Dync1li1 is required for the survival of mammalian cochlear hair cells by regulating the transportation of autophagosomes

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Abstract

Dync1li1, a subunit of cytoplasmic dynein 1, is reported to play important roles in intracellular retrograde transport in many tissues. However, the roles of Dync1li1 in the mammalian cochlea remain uninvestigated. Here we first studied the expression pattern of Dync1li1 in the mouse cochlea and found that Dync1li1 is highly expressed in hair cells (HCs) in both neonatal and adult mice cochlea. Next, we used Dync1li1 knockout (KO) mice to investigate its effects on hearing and found that deletion of Dync1li1 leads to early onset of progressive HC loss via apoptosis and to subsequent hearing loss. Further studies revealed that loss of Dync1li1 destabilizes dynein and alters the normal function of dynein. In addition, Dync1li1 KO results in a thinner Golgi apparatus and the accumulation of LC3+ autophagic vacuoles, which triggers HC apoptosis. We also knocked down Dync1li1 in the OC1 cells and found that the number of autophagosomes were significantly increased while the number of autolysosomes were decreased, which suggested that Dync1li1 knockdown leads to impaired transportation of autophagosomes to lysosomes and therefore the accumulation of autophagosomes results in HC apoptosis. Our findings demonstrate that Dync1li1 plays important roles in HC survival through the regulation of autophagosome transportation.

Author summary

Hearing loss is one of the most common sensorial disorders globally. The main reason of hearing loss is the irreversible loss or malfunction of cochlear hair cells. Identifying new
hearing loss-related genes and investigating their roles and mechanisms in HC survival are important for the prevention and treatment of hereditary hearing loss. Cytoplasmic dynein 1 is reported to play important roles in in ciliogenesis and protein transport in the mouse photoreceptors. Here, we described the expression pattern of Dync1l1 (a subunit of cytoplasmic dynein 1) in the mouse cochlea and used knockout mice to investigate its specific role in the hair cell of cochlea.

Introduction

Hearing loss is one of the most common sensorial disorders globally, and it leads to reduced quality of daily life and to huge economic costs [1]. Genetic factors, aging, intense noise exposure, and aminoglycoside treatment can cause hearing loss, mainly through the irreversible loss or malfunction of cochlear hair cells (HCs) [2]. Genetic factors causing hereditary hearing loss remain to be identified. Therefore, identifying new hearing loss–related genes and investigating their roles and mechanisms in HC survival are important for the prevention and treatment of hereditary hearing loss.

Cytoplasmic Dynein1 (hereafter referred to as dynein), responsible for intracellular retrograde transport, is a multi-subunit complex that consists of two copies of the 530 kDa dynein heavy chain (DHC), two 74 kDa dynein intermediate chains (DICs), two 53–57 kDa dynein light intermediate chains (DLICs), and three 8–21 kDa light chains (DLCs) [3,4]. The DHC harbors the ATPase and is responsible for force production. The DIC plays a key scaffolding role in the complex and is involved in communicating with other protein complexes to regulate dynein activity [3,5]. It is reported that the DLC can act as an adapter to link various proteins to the dynein motor complex [6–8]. The DLIC is an essential subunit of dynein and is highly conserved in different eukaryotic cells [9]. In vertebrates, there are two DLIC genes, Dync1l1i1 and Dync1l1i2, while there is only one DLIC gene in lower eukaryotes [10,11]. The C-terminus of the DLIC contains two conserved regions with helical propensity, and the N-terminal GTPase-like domain is also conserved and tightly binds to the DHC [7,12,13]. Knockdown (KD) or knockout (KO) of Dync1l1i1 in vitro leads to mitotic defects, fragmentation of the Golgi apparatus, and abnormal intracellular vesicle transport [14–17], and depletion of Dync1l1i1 in Drosophila cells and Aspergillus nidulans leads to destabilization of the DHC and DIC [14,18]. Mice with a point mutation in Dync1l1i1 show increased length of dendrites in cortical neurons and an increased number of dendrite branches in dorsal root ganglia neurons [19]. In the retina, Dync1l1i1 regulates the transportation of membrane proteins of rod outer segment from the Golgi to the base of the connecting cilium, thus regulating the formation of primary cilia [20]. Considering that the retina and the cochlea are both sensorial organs and might share some common mechanisms, we hypothesize that Dync1l1i1 plays important roles in the cochlea.

Autophagy is a highly conserved homeostatic process that eliminates defective organelles and misfolded proteins [21–23]. Several studies have suggested that there is a close relationship between autophagy and hearing loss in animal models [24–26]. Autophagophagic flux includes autophagosome formation, transportation and fusion with the lysosome, and finally maturation to form autolysosomes (both autophagosome and autolysosomes are called autophagic vacuoles) [27]. Disruption of autophagic flux will prevent autophagosomes from being cleared from the cell, thus resulting in increased cellular stress and ultimately leading to cell death and subsequent neurodegenerative disorders [28–30]. Current research on autophagy in the
auditory system is limited, and most such studies have focused on sensorineural hearing loss caused by exogenous HC damage [31].

It has been widely reported that there is a close relationship between dynein and autophagy [32]. When autophagy is induced due to nutrient starvation in vitro, DLCs help to release autophagic regulators from the dynein complex and thus initiate autophagosome nucleation [33,34]. In the brain, the impairment of dynein-driven autophagosome motility causes autophagosomes to accumulate in neurites and synaptic termini, and this indicates the importance of dynein and autophagy in the clearance of aggregate-prone proteins in preventing neurodegenerative diseases [35–39]. However, the relationships between dynein and autophagy in the inner ear remain to be investigated.

To understand the role of Dync1li1 in cochlear HCs and to elucidate the relationship between dynein and autophagy in the inner ear, we determined the expression pattern of Dync1li1 in the inner ear and investigated its role and mechanism in hearing function by using Dync1li1 KO mice [20]. We found that Dync1li1 is highly expressed in HCs in both neonatal and adult mice and that Dync1li1 KO leads to progressive HC loss via apoptosis and subsequently leads to hearing loss. We also found that deletion of Dync1li1 resulted in a reduced number of Golgi lamellae and the accumulation of autophagosomes both in vitro and in vivo, which suggested that deletion of Dync1li1 leads to HC apoptosis due to impaired transporta-

**Materials and methods**

**Ethics statement**

Animals were maintained following the Rutgers University Institutional Animal Use and Care Committee (Protocol 201702497), National Institutes of Health guidelines, and the policies of the Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic (Protocol 43/2015). These regulatory bodies approved all experimental procedures involving the animals.

All animal procedures were performed according to the protocols that were approved by the Animal Experimental Ethical Inspection Form of Southeast University (No.20210302028). All animal procedures were consistent with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals.

**Animals**

Dync1li1 KO mice were a gift from Prof. Wufan Tao (Fudan University, Shanghai, China) [20]. LC3-GFP reporter mice of both sexes in the C57BL/6J/Nju background (Stock D000244, Nanjing Biomedical Research Institute of Nanjing University) were used in the experiments.

