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# Otoferlin as a multirole $\text{Ca}^{2+}$ signaling protein: from inner ear synapses to cancer pathways

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Humans have six members of the ferlin protein family: dysferlin, myoferlin, otoferlin, fer1L4, fer1L5, and fer1L6. These proteins share common features such as multiple  $\text{Ca}^{2+}$ -binding C2 domains, FerA domains, and membrane anchoring through their single C-terminal transmembrane domain, and are believed to play a key role in calcium-triggered membrane fusion and vesicle trafficking. Otoferlin plays a crucial role in hearing and vestibular function. In this review, we will discuss how we see otoferlin working as a  $\text{Ca}^{2+}$ -dependent mechanical sensor regulating synaptic vesicle fusion at the hair cell ribbon synapses. Although otoferlin is also present in the central nervous system, particularly in the cortex and amygdala, its role in brain tissues remains unknown. Mutations in the OTOF gene cause one of the most frequent genetic forms of congenital deafness, DFNB9. These mutations produce severe to profound hearing loss due to a defect in synaptic excitatory glutamatergic transmission between the inner hair cells and the nerve fibers of the auditory nerve. Gene therapy protocols that allow normal rescue expression of otoferlin in hair cells have just started and are currently in pre-clinical phase. In parallel, studies have linked ferlins to cancer through their effect on cell signaling and development, allowing tumors to form and cancer cells to adapt to a hostile environment. Modulation by mechanical forces and  $\text{Ca}^{2+}$  signaling are key determinants of the metastatic process. Although ferlins importance in cancer has not been extensively studied, data show that otoferlin expression is significantly associated with survival in specific cancer types, including clear cell and papillary cell renal carcinoma, and urothelial bladder cancer. These findings indicate a role for otoferlin in the carcinogenesis of these tumors, which requires further investigation to confirm and understand its exact role, particularly as it varies by tumor site. Targeting this protein may lead to new cancer therapies.

## KEYWORDS

ferlins,  $\text{Ca}^{2+}$  sensor, membrane fusion, auditory synaptopathy, deafness, brain synapses, dysferlinopathy, cancer

## 1. Introduction

Interest in otoferlin began with the discovery of mutations in the OTOF gene that were identified as responsible for recessive profound deafness in humans (Yasunaga et al., 1999, 2000). This deafness is called DFNB9 (for autosomal recessive deafness 9). It represents about 2% of non-syndromic prelingual deafness, i.e., without any other associated health problem,

and is the most frequent cause of auditory neuropathy spectrum disorder (ANSO) (Iwasa et al., 2019). To date, about 220 mutations leading to DFNB9 deafness have been identified (Azaiez et al., 2021). Remarkably, some otoferlin mutations, notably in the C2F domain, can lead to exacerbated deafness when body temperature rises during a fever (Varga et al., 2006; Marlin et al., 2010; Matsunaga et al., 2012; Strenzke et al., 2016). This peculiar property underlines the importance of a precise functional 3D architecture of the multi-C2 protein, which is known to interact at the presynaptic active zone of the sensory hair cells with SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins such as SNAP25 (Roux et al., 2006; Ramakrishnan et al., 2014; Hams et al., 2017; Calvet et al., 2022) and with  $\text{Ca}_v1.3$   $\text{Ca}^{2+}$  channels (Ramakrishnan et al., 2009; Vincent et al., 2017, 2018; **Table 1** and **Figures 1, 2**).

DFNB9 is caused by a defect in synaptic neurotransmission from auditory inner hair cells (IHCs) to their contacting spiral ganglion afferent fibers (Roux et al., 2006; Beurg et al., 2010; Michalski et al., 2017). The sound-induced electrical analog signal of the hair cells, also called cochlear microphonic, is incorrectly transduced into nerve impulses at the auditory nerve fibers. We recall that one of the morphological characteristics of the afferent IHC synapses is the presence of a presynaptic ribbon, an electron-dense presynaptic structure 300–350 nm wide (**Figures 1, 3**). Each of the 15–20 presynaptic ribbons per IHC are facing a single postsynaptic auditory nerve structure bearing AMPA (glutamate) ionotropic receptors composed of GluA2, GluA3, and GluA4 subunits (Rutherford et al., 2023). This presynaptic ribbon, composed of RIBEYE proteins arranged in staircase pattern, allows the attachment of numerous synaptic vesicles at the membrane active zone of release. This ribbon structure is essential for spike timing precision of the auditory nerve fibers (Wittig and Parsons, 2008; Becker et al., 2018; Jean et al., 2018).

