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The ubiquitin-proteasome system in normal hearing and deafness

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ABSTRACT

Post-translational modifications of proteins are essential for the proper development and function of many tissues and organs, including the inner ear. Ubiquitination is a highly selective post-translational modification that involves the covalent conjugation of ubiquitin to a substrate protein. The most common outcome of protein ubiquitination is degradation by the ubiquitin-proteasome system (UPS), preventing the accumulation of misfolded, damaged, and excess proteins. In addition to proteasomal degradation, ubiquitination regulates other cellular processes, such as transcription, translation, endocytosis, receptor activity, and subcellular localization. All of these processes are essential for cochlear development and maintenance, as several studies link impairment of UPS with altered cochlear development and hearing loss. In this review, we provide insight into the well-oiled machinery of UPS with a focus on its confirmed role in normal hearing and deafness and potential therapeutic strategies to prevent and treat UPS-associated hearing loss.

Abbreviations

ABR	auditory brainstem response
ARHL	age-related hearing loss
Atoh1	protein atonal homolog 1
ATP	adenosine triphosphate
Cx26	connexin-26
D-gal	D-galactose
DPOAE	distortion product otoacoustic emission
DUBs	deubiquitinating enzymes
DSL	delta/Serrate/Lag-2
E1	ubiquitin activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
Fbx2	F-Box protein 2
GWAS	genome-wide association study
HCs	hair cells
HECT	homologous to E6-AP C terminus
IHCs	inner hair cells
JAMM	JAB1/MPN/Mov34 metalloenzyme
MAGUK	membrane-associated guanylate kinase
Mdm2	mouse double minute 2 homolog
MJDs	Machado-Joseph disease proteases
NEDD4-1/2	neural precursor cell expressed developmentally down-regulated protein 4 1/2
NIHL	noise-induced hearing loss
OHCs	outer hair cells
OTUs	ovarian tumor proteases
PCP	planar cell polarity
Pk	prickle1
PSD95	postsynaptic density protein 95
PSMC	proteasome 26S Subunit, ATPase
PSMD	26S proteasome non-ATPase regulatory subunit
RBBP6	retinoblastoma-Binding Protein 6
RBR	RING between RING families
RING	really interesting new gene
RNF8	RING finger protein 8
Rpn	regulatory particle of non-ATPase
ROS	reactive oxygen species
SCs	supporting cells
SCF	SKP1-cullin-F-box
Sem1	suppressor of exocytosis mutation 1
SMAD	homologs of the drosophila protein, mothers against decapentaplegic (MAD)
Smurf1/2	SMAD specific E3 ubiquitin protein ligase ½
Ub	ubiquitin
Ubr3	ub ligase n-recognin 3
UCHs	ubiquitin C-terminal hydrolases
UPS	ubiquitin-proteasome system
USPs	ubiquitin-specific proteases
Wnt	wingless-related integration site
ZO-1	zonula occludens-1

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1. Introduction

The function and stability of proteins can be finely controlled by different post-translational editing processes, the so-called post-translational modifications. This involves modification of certain specific amino acids by a wide variety of groups, including phosphates, lipids, methyls, acetyls, or carbohydrates. One of the most studied modifications is the conjugation with ubiquitin (Ub), a small protein of 76 amino acids whose primary function is to target its substrates to the proteasome, the leading cell machinery of protein degradation. Ubiquitination (or ubiquitylation) is a reversible post-translational modification regulating the stability, interaction, and localization or activity of thousands of proteins. Consequently, it impacts numerous cellular processes, including protein degradation, cell signaling, autophagy, endocytosis, and intracellular protein localization (Hershko and Ciechanover, 1998; Mukhopadhyay and Riezman, 2007). Such modifications of protein function are essential in the regulation of many cochlear functions. Our present understanding of ubiquitination in normal cochlear development and maintenance is the focus of this review. We also describe the consequences of dysfunctional machinery on hearing. Finally, we review the prospects of targeting the ubiquitin-proteasome system (UPS) to reduce the progression of deafness associated with ototoxicity and aging.

2. Ubiquitin Proteasome System

Ubiquitination is a three-step process (Fig. 1). The first step is the ATP-dependent transfer of Ub onto Ub-Activating Enzyme or E1. During the next step, the E1-Ub thiol ester is recognized by multiple Ubiquitin-Conjugating Enzymes or E2s, which conjugate the activated Ub from the E1 enzyme and interacts selectively with the third enzyme of the process (van Wijk and Timmers, 2010). This last step revolves around a Ub-Protein Ligase, or E3 ligase, which controls ubiquitination specificity by recognizing and transferring Ub to the adequate substrate (Metzger et al., 2012). The E3 ligase can be a single protein or a protein complex, such as the Skp1/Cullin1/F-Box protein (SCF) modulator complex. Before ubiquitination, substrates of E3 ligases undergo post-translational modification, including phosphorylation, glycosylation, methylation, or acetylation, to produce a specific sequence known as “degron” that an E3 ligase can recognize (Johnson et al., 1995).

Comparative genome analysis reveals few genes encoding E1, tens of E2 encoding genes, and more than 800 encoding E3s (Semple et al., 2003). E3 enzymes are grouped into three prominent families, namely, the RING (Really Interesting New Gene), HECT (homologous to E6-AP C terminus), and RBR (RING between RING) families (Weber et al., 2019). Depending on the type of E3, the ubiquitin-protein ligation mechanism may vary. The RING E3s are characterized by their RING, or U-box fold catalytic domain, promoting direct Ub transfer from an E2 to a substrate (Deshaies and Joazeiro, 2009). With more than 600 predicted members, the RING E3s constitute the most prominent family of Ub ligases. The HECT (28 members) and RBR (14 members) E3s catalyze substrate ubiquitination in a two-step reaction: in the first step, they accept the activated Ub from the E2 in a trans-thiolation reaction on their catalytic cysteine, and in the second step, the Ub moiety is transferred to the ubiquitination site, which can be a lysine, cysteine, serine or threonine on the target substrate (McDowell and Philpott, 2016).