**Genotyping PCR**

LC3-GFP reporter mice were genotyped by using genomic DNA from tail tips. Tail tips were digested by adding 180 μl 50 mM NaOH, incubating at 98°C for 1 h, and adding 20 μl 1M Tris-HCl pH 7.0. The genotyping primers were used as follows: wild type (F) 5’-TGA GCG AGC TCA AGA TAA TCA GGT-3’; wild type (R) 5’-GTT AGC ATT GAG CTG CAA GCG CCG TCT-3’; mutant (F): 5’-TCC TGC TGG AGT TCG TGA CCG-3’; mutant (R): 5’-TTG CGA ATT CTC AGC CGT CTT CAT CTC TCT CGC-3’. The PCR system for genotyping is as follows: genomic DNA 3 μl, primer of each 0.5 μl, 2× PCR mix (Vazyme, P112-03) 10 μl, and add H2O up to a total volume of 20 μl. The conditions of PCR were an initial denaturing
step of 3 min at 94°C followed by 35 cycles of 35 s denaturation at 94°C, 30 s annealing at 60°C, and 40 s extension at 72°C. Genotyping of Dync1li1 KO mice was performed according to the previous report [20]. The genotyping primers of Dync1li1 were used as follows: wild type (F) 5′-GGA AGA TGT GAC AAG ACA GAC ACG-3′; wild type (R) 5′-TGG CTC AGT GGT AAA GGT CC-3′; mutant (F): 5′-GGA AGA TGT GAC AAG ACA GAC ACG-3′; mutant (R): 5′-TCA GGA AAA GCA CTG GCT G-3′.

**Auditory brainstem response (ABR) test**

The mice were injected I.P. with 0.01 g/ml pentobarbital sodium (100 mg/kg body weight) to achieve deep anesthesia, and the closed-field ABR test were measured for thresholds is previously described [40]. The ABR test was performed in a soundproof room, and 3 fine needle-like electrodes were inserted at the cranial vertex, underneath the ear, and at the back near the tail of the mice. The frequency of ABR test are 4 kHz, 8 kHz, 16 kHz, 24 kHz, and 32 kHz. The hearing thresholds were determined by decreasing the sound intensities from 90 dB in 20 dB steps until the lowest sound intensity of the first wave could be identified. All test was measured by TDT System III workstation running SigGen32 software (Tucker-Davis Technologies). The data were analyzed by using GraphPad Prism 7 software.

**Immunostaining and image acquisition**

For P0–P7 neonatal mice, the cochlea was dissected with sharp forceps (WPI) in cold HBSS and the tissue fixed in 4% paraformaldehyde (PFA) for 1 h at room temperature (RT). When mice older than P7, the temporal bone was fixed in 4% PFA for 1 h, decalcified in 0.5 M EDTA solution for 1–3 days (it depends on the mice age) at RT, and then dissected in HBSS. The sample was washed by PBS and then blocked with blocking medium (5% donkey serum, 0.5% Triton X100, 0.02% sodium azide, and 1% bovine serum albumin in pH 7.4 PBS) for 1 h at RT. And then incubated with primary antibodies diluted in PBT1 medium (2.5% donkey serum, 0.1% Triton X100, 0.02% sodium azide, and 1% bovine serum albumin in pH 7.4 PBS) at 4°C overnight. The sample was then washed with 0.1% Triton X100 in pH 7.4 PBS for three times and incubated with fluorescence-conjugated secondary antibody (Invitrogen) or phalloidin (Invitrogen), both diluted 1:400 in PBT2 medium (0.1% Triton X100 and 1% bovine serum albumin in pH 7.4 PBS), for 1 h at RT. The sample was mounted in antifade fluorescence mounting medium (DAKO) after washing with 0.1% Triton X100 in pH 7.4 PBS for three times. The primary antibodies were anti-Myo7a (rabbit anti-myo7a; Proteus Bioscience, #25–6790; 1:1000 dilution in PBT1), phalloidin (Invitrogen, A34055), DAPI (Solarbio, C0060), anti-Dync1li1 (Abcam, ab157468, 1:400 dilution in PBT1), anti-Ctbp2 (BD Biosciences, #612044, 1:400 dilution in PBT1), anti-PSD95 (Millipore, #MAB1596, 1:400 dilution in PBT1), anti-Rab7 (abcam, ab137029, 1:400 dilution in PBT1). A TUNEL kit (Roche, 11684817910) was used to detect apoptotic cells according to the instructions. For image acquisition, all samples were scanned by Zeiss microscope (LSM 710) with the same hardware settings to enable direct comparison between treatment conditions. Since synapses are not always on the same layer, we performed Z projection with ImageJ software to capture the Ctbp2 (presynaptic marker) and PSD95 (postsynaptic marker) staining images in S3 Fig. HEI-OC1 cell immunohistochemistry protocol was the same as above.

**Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)**

As previously described [41], Temporal bones were collected and immediately fixed in 2.5% glutaraldehyde (Sigma-Aldrich, G5882) for 24 h and then in 1% OsO4 (Beijing
Zhongxingbairui Technology) for 2h, then dehydrated in ethanol, dried, and then coated with gold. For SEM, samples were mounted on stubs, and sputter coated with gold (Cressington, 108). A scanning electron microscope (FEI Quanta 200) operating at 10 kV was used to take images of the hair bundles. For TEM, the sample further penetrated with graded propylene oxide (Macklin Biochemical, P816084) series and gradually polymerized in araldite. The ultra-thin sections made by Leica powertone (Leica, Em UC6) were post-stained with uranyl acetate and lead citrate in turn, and examined by transmission electron microscope (Hitachi, H-7650). All pictures were taken by the electron microscope room of Nanjing Agricultural University.

**Real-time quantitative PCR**

Samples were dissected to extract total RNA with Trizol reagent (Life, 15596–018) as previously described [41]. Reverse transcription of RNA into cDNA by RevertAid First Strand cDNA Synthesis Kit (Life, K1622), and real-time quantitative PCR (real-time qPCR) was performed by using the FastStart Universal SYBR Green Master (ROX) kit (Roche, 17747200) on Real-Time PCR System (Thermo Fisher Scientific) to quantify the levels of gene expression. The condition of qPCR was as follows: an initial denaturing step: 15 s at 95˚C, then followed by 38 cycles of denaturation step:15 s at 95˚C, then followed by annealing step: 60 s at 60˚C, and 20 s extension at 72˚C. Gapdh was used as the reference gene and used to normalize the mRNA expression data. Using the comparative cycle threshold (ΔΔCt) method to calculate the result. The primers of qPCR are shown in S1 Table.

**Western blotting**

The basilar membranes of neonatal mice (P0–P7) and the temporal bones of mice older than P7 were dissected, and homogenized in ice-cold RIPA lysis buffer (Beyotime, P0013B) by using tissue homogenizer (Shanghai Jingxin Industrial Development Co., Ltd., XJFSTPRP-48). After centrifuged at 12,000 g for 15 min at 4˚C, the supernatant was added with 5X SDS loading buffer (Beyotime, P0015L), separated by 10% or 15% SDS-PAGE, and transferred to an immobilon PVDF membrane (ISEQ00010, Millipore). After blocking with 5% non-fat dried milk in 0.1% PBS-Tween20 for 1 h at RT, the PVDF membrane was incubated with the primary antibody at 4˚C overnight. After washing with 0.1% PBS-Tween20 for 5 times per 6 min, the membrane was incubated with HRP-conjugated second antibody (goat anti mouse HRP, M21001, goat anti rabbit HRP, M21002; ABMART) for 1 hour at RT. Signals were detected with the West Femto Trial Kit (Product #34094; Thermo Scientific) on a FluorChem M system (FM0477; ProteinSimple). The primary antibodies were anti-Dync1l1 (abcam, ab157468), anti-Dync1l2 (Proteintechn, 18885-1-AP), anti- Dync1l1/2 (DIC) (Millipore, MAB1618), anti-Dynll1 (DLC) (abcam, ab51603), anti-Rab7 (abcam, ab137029), anti-LC3 (CST, #4108), anti-Calnexin (santa cruz, sc-70481), anti-P-eIF2α (CST, 3597S), and anti-Gapdh (abcam, ab181602), anti-Dynactin p150 (santa cruz, sc-135890), anti- RILP (Abcam, ab140188)