Otoferlin-induced synaptopathy is due to a defective  $\text{Ca}^{2+}$ -evoked exocytosis of synaptic vesicles containing glutamate, the main cochlear excitatory neurotransmitter, at the IHC ribbon synapses (**Figure 3**). Like in most neurosecretory cells, vesicle trafficking and synaptic exocytosis in IHCs are thought to involve interactions of SNARE complex proteins such as SNAP25 (Calvet et al., 2022). Synaptotagmins (Syts), a large family of transmembrane proteins containing tandem  $\text{Ca}^{2+}$ -binding C2-domains, confer  $\text{Ca}^{2+}$  sensitivity to SNARE-dependent vesicle fusion in the CNS (central nervous system) (Chapman, 2008). However, the implication of Syts in hair cell synaptic exocytosis has been shown to be limited to immature cochlear developmental stages when hair cells fire action potentials (Beurg et al., 2010). At post-hearing mature stages, when hair cells display gradual depolarization upon sound stimulation, otoferlin becomes the major  $\text{Ca}^{2+}$  sensor triggering synaptic vesicle exocytosis.

DFNB9 is classified as a neuropathy and, more specifically, as an auditory cochlear synaptopathy (Moser and Starr, 2016). In addition to the cochlea, otoferlin expression has also been shown in the brain with unknown function (Schug et al., 2006) and, in the peripheral vestibular organs where hair cell  $\text{Ca}^{2+}$ -dependent fast synaptic vesicle exocytosis is also impaired (Dulon et al., 2009). In DFNB9 patients, it is important to note that the active electromechanical sound amplification function by the outer hair cells (OHCs) is normal, as indicated by measurements of the distortion products of otoacoustic emissions (DPOAEs). The

DFNB9 auditory cochlear synaptopathy is currently overlooked in clinics when the systematic hearing screening performed at birth relies only on DPOAE measurements. Since delays in the management of sensory pathology reduce the quality of language development, it is becoming essential to screen for deafness with automated auditory brainstem recordings (ABRs). For DFNB9 hearing loss, hearing aids or cochlear implantation are the only effective treatments currently available. Future clinical gene therapies are on the way, as demonstrated by successful rescue viral gene therapies in otoferlin-deficient mouse models (Al-Moyed et al., 2019; Akil, 2020; Rankovic et al., 2020). However, in these studies, for unknown reasons, there was only a partial rescue of ABR wave I amplitude (the neural response associated with the electrical activity of the IHC ribbon synapses), suggesting a non-restored loss of some ribbon synapses. This incomplete rescuing is likely due to the late developmental stage (postnatal days P1 to P3 in mice) at which AAV treatment was performed in these studies. Indeed, otoferlin seems essential for normal early prenatal development and the maintenance of ribbon synapses (Stalman et al., 2021).

## 2. Otoferlin structure and isoforms

Otoferlin is a large 1997 amino acid (aa) protein, that includes a single C-terminal transmembrane domain (TMD) anchoring the protein to the vesicular membrane and six C2 domains (A–F) oriented toward the IHC cytosol (**Figures 3, 4**). The C2 domains are structures composed of eight antiparallel  $\beta$ -sheets and negatively charged top loops due to the presence of five aspartate residues, constituting the putative binding site for  $\text{Ca}^{2+}$  ions (Sutton et al., 1995; Xue et al., 2008). The C2-A domain is the only C2 domain predicted to be unable to bind  $\text{Ca}^{2+}$  due to a shorter top loop, thus lacking aspartates coordinating calcium binding (Helfmann et al., 2011). The main function of C2 domains is to target membrane surfaces following  $\text{Ca}^{2+}$ -binding. C2 domains bind to their target membranes by using a combination of hydrophobic and electrostatic interactions, preferentially with phosphatidylinositol 4,5-bisphosphate (PIP2) or phosphatidylserine (PS) (Corbalan-Garcia and Gómez-Fernández, 2014). Otoferlin also has a FerA domain, a four-helix bundle fold with its own  $\text{Ca}^{2+}$ -dependent phospholipid-binding activity, suggesting that the interaction of this domain with the membrane is enhanced by the presence of  $\text{Ca}^{2+}$  (Harsini et al., 2018).

Otoferlin is produced mainly in the brain and inner ear (cochlea and vestibule), and in smaller amounts in other organs: the heart, liver, pancreas, kidney, and skeletal muscle (Yasunaga et al., 2000). Otoferlin appears as a globular protein when using the AlphaFold protein structure prediction software (Jumper et al., 2021; **Figure 4**). In humans, a long and short isoform of otoferlin have been identified (Yasunaga et al., 1999, 2000), with the long one comprising all six C2 domains and a C-terminal *trans*-membrane domain (TMD) (1997 aa), while the short one expresses only the last three C-terminal C2 domains (C2-DEF) with the TMD (1230 aa). The role of these two isoforms remains unknown. Of note, the short otoferlin isoform is not present in mice, whereas the human long isoform has 98% sequence similarity with murine otoferlin.

The long isoform of otoferlin is encoded by 48 exons, of which exons 6 and 31 can have alternative splicing. Exon 47

TABLE 1 The otoferlin interactome.