The cellular Ub level is tightly regulated by deubiquitinating enzymes or deubiquitinases (DUBs) (Fig. 1) (Reyes-Turcu et al., 2009). Indeed, DUBs are essential for Ub recycling and, consequently, modulate the proteasome’s substrate degradation rate (Finley et al., 2016; Livneh et al., 2016). The human genome encodes approximately 90 DUBs. The vast majority of DUBs are cysteine proteases, and these can be organized into four subclasses: ubiquitin-specific proteases (USPs), Ub C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), and Machado-Joseph disease proteases (MJDs). USPs are mainly responsible for cleaving Ub–substrate and Ub–Ub peptide connections, reversing protein ubiquitination. UCHs act precisely to remove small adducts from Ub, allowing preservation of the monomeric pool. Therefore, these DUBs can either antagonize or facilitate substrate presentation to the proteasome. The second class of DUBs (different from the cysteine proteases) presents a Ub protease domain called JAMM (JAB1/MPN/Mov34 metalloenzyme). They are the only metalloproteases known to act as DUBs (Nijman et al., 2005). Deubiquitination is also partially accomplished by one of the 19S regulatory particle subunits, Rpn11 (Verma et al., 2002).

Proteins can be monoubiquitinated on one or several of their lysine residues, respectively leading to mono-or multi-ubiquitination (Sadowski et al., 2012). For a long time, monoubiquitination was only considered a phosphorylation-like modification regulating non-proteolytic processes like histone function, gene expression, receptor endocytosis, and DNA repair (Passmore and Barford, 2004). However, it has recently been demonstrated that monoubiquitination could also act as a degradation signal (Braten et al., 2016; Haglund et al., 2003).

Because Ub has seven lysine residues, the Ub of a monoubiquitinated substrate protein can be the target of additional ubiquitination. This results in the formation of long poly-Ub chains where the C-terminus of each Ub unit is linked to a specific lysine residue of the previous Ub (Peng et al., 2003). Proteomic analyses have shown that all seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) are actively used (Swatek and Komander, 2016; Yau and Rape, 2016). Poly-Ub chains regulate virtually all biological processes, including cell division, transcription, immunity, endocytosis, and protein quality control (Ohtake and Tsuchiya, 2017). Poly-Ub chains linked through Lys48 are the principal signal for targeting substrates to the 26S proteasome, a multisubunit complex of 2.5 MDa involved in the degradation of more than 80% of proteins (Ciechanover, 2015).

The 26S proteasome, constituting 1 and 2% of the cell mass, is divided into a 20S core particle surrounded by two 19S regulatory particles. These 19S particles bind polyubiquitinated proteins and control their entry into the core particle that hydrolyze proteins. Degradation of the tagged protein by the 26S proteasome can be divided into 4 parts. First is the initial ATP-activated binding step, which involves high-affinity Ub receptor sites on the 19S regulatory particles (Peth et al., 2010). In the next step, the conjugate’s association with the 26S becomes tighter to confirm the substrate’s commitment to degradation. In the third step, proteins are deubiquitinated by one or more of the 26S-associated deubiquitinating enzymes (DUBs) (Finley, 2009). Finally, proteins are unfolded and translocated into the 20S core particle for degradation. Unlike traditional proteases, the proteasome does not degrade proteins to amino acids but instead produces a highly heterogeneous mixture of peptides (Kisselev et al., 1999). Those peptides are either used by adaptive cell-mediated immunity or further processed to generate amino acids used for de novo protein synthesis.

The 20S core consists of two beta-rings flanked by two alpha-rings. The 19S lid is split into two compartments: the base that contains six AAA-ATPases (Rpt1-6) that bind directly to the 20S core particle and four non-ATPase subunits (Rpn1, Rpn2, Rpn10, and Rpn13), and the lid itself is composed of nine non-ATPase subunits: Rpn3, Rpn5-Rpn9, Rpn11, Rpn12, and Sem1. Rpn11 (also called PSDM14 or POH1) is a JAMM-type DUB that removes, in an ATP-dependent manner, Ub chains “en block” from substrates committed for degradation.

Besides addressing substrates to proteasomal degradation, ubiquitination can also serve as a signal for autophagy. Until recently,