**Cell culture and cell transfection**

HEI-OC1 cells were cultured at 37˚C with 10% CO2 in DMEM containing 10% FBS (Pansera, #P30-2602) and 100 IU/ml penicillin (Sigma-Aldrich, A0166). The cells were digested by 0.25% trypsin/EDTA (Life Technologies, #25200056) and then subcultured at 75–80% confluency. When the cells grew to a suitable density, Lipofectamine 2000 Transfection Reagent (Invitrogen, #11668027) was used to transfect plasmids into cells according to the manufacturer’s instructions. The shNC-GFP and shDync1l1-GFP plasmids were generated by OBiO
Technology Corp., Ltd. The siNC and siDync1li1 siRNA (5’-CCAGUGCUCGUAGUCUGUATT-3’; 5’-GACAGAGGUGAAGUUGTT-3’) were generated by Shanghai GenePharma Company. The shNC-GFP plasmid and siNC siRNA were used as the negative control, and the shDync1li1-GFP (AGTATGGCGCAGCGCTGATT) plasmid and siDync1li1 siRNA were used to knock down Dync1li1 in HEI-OC1 cells (House Ear Institute-Organ of Corti 1, a cochlear HC-like cell line). The LC3-RFP plasmid, which was a kind gift from Prof. Zheng Ying (Soochow University, Jiang Su, China) [42–45], was used to label LC3+ autophagic vacuoles. The RFP-GFP-LC3 plasmid was purchased from Hanbio Biotechnology (Lot. No. TSB005062-1).

**Data quantification and statistical analysis**

The number of myo7a+ outer HCs (OHCs) and inner HCs (IHCs) per 100 μm were counted in the apical, middle, and basal turns of the cochlea. For synapse counting, Z-projection was performed to project multiple slides of a Z-stack image onto a single layer, and the numbers of Ctbp2+ and PSD95+ puncta were counted in the apical, middle, and basal turn of the cochlea per 100 μm using the ImageJ software (S3 Fig). For HEI-OC1 cells, all of the LC3-RFP fluorescent puncta were counted in each HEI-OC1 cell, and over 50 GFP-shRNA/LC3-RFP double-positive cells were counted. For each experiment, at least three independent experiments were performed. GraphPad Prism 7 software was used to analyze the data and presented as means ± standard errors of the means. A two-tailed, unpaired Student’s t-tests were performed to analyze the data, and p < 0.05 was considered statistically significant.

**Results**

**Dync1li1 is expressed in cochlear HCs in both neonatal and adult mice**

RT-PCR showed that Dync1li1 mRNA was highly expressed in postnatal day 3 (P3) mouse cochlea and in the HEI-OC1 cell line (Fig 1A). Dync1li1 protein was also detected by western blotting in P3 cochlea (Fig 1B). Next, immunofluorescent staining showed that Dync1li1 was highly expressed in HCs in both whole mount and frozen sections of P3 (newborn) and P30 (adult) mouse cochlea. In addition, we also observed that Dync1li1 is also expressed in other types of cells in the organ of Corti, such as spiral neurons and some of supporting cells (Fig 1C–1E). Fig 1F shows a diagram of the IHC and OHC of cochlea. These results suggested that Dync1li1 is highly expressed in HCs and might play important roles in HCs in both the neonatal and adult cochlea.

**Dync1li1 KO leads to progressive HC loss in vivo**

Next, we showed that Dync1li1 is indeed knocked out in Dync1li1 KO mice (Figs 2A and S4). We first sacrificed mice from neonatal to adult ages to investigate whether HC number is affected by Dync1li1 KO. We saw no significant HC loss before P21 in Dync1li1 KO mice, while slight HC loss could be observed from P21 and HC loss gradually became more and more severe as the mice aged (Figs 2B and S1). Quantification of HC loss showed no significant HC loss in P21 mice, although a few HCs were lost in the basal turn. However, significant OHC loss was seen in the apical, middle, and basal turns of the cochlea in P30 and P60 mice (Fig 2C), while the number of IHCs was not significantly changed (Fig 2D). Immunofluorescent staining and scanning electron microscopy (SEM) both showed that the hair bundles of surviving HCs in Dync1li1 KO mice had normal morphology (Fig 2E and 2F). Because HCs are important sound-sensing cells, we used auditory brainstem response to detect the hearing ability of Dync1li1 KO mice. Consistent with the degree of HC loss, the hearing thresholds of
Fig 1. The expression pattern of Dync1li1 in the cochlea of wild-type mice. (A, B) Dync1li1 mRNA (A) and protein (B) expression in P3 mouse cochlea by RT-PCR and Western blotting, respectively. Brain tissue and the HEI-OC1 cell line were used as the positive controls. (C) Whole mount immunofluorescent staining of Dync1li1 in P3 mouse cochlea. The large square image is a single XY slice, the vertical red line shows the position of the orthogonal slice, which is shown on the right side of each panel, and the blue line on the orthogonal line shows the level of the XY slice on the left. (D) Frozen section immunofluorescent staining of Dync1li1 in P3 mouse cochlea. The white boxes and the dotted lines show enlarged images. (E) Whole mount immunofluorescent staining of Dync1li1 in P30 adult mouse cochlea. For all experiments, scale bars are shown in the figure. (F) A diagram of the IHC and OHC in the cochlea.

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The Dync1li1 KO mice were not affected at P21 but were significantly increased at P30 and P60 (Fig 2G). Together these results suggest that Dync1li1 is highly expressed in HCs and that its deletion results in progressive HC loss and hearing loss in adult mice, and thus that Dync1li1 plays important roles in HC survival.

TUNEL signals, which are indicative of apoptosis, were observed in HCs of both P21 and P30 Dync1li1 KO mice, but not in the control group (Fig 3A). Real-time qPCR results also showed that the expression of apoptosis-related genes, such as Aparf and Caspase3, were significantly upregulated in the Dync1li1 KO mouse cochlea (Fig 3B). Together, these results indicate that HC loss in Dync1li1 KO mice was due to HC apoptosis.

Dync1li1 KO decrease the stability of Dynein complex in HCs

Because Dync1li1 is an important part of the dynein complex, which is crucial in all eukaryotic cells for transporting a variety of essential cargoes toward the minus end of microtubules (also called retrograde transport), we speculated that HC apoptosis caused by Dync1li1 KO might be related to impaired retrograde transportation. Thus, we first detected the expression of other
components of the dynein complex to determine the stability of dynein. The mRNA levels of
\textit{Dync1h1} (DHC), \textit{Dync1i1} (one of the DIC genes), and \textit{Dync1l1} (one of the DLC genes) were all significantly downregulated in P60 \textit{Dync1li1} KO mouse cochlea (Fig 4A), and the protein level of Dync1i1/2 (DIC) and Dyncll1 (DLC) were also significantly downregulated, with Dync1i1/2 being the most pronounced and the expression of Dync1li2 was not significantly changed in P30 \textit{Dync1li1} KO mice (Fig 4B and 4C). We then used TEM to explore the effects on transport-related organelles in cochlear HCs, such as the structure of the endoplasmic reticulum (ER) and the Golgi apparatus, and we found that the number of lamellae per Golgi was significantly reduced in \textit{Dync1li1} KO OHCs compared to the control group and that the Golgi apparatus was thinner in the \textit{Dync1li1} KO OHCs (Fig 4D–4F). Together, these results indicate that deletion of \textit{Dync1li1} leads to unstable dynein complexes and to decreased stacks of Golgi apparatus in cochlear OHCs.