Identified interacting domains of otoferlin										
Interacting factors	Putative function	Otoferlin	C2A	C2B	C2C	C2D	C2E	C2F	TMD	References
Ca <sup>2+</sup>	Bind to Asp residues in C2 domains	✓		✓	✓	✓	✓	✓		Johnson and Chapman, 2010; Michalski et al., 2017
Syntaxin 1A	SNARE complex	✓	✓	✓		✓	✓	✓		Ramakrishnan et al., 2009, 2014
SNAP-25	SNARE complex	✓						✓		Ramakrishnan et al., 2014
Ca <sub>v</sub> 1.3	L-type Ca <sup>2+</sup> channel	✓	✓	✓		✓		✓		Ramakrishnan et al., 2014; Hams et al., 2017
CaMKIIδ	CaM kinase	✓						✓		Meese et al., 2017
Endophilin-A1	Endocytic adaptors	✓								Kroll et al., 2019
PIP2	Phospholipid binding	✓			✓			✓		Roux et al., 2006; Ramakrishnan et al., 2009; Padmanarayana et al., 2014
Myosin VI	Motor for vesicular membrane traffic	✓				✓				Heidrych et al., 2009
Golgi marker GM130	Endosome- network dynamics	✓								Heidrych et al., 2008
Rab8b GTPase	Protein transport regulator	✓								Heidrych et al., 2008
NSF	SNARE chaperone membrane fusion	✓	✓	✓		✓		✓		Selvakumar et al., 2017
Ergic2 (ER/Golgi)	Brain specific partner	✓				✓				Zak et al., 2012
Dynamin	Fast endocytosis GTPase	✓				✓	✓	✓		Tertrais et al., 2019
AP2	Clathrin adaptor protein, slow endocytosis	✓	✓	✓		✓		✓		Duncker et al., 2013; Jung et al., 2015; Selvakumar et al., 2017
Calpain	Ca <sup>2+</sup> activated protease cleaving otoferlin	✓								Redpath et al., 2014
Tryptophan-rich basic protein (WRB)	TRC40 complex, transmembrane recognition complex	✓							✓	Vogl et al., 2016

Otoferlin is a multi-C2-domain transmembrane vesicular protein with mutations associated with congenital deafness. Based on the published literature, this table recapitulates the putative factors (left column) interacting with otoferlin, its specific C2 domains, or its transmembrane domain (TMD). Most of these factors interacting with otoferlin share functions with SNARE proteins or have other neurotransmission-related functions.