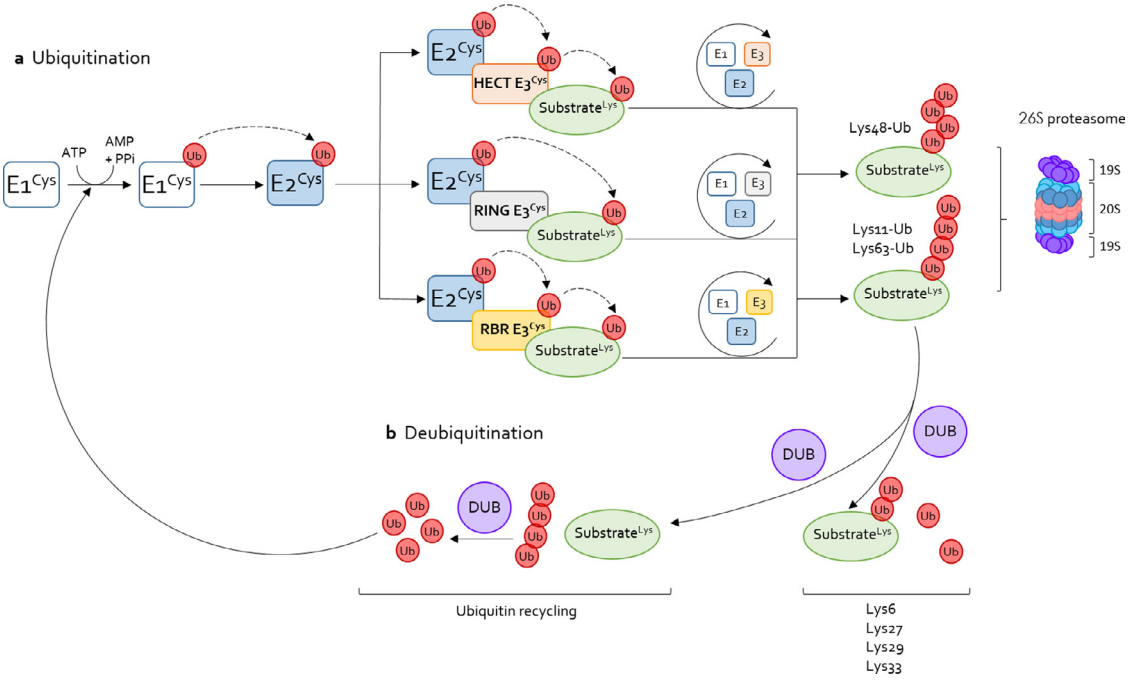

Fig. 1. Protein ubiquitination and Deubiquitination a**.** Ubiquitination is a post-translational modification that results in ubiquitin's covalent attachment to the substrate protein. Ubiquitin (Ub), a 76 amino acid polypeptide, is first attached to a cysteine residue of the E1 ubiquitin-activating enzyme in an ATP-dependent reaction, followed by its transfer to a cysteine residue of the E2 ubiquitin-conjugating enzyme. Finally, in association with a specific E3 Ub-ligase, the E2 transfers the poly-Ub to the substrate's lysine residue. RING E3 ligases catalyze Ub transfer directly from the E2 to the substrate. For HECT and RBR E3 ligases, the activated Ub is first conjugated to a cysteine of the E3 and then transferred to the substrate, which produces different kinds of Ub linkages. The substrate can be modified with a single Ub (monoubiquitination) or polymeric Ub chains such as the Lys6, 11, 27, 29, 33, 48, and 63 linkages. These poly-Ub chains can regulate all biological processes, including cell division, transcription, immunity, endocytosis, and protein quality control b. Deubiquitinating enzymes (DUBs) reverse or edit ubiquitination, a crucial process for modifying and determining the fate of substrates. DUBs change the length and type of poly-Ub chains and disassemble unanchored Ub chains to recycle Ub monomers. PPi, inorganic pyrophosphate. Adapted from Zheng et al. (2016).

UPS and autophagy were thought to be two independent pathways controlling protein degradation (Korolchuk et al., 2010). However, the recent discovery of several common regulators indicates a close relationship between them (Kocaturk and Gozuacik, 2018; Lilienbaum, 2013). Indeed, autophagy and UPS can compensate for each other. UPS inhibition by lactacystin, MG132, or bortezomib results in the upregulation of autophagic activity in cells (Fan et al., 2018; Selimovic et al., 2013; Wu et al., 2008). On the other hand, pharmacological inhibition of autophagy was correlated to UPS activation in cultured human colon cancer cells (Wang et al., 2013). Therefore, both systems intersect and communicate at multiple points to coordinate their proteostasis and organelle homeostasis (Dikic, 2017). In autophagy, the bilayer membrane vesicles formed in the cytoplasm coat the damaged proteins or organelles to form autophagosomes, which can directly be fused to a lysosome in order to be degraded. Selective autophagy is the process by which the poly-Ub chain is recognized by proteins such as p62 to recruit targets into phagosomes. The crucial function of autophagy on the inner ear homeostasis has already been reviewed elsewhere (Fujimoto et al., 2017; Magariños et al., 2017), and therefore, this review focuses on the UPS in the cochlea.

3. Ubiquitination controls key regulators of cochlea development

3.1. Mdm2 regulates several actors of inner ear development

The inner ear is divided into two compartments. The first part encompasses the vestibule and the semicircular canals, which are located dorsally and are involved in the perception of balance and send information to the brain about acceleration and the angular position of the head. The ventral part of the inner ear is the cochlea, a complex structure shaped like a spiral responsible for hearing that contains a single row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) surrounded by supporting cells (SCs).

The inner ear originates from a portion of the ectoderm near the rhombencephalon, which thickens to form the otic placode under the influence of intrinsic factors and surrounding mesodermal signals (Fig. 2). The otic placode undergoes a progressive invagination to form the otocyst or otic vesicle. Because the formation of the otic placode is the first step in a long process leading to the complete development of the inner ear, altering that first keystone is detrimental to the whole process. Mdm2 is a RING-Type E3 Ub ligase found in the mouse inner ear, and one of its substrates, p53, is critical in inner ear development. Mdm2 polyubiquitinates p53, leading to its degradation by the proteasome. While deletion of Mdm2 in the mouse developing inner ear (E8.5) induces abnormally small otic placodes filled with apoptotic cells (Laos et al., 2017), its deletion at a later stage (P6-P7) in SCs induces their death, leading to the degeneration of OHCs. These data show that p53 upregulation due to Mdm2 deletion induces the death of both SCs and hair cells (HCs). The p53 transcription factor is well-known for its role in guarding genomic integrity upon DNA damage and oncogene activation (Kruiswijk et al., 2015; Ozaki and Nakagawara, 2011). This protein is also critical during embryonic development. Indeed, if p53 levels are too low (e.g., in p53 knockout mice) or artificially stabilized, numerous developmental defects appear (Armstrong et al., 1995). However, p53 inactivation in the mouse inner ear does not lead to phenotypic changes during development and adulthood (Laos et al., 2017). Compensation by other members of the p53 family, such as p63 and p73, cannot be excluded (Kastenhuber and Lowe, 2017). In addition to promoting p53 degradation, Mdm2 might also suppress