HC apoptosis caused by \textit{Dync1li1} KO is due to impaired transportation of autophagosomes to lysosomes Considering that dynein plays crucial roles during the autophagic process (Fig 5D) and that autophagy is involved in apoptosis [46], we hypothesized that the HC apoptosis caused by \textit{Dync1li1} KO is due to impaired transportation of autophagosomes to lysosomes. To test this, we first detected the LC3 signal (autophagic vacuoles marker) in HCs of P60 \textit{Dync1li1}+/− mouse cochlea and found significantly more LC3-positive puncta in the OHCs of \textit{Dync1li1}+/− compared to the LC3-GFP–only control mouse (Fig 5A and 5B). Western blotting showed that the LC3 and Sqstm1/p62 protein level were upregulated in P60 \textit{Dync1li1} KO mouse cochlea, suggesting that autophagic vacuoles were aggregated in the HCs (Fig 5C). Moreover, Rab7, an adaptor protein during the maturation of autolysosomes [47,48], was also upregulated in the cochlea of P60 \textit{Dync1li1} KO mice (Fig 5C), which indicated that
Fig 4. Dyncll1 deficiency affects the integrity of Dynein complex and Golgi apparatus. (A) Quantification of the mRNA expression of important subunits in dynein complex (Dyncll1, Dyncll2, and Dynll1) in P60 Dyncll1 KO and the control mice by qPCR. N = 3. N refers to 3 independent qPCR experiments were performed. (B, C) Western blotting (B) and quantification of the western blotting (C) of the Dynein subunit in the cochlea of P60 Dyncll1 KO mice. Gapdh was used as the internal reference. N is indicated in the figure. (D, E) TEM of OHCs in P60 Dyncll1 KO and control mice. The Golgi apparatus is indicated by red arrows in (D) and red lines in (E). (F) Quantification of the number of lamellae per Golgi. For all experiments, scale bars are shown on the figure and N is indicated in the figure. *p < 0.05, **p < 0.01.

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Fig 5. Dync1li1 deficiency induced the accumulation of autophagosomes vacuoles in HCs. (A) Immunofluorescence of LC3 (green puncta, indicated by white arrows) in HCs of P60 Dync1li1^+/−-LC3-GFP mice and control mice. Myo7a was used as HC marker. The enlarged images are shown in the lower left corner. (B) Quantification of the number of LC3^+ OHCs. N = 3. (C) Western blotting of the LC3 (LC3-I, 16 kDa; LC3-II, 14 kDa.), Rab7, and Sqstm1/p62 in the cochlea. Gapdh
newly formed autophagosomes (with Rab7 on their surface) could not be transported to lysosomes for degradation and thus had accumulated in the HCs.

Next, we verified these results in the HC-like HEI-OC1 cell line [49]. We used shRNA to knock down Dync1li1 in the HEI-OC1 cell line and confirmed the KD efficiency (Fig 6A and 6B). We then transfected the LC3-RFP plasmid into HEI-OC1 cells and quantified the LC3 puncta, and we found that the number of LC3 puncta was significantly increased in the Dync1li1 KD group at both 24 h and 36 h after transfection compared to the controls (Fig 6C and 6D).

RFP-GFP-LC3 is a tool plasmid for detecting the level of autophagy in cells as illustrated in Fig 6E [50]. There are two fluorescent protein, red RFP and green GFP, expressed as a fusion protein with LC3, in which GFP is a pH sensitive protein. When in the autophagic vacuoles with a neutral pH (autophagosome), RFP and GFP both show fluorescent signal, and thus the LC3 dots are yellow (GFP+/RFP+). When in the autophagic vacuoles with an acidic pH (autolysosome), GFP cannot show fluorescent signal and only RFP can show red fluorescent signal, and thus the LC3 dots are red. Therefore, we can use this plasmid to measure autophagic flux in the Dync1li1 KD group and the control group. And we found that in Dync1li1 KD OC1 cells, the number of autophagosomes (GFP+/RFP+) were significantly more than that in the control group, while the number of autolysosomes (GFP-/RFP+) was less than that in the control group (Fig 6F and 6G). These data suggested that Dync1li1 KD led to accumulation of autophagosomes which cannot be eliminated by transporting to lysosomes to form autolysosomes. Moreover, we also found that the protein level of Dync1li2 (DIC) and Dyncll1 (DLC) were also significantly down regulated, the expression of Dync1li2 was not changed, and the protein level of LC3 and Sqstm1/p62 were up regulated in Dync1li1 KD group (Fig 6H and 6I).

These results are consistent with the changes in protein level in Dync1li1 KO mouse cochlea. Therefore, we conclude that knock down of Dync1li1 in vitro led to impaired transportation of autophagosomes to lysosomes and to abnormal accumulation of autophagosomes in HC-like HEI-OC1 cells.

In summary, the mechanisms we identified are shown in Fig 7. Under normal conditions, late autophagosomes with harmful substances produced in HCs are transported by dynein to be fused with lysosomes and form autolysosomes for subsequent degradation, thereby maintaining cell homeostasis. When Dync1li1 is defective or missing, the dynein complex becomes unstable and cannot effectively transport late autophagosomes to lysosome for degradation, which leads to the accumulation of these autophagosomes with harmful substances. This disruption in cell homeostasis triggers HC apoptosis and thus leads to hearing loss.

Discussion

Dynein plays critical roles in the central nervous system through its effects on nuclear migration and retrograde transport of various cargos [51]. In this study, we found that one member of the dynein complex, Dync1li1, was highly expressed in cochlear HCs, and Dync1li1 KO mice showed progressive hearing loss along with early onset of HC apoptosis. Further study revealed that Dync1li1 deletion decreased the expression level of other members of dynein complex, including the DHC, DIC, and DLC, which further reduced the number of lamellae in the Golgi apparatus and led to the accumulation of autophagosomes in HCs. Together our data suggest that the unstable dynein complex caused by Dync1li1 KO affects Golgi-related
Fig 6. Accumulation of autophagosomes in Dync1l1 KD HEI-OC1 cells. (A, B) OC1 Cells were transfected with shDync1l1-GFP shRNA, and qPCR analysis (A) and Western blotting (B) were used to test the knockdown efficiency of Dync1l1. shNC-GFP was used as control shRNA. The cells were harvested after 36h transfection. (C, D) shNC-GFP and shDync1l1-GFP were cotransfected with LC3-RFP.
plasmons into OC1 cells for 24 h and 36 h, respectively. Red dots in (C) indicate the LC3 puncta (autophagic vacuoles), which were quantified in (D). (E) Schematic of the working principle of the RFP-GFP-LC3 plasmid. In a neutral environment (autophagosome), LC3 is expressed with both GFP and RFP fluorescent proteins, and thus the autophagosome dots (RFP-GFP) are red. In an acidic environment (autolysosome), GFP fluorescence is quenched, and thus the autolysosome dots (RFP) are red. (F, G) siNC and siDync1li1 siRNA were cotransfected with RFP-GFP-LC3 plasmid for 36 h. Western blotting (H) and quantification of Dynein subunit proteins after siRNA transfection. Quantification of protein expression levels at 36 h after siRNA transfection (I). (J) Western blotting of LC3, Sqstm1/p62 and Rab7 at 36 h after siRNA transfection. For all experiments, scale bars are shown in the figure. *p < 0.05, **p < 0.01, n.s. not significant.

