimens were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.4). For immunohistochemistry, after blocking treatment with IMAGE-IT FX (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 30 min, the samples were incubated with primary antibodies at 4 °C overnight. The samples were then incubated with secondary antibodies at 37 °C for 30 min and mounted for analysis using an A1R confocal laser scanning microscope (Nikon, Tokyo, Japan). The GFP-LC3 puncta formation assay was performed in the cytoplasm of the HCs with the focal plane at the apical body of the middle cochlear turn. The average number of punctate structures per cell was calculated from 5 inner and 15 outer HCs per experiment. Quantification of GFP-LC3-associated autophagosomes was performed with

Figure 5 Damage to Atg5-deficient HCs was progressively developed. (a) Confocal imaging of the organ of Corti in Atg5^lox/lox;Pou4f3-Cre and Atg5^lox/+;Pou4f3-Cre mice. The HCs in Atg5^lox/lox;Pou4f3-Cre mice showed normal morphogenesis at P5. At P14, stereocilia were damaged in many HCs and some of the outer HC (OHC) bodies were destroyed. At 8 weeks of age, almost all of the OHC bodies and many inner HC (IHC) bodies were destroyed, and most of the stereocilia in the IHCs were damaged. MYO7A and phalloidin were used as markers of the HC cytoplasm and stereocilia, respectively. O1, the first row outer HC; O2, the second row outer HC; O3, the third row outer HC. Scale bars: 10 μm. (b) A comparison of the number of cells per 100 μm from Atg5^lox/lox;Pou4f3-Cre mice (n = 5) and Atg5^lox/+;Pou4f3-Cre mice (n = 5) was performed by Student’s t-test at P5, P14, and 8 weeks of age. At P5, there were no significant differences between these genotypes. At P14 and 8 weeks of age, Atg5^lox/lox;Pou4f3-Cre mice had significantly fewer HCs than Atg5^lox/+;Pou4f3-Cre mice. Error bars: S.E. **P < 0.01, ***P < 0.001

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ImageJ (NIH, Bethesda, MD, USA), using the basal dispersed GFP-LC3 fluorescence as the threshold.

**Cochlear surface preparation for immunohistochemistry.** The cochleae were fixed in 4% PFA in PBS. The surface of the organ of Corti was prepared by removing the bony otic capsule, the stria vascularis, the Reissner's membrane, and the tectorial membrane. For immunohistochemistry, the specimens were incubated with primary antibodies at 4 °C overnight. The specimens were then incubated with secondary antibodies at 37 °C for 30 min and mounted for analysis with the A1R confocal laser scanning microscope.

**Histological and immunohistochemical analyses for paraffin-embedded sections.** The cochleae were fixed in 4% PFA in PBS and decalcified in 10% ethylenediamine tetraacetic acid for several days. Then the specimens were dehydrated in a graded ethanol series and embedded in paraffin. The paraffin block containing the tissue was cut into 4-μm sections, and the sections were deparaffinized and then rehydrated through a xylene and ethanol series. For immunohistochemistry, after antigen retrieval and blocking treatment with 0.1% Triton X-100 and 5% goat serum in PBS at room temperature for 30 min, sections were incubated with primary antibodies at 4 °C overnight. The sections were then incubated with secondary antibodies at 37 °C for 30 min and mounted for analysis with the A1R confocal laser scanning microscope. In all images, the same threshold fluorescence intensity for each channel was fixed. An inner HC and three outer HCs were selected from each cochlear turn (i.e., the apical turn, the middle turn, or the basal turn), and the number of aggregates containing ubiquitin and p62 per 100-μm² field of the cytoplasm in the auditory HCs, the outer HCs, and the total HCs was then counted for each cochlea. For each mouse group (i.e., P5 Atg5flox/flox;Pou4f3-Cre mice, P5 Atg5flox/+;Pou4f3-Cre mice, P14 Atg5flox/flox;Pou4f3-Cre mice, or P14 Atg5flox/+;Pou4f3-Cre mice), six cochleae were used for a comparison of the number of aggregates.

**Figure 6** Stereocilia of Atg5-deficient HCs were progressively damaged. Scanning electron microscopy of the HCs in Atg5flox/flox;Pou4f3-Cre and Atg5flox/+;Pou4f3-Cre mice. The HCs in Atg5flox/flox;Pou4f3-Cre mice showed normal morphogenesis at P5. At P14, stereocilia were damaged or irregularly shaped in many HCs. At 8 weeks of age in Atg5flox/flox;Pou4f3-Cre mice, almost all of the stereocilia in the outer HCs (OHCs) were destroyed and most of the stereocilia in the inner HCs were damaged or irregularly shaped. Scale bars: 10 μm for total HCs, 1 μm for OHC.
Scanning electron microscopy. The temporal bones were fixed with 2.5% glutaraldehyde and 2% PFA in 0.1 M phosphate buffer (pH 7.4) at room temperature for 1 h. The surface of the organ of Corti was prepared by removing the bony otic capsule, the stria vascularis, the Reissner's membrane, and the tectorial membrane. The tissues were postfixed with 1% osmium tetroxide buffered with 0.1 M phosphate buffer, dehydrated in a graded ethanol series, critical-point dried, and sputter-coated with platinum. The samples were observed under a scanning electron microscope (S-4500, Hitachi, Tokyo, Japan).