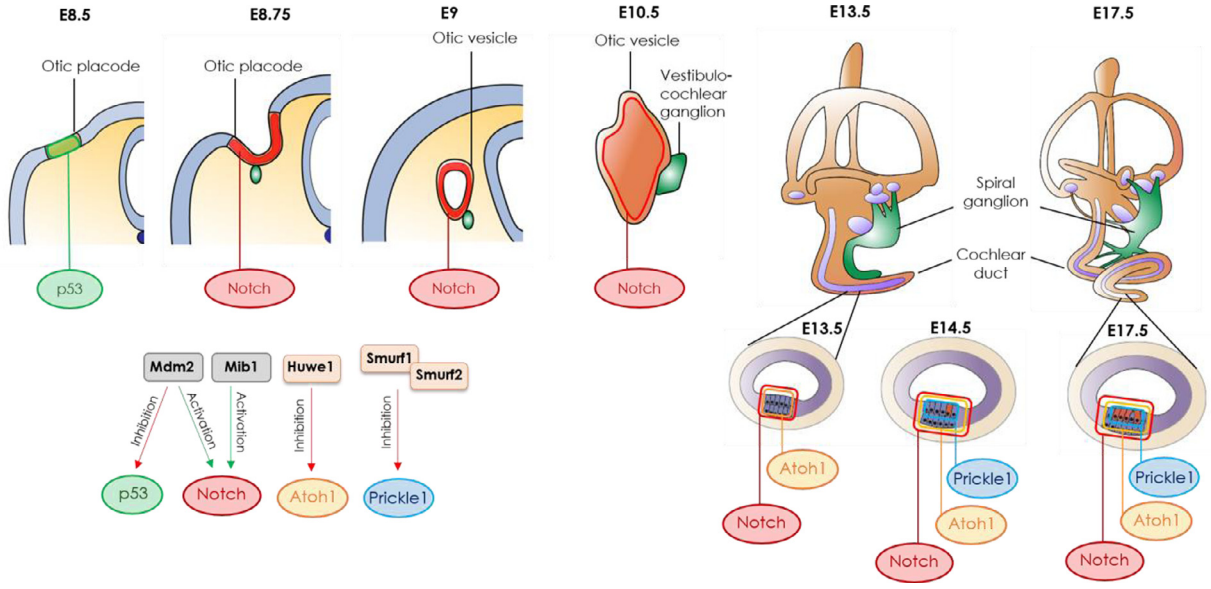

Fig. 2. Ubiquitination controls key regulators of cochlea development. At E8.5, the otic placode starts its invagination and expresses p53, a transcription factor regulated by the E3 ligase Mdm2. From E8.75 onwards, the developing inner ear is under the influence of Notch, an essential cell fate regulator modulated by the Mdm2 and Mib1 E3 ligase. From E13.5 onwards, the sensory progenitors differentiate into HCs under the impulse of the transcription factor Atoh1, ubiquitinated by Huwe1. The synchronized orientation of stereocilia in the cochlea starts at E14.5 in a base-to-apex gradient. Prickle1, a core planar cell polarity component, is regulated by the Smurfs E3 ligases. Between E15.5 and E17.5, the cochlea continues to grow, and the differentiation of IHCs and then OHCs progresses to the apical turn.

p53 transcriptional activity in several ways. Mdm2 can bind the p53 transactivation domain, thus interfering with the recruitment of the basal transcription machinery and essential coactivator(s) (Thut et al., 1997). Mdm2 was also reported to promote NEDD8 conjugation to p53, a modification that inhibits its transcriptional activity (Xirodimas et al., 2004). Finally, Mdm2 induces monoubiquitination to histone surrounding the p53-DNA binding elements resulting in transcriptional repression (Minsky and Oren, 2004).

Mdm2 is also an essential regulator of the Notch signaling pathway. Notch receptors are transmembrane proteins activated by transmembrane ligands belonging to the Delta/Serrate/Lag-2 (DSL) family. After interaction with a ligand, the Notch intracellular domain (NICD) is cleaved and translocates to the nucleus, where it forms a transcriptional activating complex with other proteins to induce the expression of target genes. Notch activity can lead to two distinct signaling modes in the developing inner ear: lateral induction and lateral inhibition. The first is essential in the early development stages of the inner ear (Hartman et al., 2010). Starting at E9.5 in mice, when the pool of sensory-competent otic placode cells must be maintained and expanded, a positive feedback loop links Notch and Jag1 ligand for lateral induction signaling. Later during development, once prosensory cells exit the cell cycle (i.e., at E12.5 in mice), their differentiation into HCs or SCs is controlled by Notch lateral inhibition through multiple DSL ligands like Dll1, Jag2, or Dll3 (Daudet and Żak, 2020). Indeed, nascent HCs, by expressing DSL ligands, inhibit their neighbors from becoming HCs, forcing them to become SCs.