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transport and the transport of autophagosomes to lysosomes and that this ultimately results in HC apoptosis in the cochlea.

The cilia of HCs (called HC bundles) show a highly ordered arrangement that makes them highly sensitive to vibration of the fluid environment, and the planar cell polarity (PCP) of HC bundles is essential for hearing function [52–55]. It is reported that Dync1li1 knockout mice show deficient ciliogenesis of photoreceptors [20]. Here we found that Dync1li1 is highly expressed in auditory HC cytoplasm, rather than in HC bundles, in both neonatal and adult mouse cochlea. Dync1li1 KO mice showed progressive HC loss and hearing loss at all frequencies, but no changes were observed in the morphology or PCP of HC bundles. These results indicated that the function of Dync1li1 in HCs was not involved in the formation or PCP of the hair bundles.

The TUNEL assay showed that HC loss caused by Dync1li1 deficiency is due to early onset of HC apoptosis. Previous study showed that separation of DLIC from the dynein complex result in unstable DHC in vertebrate [7]. In the mouse retina, loss of Dync1li1 reduced the protein levels of DHCDIC and DLC and therefore impair the transport ability of dynein [20]. Consistent with previous reports, here we also found that deletion of Dync1li1 led to decreased expression of the DHC, DIC, and DLC in mouse cochlea and thus caused the destabilization of the dynein complex in cochlear HCs. In the photoreceptor cells, LIC1 deletion also increase dynactin P150 (a dynein adaptor linking dynein to cargoes) protein expression [20], we also investigate the protein level of P150 in the KO Dync1li1 mouse cochlea (S2C Fig).

Considering that dynein is involved in maintaining the architecture of the ERGIC (ER-Golgi intermediate compartment) and that inhibition of dynein leads to fragmentation and dispersion of the Golgi apparatus [15,56–58], we also investigated the effects of Dync1li1 deletion on the morphology of the ER and Golgi apparatus in HCs. Our TEM data showed that the lamellae of the Golgi apparatus were thinner in Dync1li1 KO OHCs, which suggested that Dync1li1 deletion led to disorganization of transport processes in the Golgi apparatus. We also measured the ER stress level by measuring the protein level of Calnexin and P-eIF2α, and we found that neither of them were significantly changed (S2A and S2B Fig). Since RILP is also an important adapter for dynein, we investigated the expression of RILP in the KO Dync1li1 mouse cochlea. Interestingly, we found that the protein level of RILP was not significantly changed (S2D Fig). We also investigated the location and the number of auditory ribbon synapses in Dync1li1 KO mice, but we did not observe any abnormalities. Both the location and the number of ribbon synapses were normal in HCs of Dync1li1 KO mice (S3 Fig), which indicated that Dync1li1 deficiency did not affect the formation of ribbon synapses.

Accumulating studies have shown that the dynein complex is involved in autophagic processes and dynein-dependent retrograde transport of autophagic vacuoles is essential for the survival of neurons [37,38,59–61]. Dynein inhibition impair the process of clearance of aggregate-prone proteins in the drosophila and mouse model of Huntington’s disease [35]. The mutation of dynein weaken the clearance of mutant huntingtin fragments by cross the
HDhHD mice (mouse model of Huntington disease [62]) with Dnchc1Loa (ethylnitrosourea-induced missense mutation in the dynein heavy chain 1[63]), and the level of LC3-II expression in Hdh+/+ Dnchc1Loa/+ mice was increased [35].

Basal autophagy is important for homeostasis in postmitotic cells [64,65]. In the inner ear, basal autophagic flux can be detected in cochlear HCs and is essential for hearing in mice [66,67], and autophagy-deficient mice show impaired biogenesis of otoconia [68]. However,
there is no research about the relation between dynein and autophagy in cochlear HCs. In our study, we observed that the endogenous LC3 puncta were significantly increased in both HCs and HC-like HEI-OC1 cells after Dync1li1 KO or KD, which suggested that without dynein the process of autophagosome clearance was abnormal and that this led to the accumulation of autophagosomes in HCs that in turn triggered apoptosis in HCs and subsequent hearing loss.

Many studies have shown that Rab7 is required for the complete autophagic flux and that it regulates the process of autophagosome-lysosome fusion [69,70]. These studies indicated the strong interaction between dynein and Rab7 in the process of late autophagosome-lysosome fusion. Here, our results showed that both the protein level of Rab7 and LC3 were significantly increased in cochlear HCs of Dync1li1 KO mice and Dync1li1 KD HEI-OC1 cells in vitro, which suggested that late autophagosomes accumulated in Dync1li1-deficient cells. Therefore, we speculated that deletion of Dync1li1 impaired the transportation of late autophagosomes to lysosomes, such that LC3+ autophagosomes could not be cleared and therefore accumulated in the HCs. However, it is worth noting that Rab7 is also a marker of late endosomes [71] and that Rab7 is required for mitophagosome formation by regulating phagophore transport [72,73]. Therefore, in future work we will further explore the effects of Dync1li1 deletion on other Rab7-mediated transport processes in the mouse cochlea.

In summary, we have identified new roles for Dync1li1 in maintaining the survival of mammalian cochlear HCs. We show that Dync1li1 KO leads to destabilization of the dynein complex and that this results in impaired transport of late autophagosomes to lysosomes. Therefore, LC3+ autophagosomes cannot be cleared and thus accumulate in HCs, which leads to HC apoptosis and hearing loss in adult mice.

Supporting information

S1 Fig. No HC loss is observed in the cochleae of Dync1li1 KO mice at P1 and P14. (A, B) Immunofluorescent staining of Myo7a and Phalloidin in P1 (A) and P14 (B) Dync1li1 KO mice and WT control mice cochleae, respectively. Myo7a was used as HC marker. Phalloidin was used as HC bundle marker. (C, D) Quantification of OHC (C) and IHC (D) number in the apical (APEX), middle (MID), and basal (BASE) turns of P1 Dync1li1 KO and control mice cochleae. (E, F) Quantification of OHC (E) and IHC (F) number in the apical (APEX), middle (MID), and basal (BASE) turns of P14 Dync1li1 KO and control mice cochleae. For all experiments, scale bar are shown on the figure, N is indicated in the figure, n.s. not significant. (TIF)

S2 Fig. The analysis protein level of expression of ER stress-related proteins, P150 and RILP in Dync1li1 KO mice at P30. (A, B,C,D) Western blotting of Calnexin (A), P-eIF2α (B), P150 (C) and RILP (D) in the cochleae of P30 Dync1li1 KO mice and WT mice. Calnexin was used as the ER marker and P-eIF2α was used as the ER stress marker. Gapdh was used as the internal control. (TIF)

S3 Fig. The ribbon synapses of IHCs in P21 Dync1li1 KO mice. (A) Immunofluorescent staining of Ctbp2 and PSD95 in P21 Dync1li1 KO mice and WT control mice IHCs, respectively. Images were taken from the MID turn of the cochlea. The enlarged image in the white box is shown in the lower right corner. Ctbp2 was used as presynaptic marker. PSD95 was used as postsynaptic marker. (B) Quantification of the number of synapse from MID turn of Dync1li1 KO and WT mice. For all experiments, scale bars are shown on the figure, n.s. not significant. (TIF)
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References