Auditory brainstem response. ABR was evaluated at P14, 4 weeks of age, and 8 weeks of age as previously described.37 Before the ABR measurements, the external auditory canals and tympanic membranes were confirmed to be normal in all mice. Subdermal electrodes were placed on the vertex (active electrode), in the postauricular area of the measured ear (reference electrode), and in the postauricular area of the opposite ear (ground electrode). The speaker was placed 10 cm from the tragus of the stimulated ear. A tone-burst sound (2, 4, 8, 16, and 32 kHz) was produced by a sound stimulator (Neuropack MEB-2200, Nihon Kohden, Tokyo, Japan). The stimulus duration was 15 ms, the presentation rate was 11/s, and the rise/fall time was 1 ms. At each stimulus level, ABR was obtained by averaging 500 responses. The threshold of ABR was determined as the lowest intensity level at which a clear reproducible waveform was obtained in the trace. If no response was obtained, we used 100 dB sound pressure level as the threshold value for further calculations.

FM1-43 uptake. Cochleae were dissected from P5 mice in PBS and explanted onto μ-Slide eight-well tissue culture plates (Ibidi, Martinsried, Germany). Explants were incubated in MEM (Invitrogen), supplemented with 3 g/l glucose (Wako Pure Chemicals) and 0.3 g/l penicillin G (Wako Pure Chemicals), at 37 °C for 2 h in a humidified incubator with 95% air and 5% atmospheric carbon dioxide. FM1-43 (Life Technologies, Waltham, MA, USA) was applied to the mounted tissue according to a previously described method.38 Explants were washed three times with Hanks balanced salt solution (HBSS) (Nacalai Tesque, Kyoto, Japan) containing 10 mM 4-
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Figure 8 Schematic view of the phenotype in the HCs of Atg5flox/flox;Pou4f3-Cre mice based on morphological and functional analyses. In P5 HCs, no morphological changes were observed and mechanotransduction was normal, although aggregates containing ubiquitin and p62 were observed in the cytoplasm. At P14, aggregates became more massive. Sterocilia were damaged in many HCs and some of HC bodies were destroyed. P14 mice showed profound hearing loss. At 8 weeks of age, loss of HCs was progressively developed.

Statistical analyses. Data are expressed as the mean ± S.E. Statistical analyses were conducted by using IBM SPSS statistics version 22 (IBM, Tokyo, Japan) or R version 3.0.2 (R Development Core Team, Auckland, New Zealand). A comparison of the number of punctate structures between Atg5flox/flox;Pou4f3-Cre and Atg5flox/+;Pou4f3-Cre mice was performed by Student’s t-test. For a comparison of the number of aggregates containing ubiquitin and p62, two-way analysis of variance was performed with genotype × age as variables, followed by Bonferroni post hoc tests, wherever appropriate. A comparison of the ABR threshold between Atg5flox/flox;Pou4f3-Cre and Atg5flox/+;Pou4f3-Cre mice was performed by Student’s t-test.

Conflict of Interest
The authors declare no conflict of interest.

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(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and 1.3 mM Ca2+ (HEPES-HBSS-Ca). After an additional 20-min incubation in HEPES-HBSS-Ca, 5 μM FMI-43 in HEPES-HBSS was applied for 10 s at room temperature, followed immediately by 4 washes (within 1 min) with HEPES-HBSS-Ca. When testing the effects of 1,2-bis-(2-aminophenoxyethane)-N,N,N′,N′-tetraacetic acid (BAPTA), HEPES-HBSS-Ca was replaced with HEPES-HBSS containing 5 mM BAPTA at every step. FMI-43 fluorescence of magnified images from the cochleae was captured with a 40× objective lens 5 min after FM1-43 treatment. Images were obtained with the A1R confocal laser scanning microscope.

Author contributions
CF, SI and TY conceived of the study. MF, HM, KK and NM supervised experimental design. CF, SI, SU and YS conducted the experiments. CF performed statistical analysis. HM, NM and TY supervised interpretation of data. CF wrote the manuscript. All authors edited the manuscript for content.

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