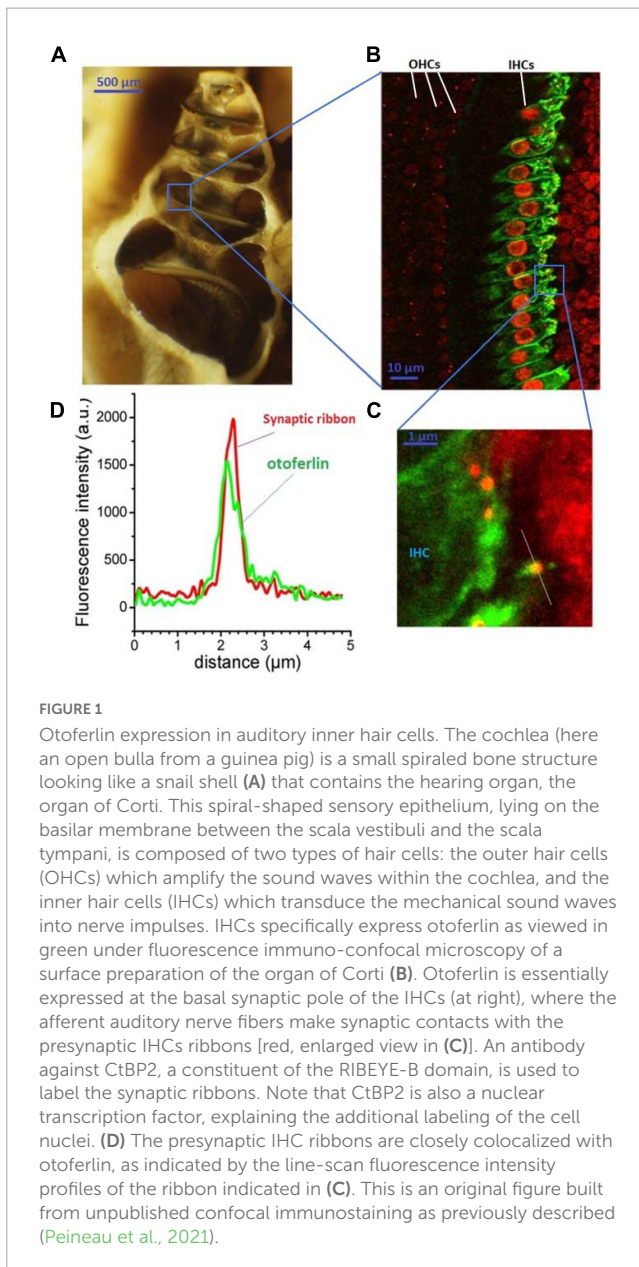


FIGURE 1

Otoferlin expression in auditory inner hair cells. The cochlea (here an open bulla from a guinea pig) is a small spiraled bone structure looking like a snail shell (A) that contains the hearing organ, the organ of Corti. This spiral-shaped sensory epithelium, lying on the basilar membrane between the scala vestibuli and the scala tympani, is composed of two types of hair cells: the outer hair cells (OHCs) which amplify the sound waves within the cochlea, and the inner hair cells (IHCs) which transduce the mechanical sound waves into nerve impulses. IHCs specifically express otoferlin as viewed in green under fluorescence immuno-confocal microscopy of a surface preparation of the organ of Corti (B). Otoferlin is essentially expressed at the basal synaptic pole of the IHCs (at right), where the afferent auditory nerve fibers make synaptic contacts with the presynaptic IHCs ribbons (red, enlarged view in (C)). An antibody against CtBP2, a constituent of the RIBEYE-B domain, is used to label the synaptic ribbons. Note that CtBP2 is also a nuclear transcription factor, explaining the additional labeling of the cell nuclei. (D) The presynaptic IHC ribbons are closely colocalized with otoferlin, as indicated by the line-scan fluorescence intensity profiles of the ribbon indicated in (C). This is an original figure built from unpublished confocal immunostaining as previously described (Peineau et al., 2021).

also carries an alternative splice site, and upon splicing, exon 48 is expressed and encodes a transmembrane domain called TMD1. If exon 47 is not spliced, it bears a stop codon at its end, so the translation of exon 48 is excluded, and otoferlin has a different TMD domain, TMD2 (Figure 4). In humans and mice, the TMD2 otoferlin isoform (exon 47) is preferentially expressed in the brain, while the TMD1 isoform (exon 48) is specifically found in the cochlea (Yasunaga et al., 2000; Schug et al., 2006). Specific mutations in exon 48 cause hearing loss (Choi et al., 2009), underscoring the importance of TMD. According to AlphaFold3D structure predictions, the differences between the two TMD isoforms are partly based on the angulation of the transmembrane domain relative to the rest of the protein (Figure 4E). These two TMDs only share less than 57% amino acid sequence similarity (Figure 4D). Notably, the brain TMD2 isoform contains a large peptidic sequence with numerous contiguous Leu residues (Leu-block of more than 9 Leu) in comparison