1. Deafness and hearing loss. 2020; Available from: https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss.

2. Dror A.A. and Avraham K.B., Hearing Loss: Mechanisms Revealed by Genetics and Cell Biology. Annual Review of Genetics. 2009; 43(1): p. 411–437. https://doi.org/10.1146/annurev-genet-102108-134135 PMID: 19694516

3. Bhabha G., Johnson G.T., Schroeder C.M., and Vale R.D., How Dynein Moves Along Microtubules. Trends in biochemical sciences. 2016; 41(1). https://doi.org/10.1016/j.tibs.2015.11.004 PMID: 26678005

4. Reck-Peterson S.L., Redwine W.B., Vale R.D., and Carter A.P. The cytoplasmic dynein transport machinery and its many cargoes. Nat Rev Mol Cell Biol. 2018; 19(8): p. 382–398. https://doi.org/10.1038/s41580-018-0004-3 PMID: 29662141

5. Belyy V., Hendel N.L., Chien A., and Yildiz A. Cytoplasmic dynein transports cargos via load-sharing between the heads. Nature communications. 2014; 5: p. 5544. https://doi.org/10.1038/ncomms6544 PMID: 25424027
6. Williams J.C., Roulhac P.L., Roy A.G., Vallee R.B., Fitzgerald M.C., and Hendrickson W.A. Structural and thermodynamic characterization of a cytoplasmic dynein light chain-intermediate chain complex. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104(24): p. 10028–10033. https://doi.org/10.1073/pnas.0703614104 PMID: 17551010

7. King S.J., Bonilla M., Rodgers M.E., and Schroer T.A. Subunit organization in cytoplasmic dynein sub-complexes. Protein science: a publication of the Protein Society, 2002; 11(5): p. 1239–1250. https://doi.org/10.1110/ps.2520102 PMID: 11967361

8. Pfister K.K. Dynein cargo gets its groove back. Structure (London, England: 1993), 2005; 13(2): p. 172–173. https://doi.org/10.1016/j.str.2005.01.003 PMID: 15698564

9. Celestino R., Henen M.A., Gama J.B., McCabe M., Barbosa D.J., et al. A transient helix in the disordered region of dynein light intermediate chain links the motor to structurally diverse adaptors for cargo transport. PLoS biology. 2019; 17(1): p. e3000100. https://doi.org/10.1371/journal.pbio.3000100 PMID: 30615611

10. Pfister K.K., Shah P.R., Hummerich H., Russ A., Cotton J., Annuar A.A., et al. Genetic analysis of the cytoplasmic dynein subunit families. PLoS genetics. 2006; 2(1): p. e1. https://doi.org/10.1371/journal.pgen.0020001 PMID: 16440056

11. Tynan S.H., Gee M.A., and Vallee R.B. Distinct but overlapping sites within the cytoplasmic dynein heavy chain for dimerization and for intermediate chain and light intermediate chain binding. The Journal of biological chemistry. 2000; 275(42): p. 32769–32774. https://doi.org/10.1074/jbc.M001537200 PMID: 10893223

12. Schroeder C.M., Ostrem J.M.L., Hertz N.T., and Vale R.D. A Ras-like domain in the light intermediate chain bridges the dynein motor to a cargo-binding region. eLife, 2014; 3: p. e03351. https://doi.org/10.7554/eLife.03351 PMID: 25272277

13. Lee I.-G., Olenick M.A., Boczkowska M., Franzini-Armstrong C., Holzbaur E.L.F., and Dominguez R. A conserved interaction of the dynein light intermediate chain with dynein-dynactin effectors necessary for processivity. Nature communications. 2018; 9(1): p. 886. https://doi.org/10.1038/s41467-018-03412-8 PMID: 29515126

14. Mische S., He Y., Ma L., Li M., Serr M., and Hays T.S. Dynein light intermediate chain: an essential subunit that contributes to spindle checkpoint inactivation. Molecular biology of the cell. 2008; 19(11): p. 4918–4929. https://doi.org/10.1091/mbc.e08-05-0483 PMID: 18799620

15. Palmer K.J., Hughes H., and Stephens D.J. Specificity of cytoplasmic dynein subunits in discrete membrane-trafficking steps. Molecular biology of the cell. 2009; 20(12): p. 2885–2899. https://doi.org/10.1091/mbc.e08-12-1160 PMID: 19386764

16. Horgan C.P., Hanscom S.R., Jolly R.S., Futter C.E., and McCaffrey M.W. Rab11-FIP3 links the Rab11 GTPase and cytoplasmic dynein to mediate transport to the endosomal-recycling compartment. Journal of cell science. 2010; 123(P12): p. 181–191. https://doi.org/10.1242/jcs.052670 PMID: 20296645

17. Jones L.A., Villema nt C., Starborg T., Salter A., Goddard G., Ruane P., Woodman P.G., Papalopulu N., et al. Dynein light intermediate chains maintain spindle bipolarity by functioning in centriole cohesion. The Journal of cell biology. 2014 207(4): p. 499–516. https://doi.org/10.1083/jcb.201406025 PMID: 25422374

18. Zhang J., Li S., Musa S., Zhou H., and Xiang X. Dynein light intermediate chain in Aspergillus nidulans is essential for the interaction between heavy and intermediate chains. The Journal of biological chemistry. 2009; 284(50): p. 34760–34768. https://doi.org/10.1074/jbc.M109.026872 PMID: 19837669

19. Banks G.T., Haas M.A., Line S., Shepherd H.L., Alqatari M., Stewart S., et al. Behavioral and other phenotypes in a cytoplasmic Dynein light intermediate chain 1 mutant mouse. The Journal of neuroscience: the official journal of the Society for Neuroscience. 2011; 31(14): p. 5483–5494.

20. Kong S., Du X., Du X., Peng C., Wu Y., Li H., et al. Dlic1 deficiency impairs ciliogenesis of photoreceptors by destabilizing dynein. Cell research. 2013; 23(6): p. 835–850. https://doi.org/10.1038/cr.2013.59 PMID: 23628724

21. Whyte L.S., Lau A.A., Hemsley K.M., Hopwood J.J., and Sargeant T.J. Endo-lysosomal and autophagic dysfunction: a driving factor in Alzheimer’s disease? Journal of neurochemistry. 2017; 140(5): p. 703–717. https://doi.org/10.1111/jnc.13935 PMID: 28027395

22. Vijayan V. and Verstreken P. Autophagy in the presynaptic compartment in health and disease. The Journal of cell biology. 2017; 216(7): p. 1895–1906. https://doi.org/10.1083/jcb.201611113 PMID: 28515275

23. Muller S., Brun S., René F., de Sèze J., Loeffler J.-P., and Jeltsch-David H. Autophagy in neuroinflammatory diseases. Autophagy reviews. 2017; 16(8): p. 856–874. https://doi.org/10.1016/j.autrev.2017.05.015 PMID: 28572049
24. Fu X., Sun X., Zhang L., Jin Y., Chai R., Yang L., et al. Tuberous sclerosis complex-mediated mTORC1 overactivation promotes age-related hearing loss. The Journal of clinical investigation. 2018; 128(11): p. 4938–4955. https://doi.org/10.1172/JCI98058 PMID: 30247156

25. He Z., Guo L., Shu Y., Fang Q., Zhou H., Liu Y., et al. Autophagy protects auditory hair cells against neomycin-induced damage. Autophagy. 2017; 13(11): p. 1884–1904. https://doi.org/10.1080/15548627.2017.1359449 PMID: 28968134