Mdm2 can positively regulate the Notch pathway directly and indirectly. Direct regulation involves non-degradative ubiquitination of the NICD, leading to activation of the Notch signaling pathway (Pettersson et al., 2013). The indirect pathway occurs by degradative ubiquitination of NUMB (Yogosawa et al., 2003). NUMB is a central negative regulator of the Notch pathway (McGill and McGlade, 2003), whose primary function is to antagonize Notch signaling by inducing its selective endocytosis and degradation (Couturier et al., 2012). Furthermore, NUMB can enter a tricomplex with p53 and Mdm2, thereby preventing ubiquitination and degradation of p53 (Colaluca et al., 2008).

The role of Mdm2 on the Notch pathway in cochlear development is not yet described. However, Mdm2 deletion in the early or late-developing cochlea would likely have significant consequences on the lateral induction and lateral inhibition pathways. Indeed, without Mdm2 to activate Notch and lateral induction, there would be fewer sensory progenitors, resulting in a reduced number of HCs and SCs. Without functional lateral inhibition, there would be an overproduction of HCs at the expense of SCs.

3.2. Mind bomb 1 ubiquitinates key regulators of the Notch pathway

Another E3 ligase known to regulate the Notch pathway is Mind bomb 1 (Mib1) (Itoh et al., 2003). Mib1 is a RING-type E3 Ligase that ubiquitinates Delta's intracellular domain, promoting its internalization and degradation. Because Notch signaling initiates upon transcellular engagement of a Notch ligand with a Notch receptor, it relies on ubiquitin-dependent internalization of the ligand (Guo et al., 2016). In the Mib1 loss-of-function mutant zebrafish, the lateral wall sensory patches consist solely of HCs produced in significant excess and prematurely. These findings are strong evidence that lateral inhibition mediated by Mib1-Delta-Notch signaling controls the pattern of HCs differentiation in the inner ear (Haddon et al., 1998). In the mouse, Mib1 has been identified as an essential regulator of DSL ligand activation, but its effect on cochlear development has not been studied since KO mice die at E11.5 (Koo et al., 2005).

3.3. Control of Atoh1 levels by Huwe1

From E13.5, sensory progenitors differentiate into HCs under the impulse of the basic helix-loop-helix (bHLH) transcription factor Atoh1 that activates HC-specific genes such as Pou4f3 (Chonko et al., 2013). In parallel, following Notch pathway activation in adjacent cells, expression of Hes (Hairy and enhancer of split) transcription factors is induced to turn off Atoh1 in future SCs (Kelley, 2006). Post-translational regulation of Atoh1 is performed by the HECT E3 ligase Huwe1 that targets Atoh1 for degradation via polyubiquitination (Cheng et al., 2016; Rotin and Kumar, 2009).

Identification of Huwe1 as the E3 ligase responsible for Atoh1 regulation opens new perspectives regarding HC regeneration. Unlike non-mammalian vertebrates, mammals suffer permanent hearing loss after HC damage, having lost the capacity to spontaneously turn on Atoh1 and generate new HCs (Brigande and Heller, 2009; Cafaro et al., 2007). Forced expression of Atoh1 has been shown to induce the production of new HCs in embryonic, post-natal and even adult stages in rodents (Izumikawa et al., 2005). Consequently, this makes Atoh1 an exciting target for HC regeneration strategies. Preclinical *in vivo* work successfully proves that transdifferentiation of SCs into HCs is possible (Shibata and Raphael, 2010). Therefore, one strategy to regenerate lost HCs could be the inhibition of Huwe1 to stabilize and upregulate Atoh1.

In the murine developing cochlea, depending on the cell type affected by Huwe1 knockdown, two different phenotypes are observed (Cheng et al., 2016). Disruption of Huwe1 in progenitor cells at E16 generates a single extra row of IHCs in the inner pillar cell region. In contrast, deletion of Huwe1 at P1 induces the production of new HCs that are normally innervated with typical auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) thresholds at P30. In humans, HUWE1 copy-number gains have been linked to hearing impairment in one patient (Froyen et al., 2012), while missense variants in the HUWE1 gene have been found in four patients who have sensorineural hearing loss (Moortgat et al., 2018). The identified variants were located in five out of six different functional domains of the protein, with an overrepresentation (7/16) in the catalytic HECT domain of HUWE1. Whether such mutations in the ubiquitin ligase domain of HUWE1 are responsible for hearing loss through perturbations in the post-translational regulation of Atoh1 or other genes involved in HC development remains to be investigated.

3.4. Smurf1 and Smurf2 regulate the core PCP pathway via the non-canonical Wnt pathway

Each HC has several rows of apical protrusions formed of actin-rich stereocilia in a staircase-like arrangement. These V-shaped stereocilia bundles are essential for mechanotransduction. The coordinated orientation of stereocilia in the cochlea - starting at E14.5 in mice - following a base-to-apex gradient manifests a specific form of tissue polarity, known as planar cell polarity (PCP) (Ezan et al., 2013; Tarchini et al., 2013). Important factors in this hair bundle development are the two HECT E3 Ub ligases, Smad ubiquitination regulatory factors 1 and 2 (Smurf 1 and 2). The loss of three Smurf alleles (Smurf1$^{-/-}$; Smurf2$^{+/-}$ or Smurf1$^{+/-}$; Smurf2$^{-/-}$) in E18.5 cochleae perturbs PCP in the organ of Corti (Narimatsu et al., 2009). Such PCP defects are illustrated by the deviation of almost all OHC hair bundles. Moreover, the loss of three Smurf alleles during cochlear development induces abnormal cell shape and cell-cell contacts, resulting in the appearance of pentagonal or heptagonal-shaped cells (Narimatsu et al., 2009). Smurfs form a complex with Par6 and Dishevelled 2 (Dvl2), two components of the core PCP pathway. This signaling complex targets Prickle1 (Pk1) for localized degradative ubiquitination. Pk1 is a core PCP component with an asymmetric distribution in HCs and establishes cell polarity via cell-cell communication (Axelrod, 2009). Localized targeting of Pk1 for degradation by Smurf1 and 2 is an effective mechanism to establish and maintain its asymmetric distribution during PCP signaling.