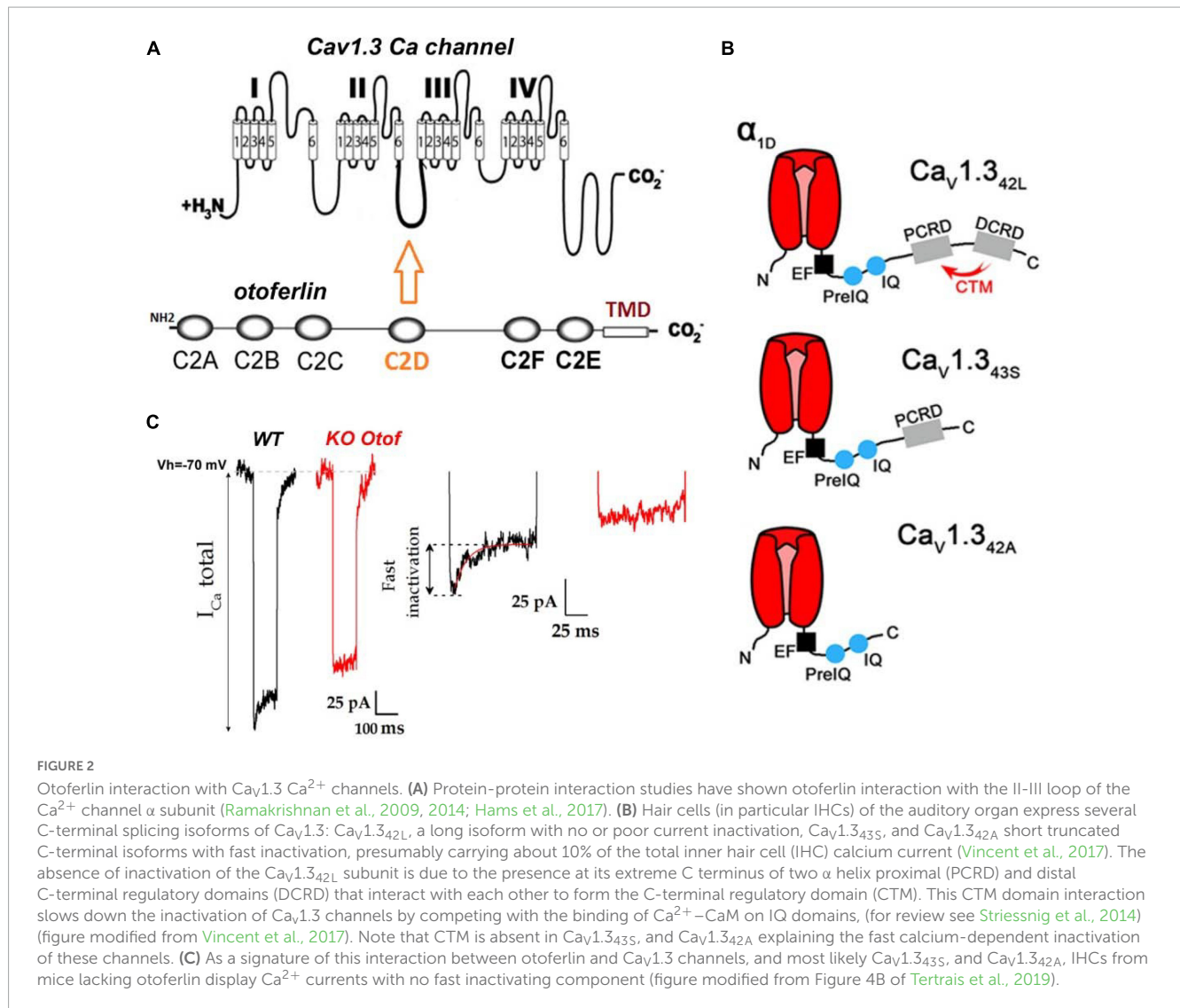
to the cochlear TMD isoform, which contains discontinuous stretches of Leu residues (Figure 4D). Large hydrophobic Leu-blocks in TMD peptides confer greater helicity and circumferential hydrophobicity that facilitate biological membrane insertion (Stone et al., 2015). The use of specific TMD should be important for targeting the protein to specific subcellular locations, such as particular glutamatergic excitatory presynaptic active zones, and specifying the interaction with specific organelles and ligands (Sharpe et al., 2010). Mutations in exon 48, such as the in-frame deletion of a conserved isoleucine in the cochlear TMD1 at position 1967 (p.Ile1967del), lead to hearing impairment and underline the importance of this domain in targeting the protein to the endoplasmic reticulum (ER) membrane in hair cells (Vogl et al., 2016).

Another isoform, with an alternative splice in exon 31 might explain deafness caused by heat sensitivity, but its presence in the human cochlea remains uncertain (Strenzke et al., 2016). A recent study, by Liu et al. (2023) provides new insights into the role of alternative otoferlin isoforms in auditory function and their modulation by environmental factors such as noise and aging. This study discovered a new short transcript of otoferlin derived from an unannotated exon 6b whose expression is increased in IHCs encoding high frequency sounds and varies under noise and aging conditions.

### 3. Genetic mutations and DFNB9

Approximately 220 mutations causing DFNB9-type deafness have been identified, some of which cause deafness during a febrile episode (Vona et al., 2020; Azaiez et al., 2021). Interestingly, there is no described pathological mutation of the C2A domain (Figure 5; Stenson et al., 2003). All other areas of the protein can be affected. According to the Human Gene Mutation Database, 41% of the variants are missense, 22% nonsense, 15% deletions, 14% splicing, 6% insertions, and 2% copy number variants. The missense variants alter the folding, stability, or function of the protein. Truncating variants (nonsense, deletion, splicing, insertion, and copy number variation) result in a missing or truncated non-functional protein. Both types of variants result in severe to profound hearing loss. One of the most common OTOF mutations (p.R1939Q) is located at the junction between the calcium binding C2F domains and the C-terminal TMD (Matsunaga et al., 2012; Kim et al., 2018). As mentioned above, some of the mutations lead to a disorder of the temperature-sensitive auditory neuropathy spectrum. Elevated body temperature causes severe to profound hearing loss, whereas normal body temperature results in normal to moderate hearing. Interestingly, all identified thermosensitive mutations are located between the C2C and C2F domains, underscoring the functional importance of these regions (Zhu et al., 2021).