26. Wu F., Xiong H., and Sha S. Noise-induced loss of sensory hair cells is mediated by ROS/AMPKα pathway. Redox biology. 2020; 29: p. 101406 –101406. https://doi.org/10.1016/j.redox.2019.101406 PMID: 31926629

27. Eskelinen E.-L. Maturation of autophagic vacuoles in Mammalian cells. Autophagy. 2005; 1(1). https://doi.org/10.4161/auto.1.1.1270 PMID: 16874026

28. Kurtishi A., Rosen B., Patil K.S., Alves G.W., and Møller S.G. Cellular Proteostasis in Neurodegeneration. Molecular neurobiology. 2019; 56(5): p. 3676–3689. https://doi.org/10.1007/s12035-018-1334-z PMID: 30182337

29. Lumkwa D., du Toit A., Kinnear C., and Loos B. Autophagic flux control in neurodegeneration: Progress and precision targeting-Where do we stand? Progress in neurobiology. 2017; 153: p. 64–85. https://doi.org/10.1016/j.pneurobio.2017.03.006 PMID: 28385648

30. Liu J., Liu W., Lu Y., Tian H., Duan C., Lu L., et al. Piperlongumine restores the balance of autophagy and apoptosis by increasing BCL2 phosphorylation in rotenone-induced Parkinson disease models. Autophagy. 2018; 14(5): p. 845–861. https://doi.org/10.1080/15548627.2017.1390636 PMID: 29433359

31. Ye B., Fan C., Shen Y., Wang Q., Hu H., and Xiang M. The Antioxidative Role of Autophagy in Hearing Loss. Frontiers in neuroscience. 2019; 12: p. 1010–1010. https://doi.org/10.3389/fnins.2018.01010 PMID: 30686976

32. Cason S.E., Carman P.J., Van Duyne C., Goldsmith J., Dominguez R., and Holzbaur E.L.F., Sequential dynein effectors regulate axonal autophagosomal motility in a maturation-dependent pathway. J Cell Biol. 2021; 220(7).

33. Di Bartolomeo S., Corazzari M., Nazio F., Oliverio S., Lisi G., Antonioli M., et al. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. The Journal of cell biology. 2010; 191(1): p. 155–168. https://doi.org/10.1083/jcb.201002100 PMID: 20921139

34. Fimia G.M., Di Bartolomeo S., Piacentini M., and Cecconi F. Unleashing the Ambra1-Beclin 1 complex from dynein chains: Ulk1 sets Ambra1 free to induce autophagy. Autophagy. 2011; 7(1): p. 115–117. https://doi.org/10.4161/auto.7.1.14071 PMID: 21079415

35. Ravikumar B., Acevedo-Arozena A., Imarisio S., Berger Z., Vacher C., O’Kane C.J., et al. Dynein mutations impair autophagic clearance of aggregate-prone proteins. Nature Genetics. 2005; 37(7): p. 771–776. https://doi.org/10.1038/ng1591 PMID: 15980862

36. Rubinsztain D.C., Ravikumar B., Acevedo-Arozena A., Imaisio S., Berger Z., Vacher C., O’Kane C.J., and Brown S.D.M. Dyneins, autophagy, aggregation and neurodegeneration. Autophagy. 2005; 1(3): p. 177–178. https://doi.org/10.4161/auto.1.3.2050 PMID: 16874055

37. Cristofani R., Crippa V., Rusmini P., Cicardi M.E., Meroni M., Licata N.V., et al. Inhibition of retrograde transport modulates misfolded protein accumulation and clearance in motoneuron diseases. Autophagy. 2017; 13(8): p. 1280–1303. https://doi.org/10.1080/15548627.2017.1308985 PMID: 28402699

38. Tammineni P. and Cai Q. Defective retrograde transport impairs autophagic clearance in Alzheimer disease neurons. Autophagy. 2017; 13(5): p. 982–984. https://doi.org/10.1080/15548627.2017.1291114 PMID: 28318364

39. Tammineni P., Ye X., Feng T., Aikal D., and Cai Q. Impaired retrograde transport of axonal autophagosomes contributes to autophagic stress in Alzheimer’s disease. Elife. 2017; 6: e21776. https://doi.org/10.7554/elife.21776 PMID: 28085665

40. Chen Y., Li L., Ni W., Zhang Y., Sun S., Miao D., et al. Bmi1 regulates auditory hair cell survival by maintaining redox balance. Cell Death Dis. 2015; 6: p. e1605. https://doi.org/10.1038/cddis.2014.549 PMID: 25611380

41. Pauley S., Lai E., and Fritzsch B. Foxg1 is required for morphogenesis and histogenesis of the mammalian inner ear. Dev Dyn. 2006; 235(9): p. 2470–82. https://doi.org/10.1002/dvdy.20839 PMID: 16691564

42. Zhang S., Zhang Y., Dong Y., Guo L., Zhang Z., Shao B., et al. Knockdown of Foxg1 in supporting cells increases the trans-differentiation of supporting cells into hair cells in the neonatal mouse cochlea. Cellular and molecular life sciences. 2020; 77(7): p. 1401–1419. https://doi.org/10.1007/s00018-019-03291-2 PMID: 31485717
43. Li B., Hu Q., Wang H., Man N., Ren H., Wen L., et al. Omi/HtrA2 is a positive regulator of autophagy that facilitates the degradation of mutant proteins involved in neurodegenerative diseases. Cell death and differentiation. 2010; 17(11): p. 1773–1784. https://doi.org/10.1038/cdd.2010.55 PMID: 20467442

44. Ren H., Fu K., Mu C., Li B., Wang D., and Wang G. DJ-1, a cancer and Parkinson’s disease associated protein, regulates autophagy through JNK pathway in cancer cells. Cancer letters. 2010; 297(1): p. 101–108. https://doi.org/10.1016/j.canlet.2010.05.001 PMID: 20510502

45. Xia Q., Hu Q., Wang H., Yang H., Gao F., Ren H., et al. Induction of COX-2-PGE2 synthesis by activation of the MAPK/ERK pathway contributes to neuronal death triggered by TDP-43-depleted microglia. Cell death & disease. 2015; 6: p.e1702. https://doi.org/10.1038/cddis.2015.69 PMID: 25811799

46. Yang J., Zhang Y., Hamid S., Cai J., Liu Q., Li H., et al. Interplay between autophagy and apoptosis in selenium deficient cardiomyocytes in chicken. Journal of inorganic biochemistry. 2017; 170: p. 17–25. https://doi.org/10.1016/j.jinorgbio.2017.02.006 PMID: 28214429

47. Khobrekar N.V. and Valle R.B. A RILP-regulated pathway coordinating autophagosome biogenesis with transport. Autophagy. 2020; 16(8): p. 1537–1538. https://doi.org/10.1080/15548627.2020.1778294 PMID: 32597306

48. Khobrekar N.V., Quintremil S., Dantas T.J., and Valle R.B. The Dynein Adaptor RILP Controls Neuro-Nuclear Autophagosome Biogenesis, Transport, and Clearance. Dev Cell. 2020; 53(2): p. 141–153.e4. https://doi.org/10.1016/j.devcel.2020.03.011 PMID: 32275887

49. Kim H.-J., Oh G.-S., Lee J.-H., Lyu A.-R., Ji H.-M., et al. Cisplatin ototoxicity involves cytokines and STAT6 signaling network. Cell research. 2011; 21(6): p. 944–956. https://doi.org/10.1038/cr.2011.27 PMID: 21321603