4. Ubiquitin modifiers in the postnatal inner ear

Maintaining the proteome's integrity is vital for all cell types including in the cochlea. Because UPS is the central mechanism regulating protein turnover, cells are highly vulnerable to damage to this system. Therefore, any modification of the ubiquitination/deubiquitination machinery would impair protein homeostasis regulation in major cochlear cell types (HCs, spiral ganglion neurons). Several studies have also shown that the UPS machinery is an essential regulator of synaptic plasticity (Lottes and Cox, 2020) and is activated in the cochlea following excessive noise exposure (Jongkamonwiwat et al., 2020).

4.1. E3 ligases

The first E3 ligase associated with deafness was Fbx2 (F-Box protein 2), an F-box protein, and SCF Ub ligase complex member. Fbx2 is one of the earliest genes detected in otic development, as well as one of the most abundant proteins in the cochlea (Thalmann et al., 1980). As a member of the SCF complex, Fbx2 differs from the majority of other E3 ligases as its role is to bind N-glycosylated and not phosphorylated substrates through its F-box-associated domain (Yoshida et al., 2002). Interestingly, Fbx2 is expressed robustly from early otic development, but the knockout mouse line does not display a detectable phenotype until months later. Indeed, in Fbx2$^{-/-}$ mice, hearing loss starts at two months of age (Nelson et al., 2007). At first glance, we might attribute the cause of deafness to E3's substrates, which accumulate with maladaptive effects. One possible substrate could be Connexin 26 (Cx26), a gap junction protein highly expressed in SCs of the cochlea. However, while Cx26 was shown to precipitate with Fbx2 (Henzl et al., 2004), a direct interaction was never demonstrated between these two proteins. Other candidate substrates are glutamate receptors and BACE1. Indeed, there is evidence that Fbx2 ubiquitinates both in the brain (Atkin et al., 2015; Gong et al., 2010), and both are important for cochlear function (Dierich et al., 2019; Takago and Oshima-Takago, 2018). It would be interesting to identify which of these substrates are regulated by Fbx2 in the cochlea, as it could help decipher the mechanism of cochlear cell death in the absence of Fbx2.

Two other RING E3 ligases have been associated with hearing, namely RING finger protein 8 (RNF8) and Ub ligase n-recognin 3 (Ubr3). Deletion of RNF8 in mice accelerates biomarkers of aging of cells and increases DNA damage in the cochlea, suggesting a role of RNF8 in aging cochlea apoptosis (Li et al., 2019). Further studies investigating specific substrates of RNF8 in the cochlea might open new perspectives regarding age-related hearing loss (ARHL) treatment. Ubr3 is specifically present in the spiral ganglion and the HCs (Tasaki et al., 2007). It has been recently shown to have a complex function in audition through its E3 ligase activity (Li et al., 2016). Ubr3 regulates Cul1-SCF E3 ligase, and this complex regulates an unknown E3 ligase that directly ubiquitinates Myosin II (MyoII). Taken together, Ubr3 negatively regulates the mono-ubiquitination of MyoII and modulates Myosin II-Myosin VIIa interactions that are both essential for HCs mechanotransduction.

Further to its role during cochlear development, Mdm2 is also essential in the adult cochlea. Indeed, it is known to ubiquitinate and degrade PSD95, a membrane-associated guanylate kinase (MAGUK) scaffolding protein associated with glutamatergic synapses (Colledge et al., 2003). Deletion of Mdm2 might lead to the accumulation of PSD95, and it has been shown that level of PSD95 correlates with synaptic strength and maturation (El-Husseini et al., 2000; Elias et al., 2006). Therefore, it would be interesting to study the effect of Mdm2 deletion on PSD95 and synaptic function in the cochlea.

In addition to RING E3 ligases, an increasing number of HECT E3 ligase genes are associated with deafness. Indeed, NEDD4-1 and NEDD4-2 are known to be highly expressed in the inner ear of rats and guinea pigs (Zhong et al., 2014; Zhong and Liu, 2009) , and NEDD4-1 has been identified as a new candidate gene involved in adult-onset progressive hearing loss (Lewis et al., 2018). Meanwhile, NEDD4-2 is one of the 67 candidate hearing loss

genes identified in a large-scale hearing loss screen in the mouse (Bowl et al., 2017). Furthermore, variants with missense mutations in the catalytic domain of NEDD4-2 have been associated with hearing impairment in two patients aged four months and four years (Broix et al., 2016). The exact role and substrates of these two E3 ligases remain to be determined in the cochlea.