The mechanisms underlying the temperature sensitive hearing loss remain essentially unknown, and they may vary with their site of location. These mutations probably alter the folding or unfolding properties of the long multi-C2-domain protein, impairing specific  $\text{Ca}^{2+}$ -dependent C2-domain interactions and synaptic vesicle fusion, as temperature rises. Another hypothesis is that there is increased degradation and loss of the protein. This is caused by the heat sensitivity of otoferlin, especially in the presence of variants



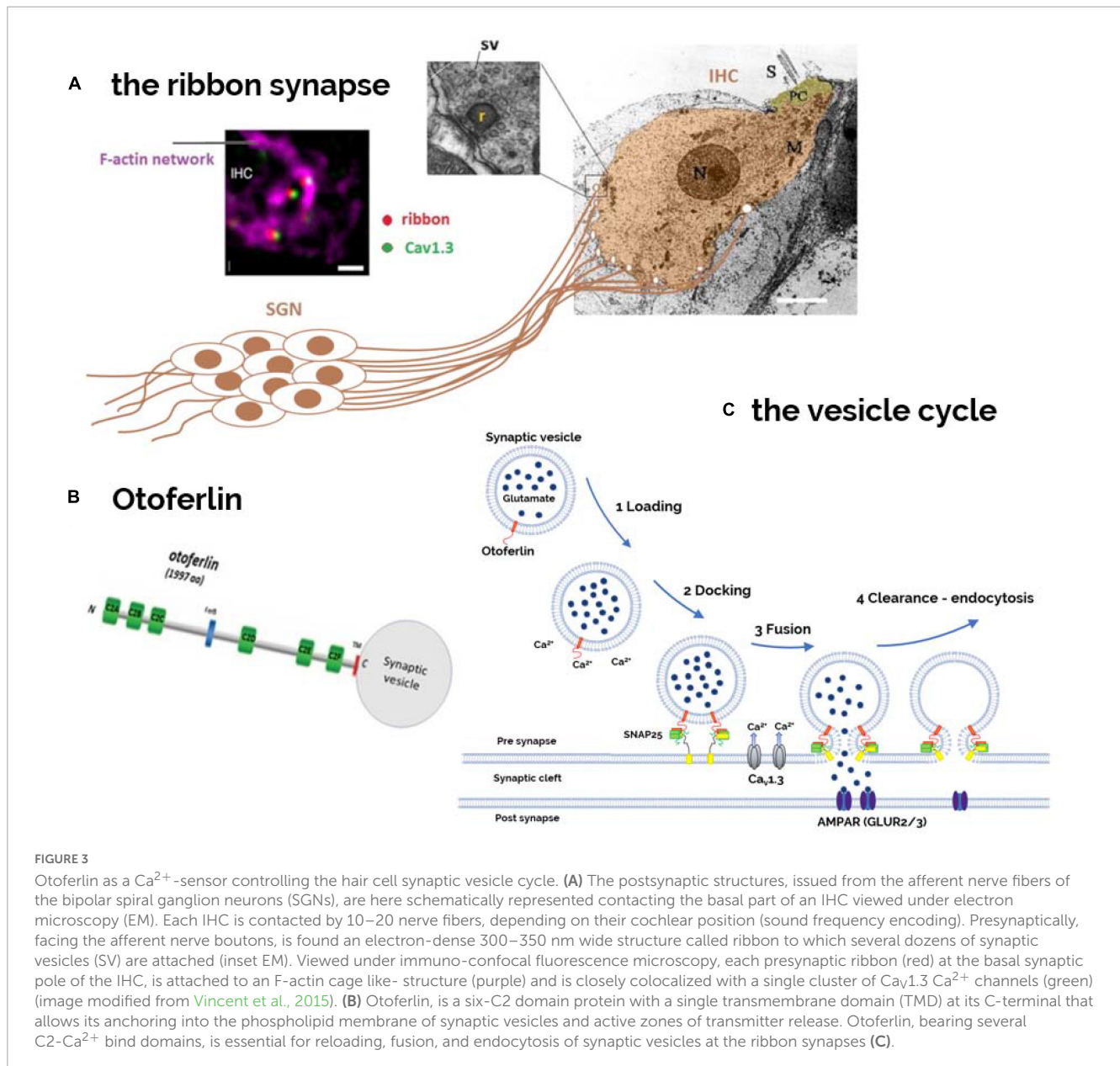
such as Ile515Thr (Strenzke et al., 2016). The protein is then degraded more quickly.

### 4. Otoferlin tissue expression

The tissue distribution of otoferlin is primarily observed in the brain and inner ear (Schug et al., 2006). In the brain it is mainly found in the amygdala and cerebral cortex (Figure 6). We recall that the two TMD otoferlin isoforms have different distributions: one is almost exclusively found in the cochlea (TMD1, exon 48), and the other is mainly found in the brain (TMD2, exon 47) (Yasunaga et al., 2000). However, this TMD variant distribution in the brain and inner ear, only demonstrated by RT-PCR and Northern blot analysis at the tissue level, needs to be confirmed at the protein and cellular levels.