50. Mizushima N., Yoshimori T., and Levine B. Methods in Mammalian Autophagy Research. Cell. 2010; 140(3): p. 313–326. https://doi.org/10.1016/j.cell.2010.01.028 PMID: 20144757

51. Perlson E., Maday S., Fu M.-M., Moughamian A.J., and Holzbaur E.L.F. Retrograde axonal transport: pathways to cell death? Trends in neurosciences. 2010; 33(7): p. 335–344. https://doi.org/10.1016/j.tins.2010.03.006 PMID: 20434225

52. O Maoiléidigh D. and Ricci A.J. A Bundle of Mechanisms: Inner-Ear Hair-Cell Mechanotransduction. Trends in neurosciences. 2019; 42(3): p. 221–236. https://doi.org/10.1016/j.tins.2018.12.006 PMID: 30661717

53. Okamoto S., Chaya T., Omori Y., Kuwahara R., Kubo S., Sakaguchi H., et al. Ick Ciliary Kinase Is Essential for Planar Cell Polarity Formation in Inner Ear Hair Cells and Hearing Function. The Journal of Neuroscience: the official journal of the Society for Neuroscience. 2017; 37(8): p. 2073–2085. https://doi.org/10.1523/JNEUROSCI.3067-16.2017 PMID: 28115485

54. Zampini V., Rüttiger L., Johnson S.L., Franz C., Furness D.N., Waldhaus J., Xiong H., et al. Eps8 regulates hair bundle length and functional maturation of mammalian auditory hair cells. PLoS biology. 2011; 9(4): p. e1001048. https://doi.org/10.1371/journal.pbio.1001048 PMID: 21526224

55. Landin Malt A., Dailey Z., Holbrook-Rasmussen J., Zheng Y., Hogan A., Du Q., and Lu X. Par3 is essential for the establishment of planar cell polarity of inner ear hair cells. Proceedings of the National Academy of Sciences of the United States of America. 2019; 116(11): p. 4999–5008. https://doi.org/10.1073/pnas.1816333116 PMID: 30814219

56. Jaarsma D. and Hoogenraad C.C. Cytoplasmic dynein and its regulatory proteins in Golgi pathology in nervous system disorders. Frontiers in neuroscience. 2015; 9: p. 397–397. https://doi.org/10.3389/fnins.2015.00397 PMID: 26578860

57. Yadav S. and Linstedt A.D. Golgi positioning. Cold Spring Harbor perspectives in biology. 2011; 3(5). https://doi.org/10.1101/cshperspect.a005322 PMID: 21504874

58. Lu L. and Hong W. From endosomes to the trans-Golgi network. Seminars in cell & developmental biology. 2014; 31: p. 30–39.

59. Corona A.K. and Jackson W.T. Finding the Middle Ground for Autophagic Fusion Requirements. Trends in cell biology. 2018; 28(11): p. 869–881. https://doi.org/10.1016/j.tcb.2018.07.001 PMID: 30115558

60. Xu M., Li X.-X., Xiong J., Xia M., Guibins E., Zhang Y., et al. Regulation of autophagic flux by dynemin-mediated autophagosomes trafficking in mouse coronary arterial myocytes. Biochimica et biophysica acta. 2013; 1833(12): p. 3228–3236. https://doi.org/10.1016/j.bbamcr.2013.09.015 PMID: 24095928

61. Katsumata K., Nishiyama J., Inoue T., Mizushima N., Takeda J., and Yuzaki M. Dynein- and activity-dependent retrograde transport of autophagosomes in neuronal axons. Autophagy. 2010; 6(3): p. 378–385. https://doi.org/10.4161/auto.6.3.11262 PMID: 20150763

62. Schilling G., Becher M.W., Sharp A.H., Jinnah H.A., Duan K., Kotzuk J.A., et al. Intranuclear Inclusions and Neuritic Aggregates in Transgenic Mice Expressing a Mutant N-Terminal Fragment of Huntingtin.
63. Hafezparast M., Klocke R., Ruhrberg C., Marquardt A., Ahmad-Annuar A., Bowen S., Lalli G., et al. Mutations in dynein link motor neuron degeneration to defects in retrograde transport. Science. 2003; 300(5620): p. 808–812. https://doi.org/10.1126/science.1083192 PMID: 12730604

64. Menzies F.M., Fleming A., Caricasole A., Bento C.F., Andrews S.P., Ashkenazi A., et al. Autophagy and Neurodegeneration: Pathogenic Mechanisms and Therapeutic Opportunities. Neuron. 2017; 93(5): p. 1015–1034. https://doi.org/10.1016/j.neuron.2017.01.022 PMID: 28279350

65. Clark S.G., Graybeal L.L., Bhattacharjee S., Thomas C., Bhattacharya S., and Cox D.N. Basal autophagy is required for promoting dendritic terminal branching in Drosophila sensory neurons. PloS one. 2018; 13(11): p. e0206743–e0206743. https://doi.org/10.1371/journal.pone.0206743 PMID: 30395636

66. Fujimoto C., Iwasaki S., Urata S., Morishita H., Sakamaki Y., Fujikawa M., et al. Autophagy is essential for hearing in mice. Cell death & disease. 2017; 8(5): p. e2780. https://doi.org/10.1038/cddis.2017.194 PMID: 28492547

67. Magariños M., Pulido S., Aburto M.R., de Iriarte Rodríguez R., and Varela-Nieto I. Autophagy in the Vertebrate Inner Ear. Frontiers in cell and developmental biology. 2017; 5: p. 56. https://doi.org/10.3389/fcell.2017.00056 PMID: 28603711

68. Mariño G., Fernández A.F., Cabrera S., Lundberg Y.W., Cabanillas R., Rodríguez F., et al. Autophagy is essential for mouse sense of balance. The Journal of clinical investigation. 2010; 120(7): p. 2331–2344. https://doi.org/10.1172/JCI42601 PMID: 20577052

69. Hyttiinen J.M.T., Niittylä M., Salminen A., and Kaarniranta K. Maturation of autophagosomes and endosomes: a key role for Rab7. Biochimica et biophysica acta. 2013; 1833(3): p. 503–510. https://doi.org/10.1016/j.bbamcr.2012.11.018 PMID: 23220125

70. Wen H., Zhan L., Chen S., Long L., and Xu E. Rab7 may be a novel therapeutic target for neurologic diseases as a key regulator in autophagy. Journal of neuroscience research. 2017; 95(10): p. 1993–2004. https://doi.org/10.1002/jnr.24034 PMID: 28186670

71. Vanlandingham P.A. and Ceresa B.P. Rab7 regulates late endocytic trafficking downstream of multivesicular body biogenesis and cargo sequestration. The Journal of biological chemistry. 2009; 284(18): p. 12110–12124. https://doi.org/10.1074/jbc.M809277200 PMID: 19265192

72. Wong Y.C., Kim S., Peng W., and Krainc D. Regulation and Function of Mitochondria-Lysosome Membrane Contact Sites in Cellular Homeostasis. Trends in cell biology. 2019; 29(6): p. 500–513. https://doi.org/10.1016/j.tcb.2019.02.004 PMID: 30898429

73. Tan E.H.N. and Tang B.L. Rab7a and Mitophagosome Formation. Cells. 2019; 8(3): p. 224. https://doi.org/10.3390/cells8030224 PMID: 30857122