4.2. Deubiquitinases

Usp53 is located at the apical portion of polarized epithelial cells, including HCs and SCs in the organ of Corti (Kazmierczak et al., 2015). It forms a complex with ZO-1 and ZO-2 but does not directly interact with occludin or claudins in tight junctions. A mouse model carrying a point mutation in the Usp53 catalytic domain, the so-called "mambo" mouse line, exhibits rapidly progressive hearing loss. The presence of this mutation induces early degeneration of OHCs (starting at P8) and SCs such as Deiters' cells, while IHCs are normal. Other findings include a decline of the endocochlear potential and increased susceptibility of heterozygous mambo mice to noise-induced hearing loss (NIHL). In humans, a single novel homozygous truncating variant in USP53 (c.951delT) has been reported to cause late-onset bilateral hearing loss in two patients (aged 9 and 14 years old) (Maddirevula et al., 2019). two other mutations predicted to lead to mRNA decay (p.Arg520Ter and p.His132Arg) were found in one patient who was deaf at birth (Zhang et al., 2020). Although the precise mechanism by which Usp53 regulates cochlear homeostasis remains to be elucidated, it is clear that the inability to shift the dynamic balance between ubiquitination and deubiquitination in the inner ear leads to early-onset hearing loss.

Another DUB linked to deafness is Ub C-terminal hydrolase L1 (Uchl1). Uchl1 cleaves the Ub C-terminal from the poly-Ub chain before proteasomal activity. Thus, Uchl1 helps preserve the free monomeric level of Ub for reuse and maintaining proteasomal activity. Mutation of Uchl1 has been discovered in many age-related neurodegenerative diseases, e.g., Parkinson's disease and Alzheimer's disease (Choi et al., 2004; Gong and Leznik, 2007). Indeed, the absence of Uchl1 induces protein accumulation, impaired proteasomal activity, and aging in the nervous system (Costes et al., 2014; Coulombe et al., 2014; Saigoh et al., 1999). Regarding its link with the inner ear, Uchl1 is expressed in the cochlear duct starting at E14.5 (Gene paint atlas, set ID: EH2687) and in the adult cochlea, predominantly in spiral ganglion neurons (Kim et al., 2019). Following gentamicin ototoxic treatment *in vitro* or *in vivo*, Uclh1 is downregulated in cochlear cells, mostly spiral ganglion neurons. Deficiency in Uchl1 can cause protein accumulation and a blockade of the autophagic flux by impaired autophagosome clearance leading to an exacerbated gentamicin-induced auditory cell death (Kim et al., 2019). These results highlight once again the critical role of DUBs in audition.

5. Proteasome alteration in hearing loss

Being at the core of the UPS process, inhibition of the proteasome is detrimental and would lead to severe defects in the mature cochlea (Engelhardt et al., 2005). In support of this, it has been shown that multiple myeloma treatment using proteasome inhibitors (the so-called "omib" molecules, with the first-in-class bortezomib) may induce neurosensory deafness (Chim and Wong, 2008; Engelhardt et al., 2005). These drugs inhibit both 20S and 26S proteasomes by targeting the core proteolytic catalytic activity of the 20S subunits (Goldberg, 2012; Kisselev et al., 2012, 2006). Although the precise mechanism by which proteasome inhibitors induce ototoxicity requires further studies, peroxisome damage caused by proteasome inhibition might be related to ototoxicity through reactive oxygen species (ROS) accumulation (Lee et al., 2015).

More recently, studies relate the identification of pathogenic variants in proteasome genes associated with neurosensory deafness. One report identified a unique pathogenic variant in the PSMC3 gene encoding one of the ATPase subunits of the 19S proteasome complex in patients who have severe sensory-neural hearing loss (Kröll-Hermi et al., 2020). This protein is involved in substrate recognition, unfolding, and translocation to the 20S. The patients carrying that PSMC3 mutation showed deafness within the early months of life, with auditory brainstem response indicating profound deafness (no response at 110 Db). It is also worth noting that temporal bone CT scan analysis of one patient revealed lateral semicircular canal malformations with the absence of the bony island in the right ear and a small bony island in the left ear. An increased level of ubiquitinated proteins in patient's fibroblasts was observed suggesting that PSMC3 mutation impairs proteasome functions and generate proteostatic stress. Also, the generation of PSMC3 loss of function in zebrafish reproduced the human phenotype with the psmc3 morphants displaying smaller ear and semicircular canal anomalies. These results confirm a major role of PSMC3, and more generally the proteasome, in hearing.

Another report identified three *de novo* mutations in PSMD12 (also called Rpn5) in patients suffering from hearing impairment (Küry et al., 2017). PSMD12 is one of the nine subunits of the 19S lid involved in substrate recognition, deubiquitination, and scaffolding (Sharon et al., 2006). These functions are thought to be substantially altered in patients with PSMD12 mutations and may contribute to their deafness. It would be interesting to study how proteome remodeling caused by loss of function of PSMD12 can lead to clinical features such as deafness.

The role of the proteasome within the context of NIHL remains poorly characterized (Mateo Sánchez et al., 2016). However, a recent study reported increased proteasome activation after noise exposure (Jongkamonwiwat et al., 2020). It is believed that auditory overstimulation acutely unbalances the cochlear proteome by driving the accumulation of hundreds of proteins. This disequilibrium activates the proteostasis network by elevating protein chaperones and proteasome levels in multiple inner ear cell types, increasing protein ubiquitination. Given the importance of ubiquitination for NIHL, future investigations into the role of ubiquitinated proteins following noise exposure could be of great interest in developing new strategies for cochlear regeneration following NIHL.

5.1. UPS impairment in age-related hearing loss

Alterations in the UPS have been documented in aging-associated diseases, including ARHL (presbycusis). The exact mechanisms causing ARHL are unknown, but the accumulation of ubiquitinated proteins has been demonstrated (Wang et al., 2015). Age-related changes affect the entire auditory system, including the peripheral and central auditory systems (Youn et al., 2020).