Otoferlin expression increases in hair cells as they mature, remaining permanently expressed in IHCs and only transiently expressed in immature OHCs (largely decreasing after P4-P6 in mice) (Roux et al., 2006; Schug et al., 2006). This transient expression in OHCs is essential for spontaneous synaptic exocytosis

by immature OHCs, a process likely involved in the establishment of frequency tonotopy of the central auditory nuclei during development (Beurg et al., 2008). In IHCs and vestibular cells, otoferlin was localized in synaptic vesicles by immunogold labeling (Roux et al., 2006; Dulon et al., 2009) or in the Golgi apparatus and presynaptic zone by immunohistochemistry (Schug et al., 2006; Heidrych et al., 2008). In HEK cell expression, otoferlin is found at the plasma membrane and colocalizes with some of the trans-Golgi markers (GM130 or TGLON2) (Redpath et al., 2016). In IHCs, otoferlin is distributed throughout the cytoplasm and plasma membrane, except for the apical portion that forms the cuticular plate and tight junctions with neighboring cells. It is also interesting to note that the expression of the protein differs according to its two isoforms, TMD1 and TMD2, when expressed in heterologous cell lines. In HEK293 cell cultures, TMD1 showed a dispersed pattern within the cell, while TMD2 was predominantly found at the plasma membrane (Redpath et al., 2016). Otoferlin is also expressed by type I and type II vestibular utricular hair cells (Dulon et al., 2009). Note that truncation of the TMD of otoferlin alters the development of hair cells and reduces membrane docking (Manchanda et al., 2021). This tail-anchored protein can be inserted



into the endoplasmic reticulum (ER) of hair cells via the TRC40 receptor tryptophan-rich basic protein (Wrb) (Vogl et al., 2016).

## 5. Otoferlin functions and mechanisms of action

### 5.1. Otoferlin multirole at the IHC synaptic vesicle cycle

Remarkably, mature inner hair cells (IHCs) lack the  $Ca^{2+}$ -sensors synaptotagmin (I and II) and Munc13, which are known to play a crucial role in vesicle exocytosis at conventional central neuronal synapses (Beurg et al., 2010; Reisinger et al., 2011; Vogl et al., 2015). It is worth recalling that mature IHCs, contrary to central neurons, do not fire action potentials but are

capable responding to a large dynamic range of sound intensity (>100 dB) by graded depolarization that is encoded into an increasing discharge rate at the postsynaptic nerve fibers. It is now established that otoferlin plays a role at several essential stages of the vesicle cycle in IHCs, including functional docking, priming, fusion, endocytosis, and possibly transport and maturation of vesicles (Figure 3; Michalski et al., 2017; Tertrais et al., 2019). In otoferlin knockout mice, the average tether length between vesicles and the active zone is increased, suggesting a possible defect in vesicle attachment and priming (Vogl et al., 2015). The downstream calcium-triggered exocytosis of vesicles is almost completely blocked in otoferlin knockout mice. Otoferlin is also involved in active zone clearance, including the transport of exocytic material, through interactions with adaptor protein 2  $\mu$ , a motor protein, and a GTPase (Jung et al., 2015). Vesicle size may indicate a role for otoferlin in vesicle reformation and maturation, and the functions of otoferlin may be regulated by phosphorylation.