It has been shown that D-galactose (D-gal) administration induces oxidative stress and senescence markers such as mitochondrial DNA-4834 deletion and senescence-associated b-galactosidase (SA-B-gal) in the rat cochlea and central auditory system (Du et al., 2014; Sun et al., 2015; Zhao et al., 2013). When D-gal is used to induce presbycusis in neurons of the auditory cortex, Uchl1 (a DUB already known to be involved in gentamicin-induced auditory cell death, see Section 5.2) is upregulated, increasing proteasomal activity to provide a compensatory action to the mild oxidative stress. However, the continuous production of ROS decreases Uchl1 and proteasomal activity (Zhang et al., 2017). These results indicate that Uchl1 may play an essential role in maintaining the mono-Ub pool to activate the UPS during auditory cortex aging. Fu-

Table 1
Major roles of UPS actors in cochlear development and function, otoprotection and involving mouse or human deafness and presbycusis.

Actor	Type	Substrate(s)	Associated role/Disease	References
Development				
Mdm2	RING E3 ligase	p53	(E8.5) Otic vesicle development; (P6-7) Supporting cells survival	Laos et al. (2017)
		NUMB/Notch	Hair cell development and differentiation ?	N/A
Mind bomb 1	RING E3 ligase	Notch	Hair cell differentiation	Haddon et al. (1998)
Huwe1	HECT E3 ligase	Atoh1	Hair cell differentiation	Cheng et al. (2016)
Smurf1/2	HECT E3 ligase	Prickle 1	Cell polarity and planar cell polarity	Narimatsu et al. (2009)
Cochlear function				
Fbx2	RING E3 ligase	Connexin 26	Deafness	Henzl et al. (2004), Nelson et al. (2007)
RNF8	RING E3 ligase	N/A	Age-related hearing loss	Li et al. (2019)
Ubr3	RING E3 ligase	MyoII	Deafness	Li et al. (2016)
NEDD4-1/2	HECT E3 ligase	N/A	Deafness	Bowl et al. (2017), Lewis et al. (2018)
USP53	USP deubiquitinase	N/A	Deafness	Kazmierczak et al. (2015)
UCHL1	UCH deubiquitinase	N/A	Susceptibility to Gentamicin-induced auditory cell death and otoprotection	Kim et al. (2019)
PSMC3	19S Proteasome subunit	N/A	Deafness	Kröll-Hermi et al. (2020)
PSMD12	19S Proteasome subunit	N/A	Deafness	Küry et al. (2017)
PSMD11	19S Proteasome subunit	N/A	Age-related hearing loss	Wu et al. (2020)
RAD6B	E2 conjugating enzyme	N/A	Age-related hearing loss	Ma et al. (2020)
RBBP6	RING E3 ligase	N/A	Age-related hearing loss	Yokoyama et al. (2012)

ture studies should investigate whether there is a protective effect of overexpression of Uchl1 in a presbycusis model.

The same D-Gal-induced-presbycusis model revealed that proteasome degradation activity was impaired by ROS and the accumulation of toxic proteins (Wu et al., 2020). PSMD11 (also called Rpn6) is a subunit of the 19S proteasome regulatory particle whose levels are associated with proteasome activity in D-Gal induced groups. Indeed, PSMD11 levels are decreased in ARHL, and its knockdown accelerated ROS accumulation. On the contrary, PSMD11 overexpression decreased ROS damage after D-Gal treatment, suggesting a protective role of the 19S proteasome subunit in ARHL, opening avenues towards novel ARHL treatment.

Another report linked accelerated ARHL with RAD6B, an E2 Ub-conjugated enzyme. RAD6B is known to promote DNA damage repair (DDR), and its absence in mice leads to significant degenerative changes in the aging cochlea (Ma et al., 2020). It would be interesting to study whether the consequences of RAD6B deletion in the cochlea are specific to its role in DDR or if and how it might also affect the UPS pathway.

Finally, a Genome-Wide Association Study (GWAS) study has allowed the discovery of RBBP6 (Retinoblastoma-Binding Protein 6), a RING-type E3 ligase, as associated with adult-onset deafness in Border Collies dogs (Yokoyama et al., 2012). Although RBBP6 has been well-studied in colorectal cancer induction of epithelial-mesenchymal transition (Xiao et al., 2019), nothing is known about its expression and specific role in the inner ear.

With age, the ability of the protein quality control machinery to preserve proteostasis is gradually compromised (Kaushik and Cuervo, 2015), and aging systems have previously been used to highlight the link between proteome integrity and hearing loss (Mikuriya et al., 2008; Wang et al., 2015). Moreover, a recent report raised the possibility that genes encoding proteostasis regulators could also be implicated in deafness with developmental origins (Freeman et al., 2019). Future studies will likely shed light on the extent to which other players are involved in early-onset hearing loss and ARHL.

6. Conclusion

UPS is an essential component of the global architecture that sustains protein homeostasis. The importance of this system is underscored by the fact that perturbation of proteostasis is central to diverse human diseases (Balch et al., 2008; Labbadia and Morimoto, 2015).

There is abundant evidence showing the importance of UPS during and after the inner ear development (Table 1). As most cells in the inner ear are postmitotic and long-lived cells, regulation of protein homeostasis by ubiquitination and deubiquitination is essential in acquiring and preserving hearing function. Several studies have also shown that the UPS machinery is an essential regulator of synaptic plasticity in the brain (Cajigas et al., 2010). Whether this is the case in the cochlea remains to be demonstrated.

Declaration of Competing Interest

none

CRediT authorship contribution statement

Ronald Pouyo: Conceptualization, Writing – original draft, Writing – review & editing. **Keshi Chung:** Writing – review & editing. **Laurence Delacroix:** Conceptualization, Writing – review & editing. **Brigitte Malgrange:** Conceptualization, Writing – review & editing.

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