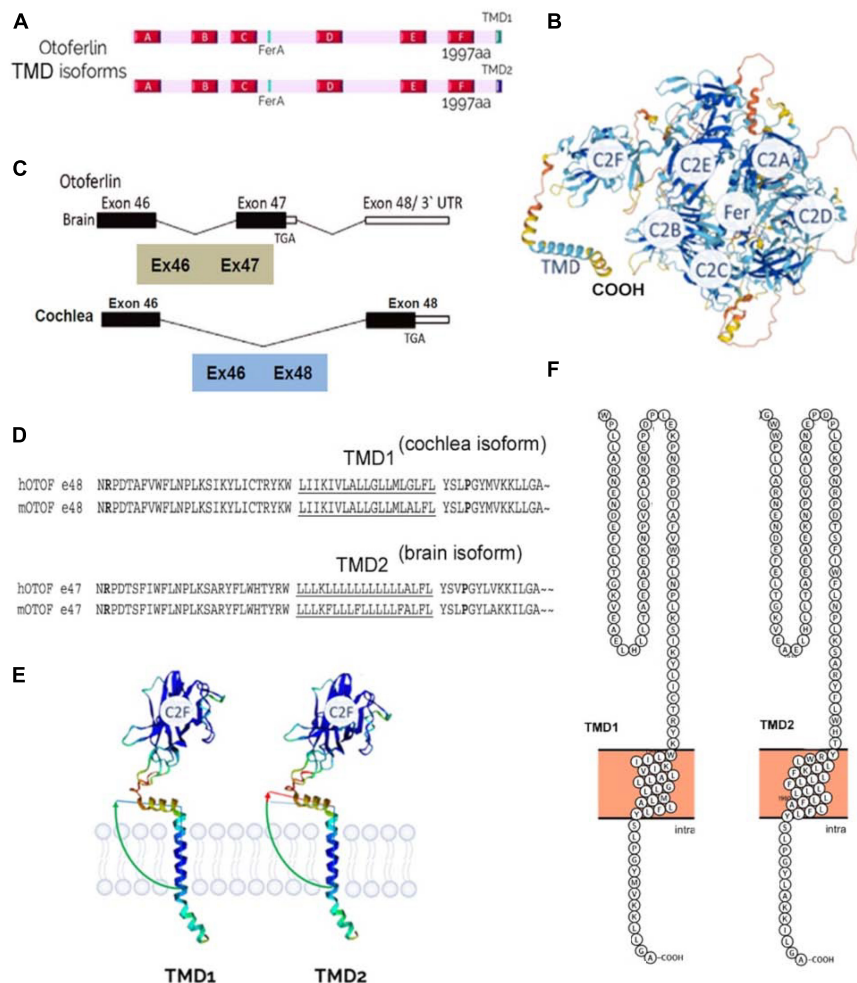


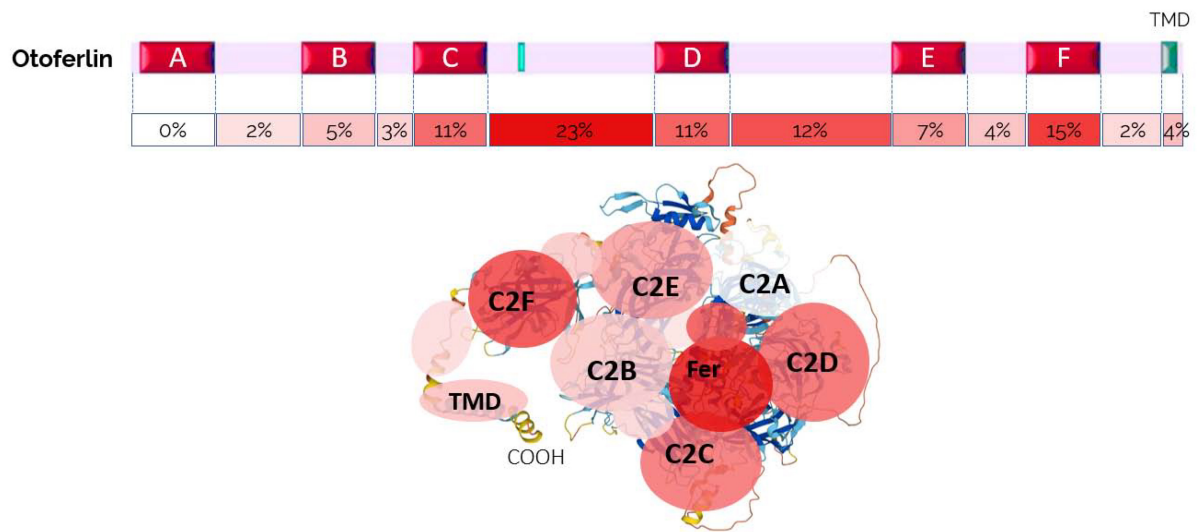
FIGURE 4

Otoferlin structure. (A) C2-domain structures of the two transmembrane domain (TMD) isoforms of otoferlin, TMD1 (cochlea) and TMD2 (brain). (B) AlphaFold prediction of otoferlin 3D-structure as a globular protein with six C2 domains (C2A-F), a Fer domain, and a C-ter TMD (structure built from AlphaFold Protein Structure Database). (C) Brain and inner ear tissues have been reported to express two different specific alternatively spliced forms of the otoferlin TMD, encoded by either exon-47 or exon-48, respectively (Fig adapted from Yasunaga et al., 2000). Exon-47 is skipped in the cochlea, leading to a different carboxy-terminal peptide sequence from that of the brain isoform, which expresses exon-47. In mRNA with exon-47 (neuronal otoferlin), translation terminates in that exon, leaving exon-48 untranslated. (D) Exon-47 of the brain isoform and exon-48 of the cochlear isoform specify the C-terminal TMD 60 amino acids of otoferlin. The amino acid sequences of the two isoforms differ essentially in the predicted transmembrane sequence (underlined). (E) Analysis of protein structure with AlphaFold software online (developed by DeepMind and EMBL-EBI) predicts a different angle of insertion of cochlear TMD1 and neuronal TMD2 otoferlin isoforms within phospholipid membranes. The use of a specific TMD is likely important for targeting the protein to specific subcellular locations, such as particular glutamatergic excitatory presynaptic active zones, and/or for specifying the interaction with ligands. (F) TMD structure analysis and representation using Protter software (Omasits et al., 2014) predicts a 21 aa TMD1 for the cochlear isoform (gi|154240702|ref|NP\_001093865.1|otoferlin [Mus musculus]) and a 23 aa for the brain isoform (gi|154240679|ref|NP\_114081.2|otoferlin [Mus musculus]).

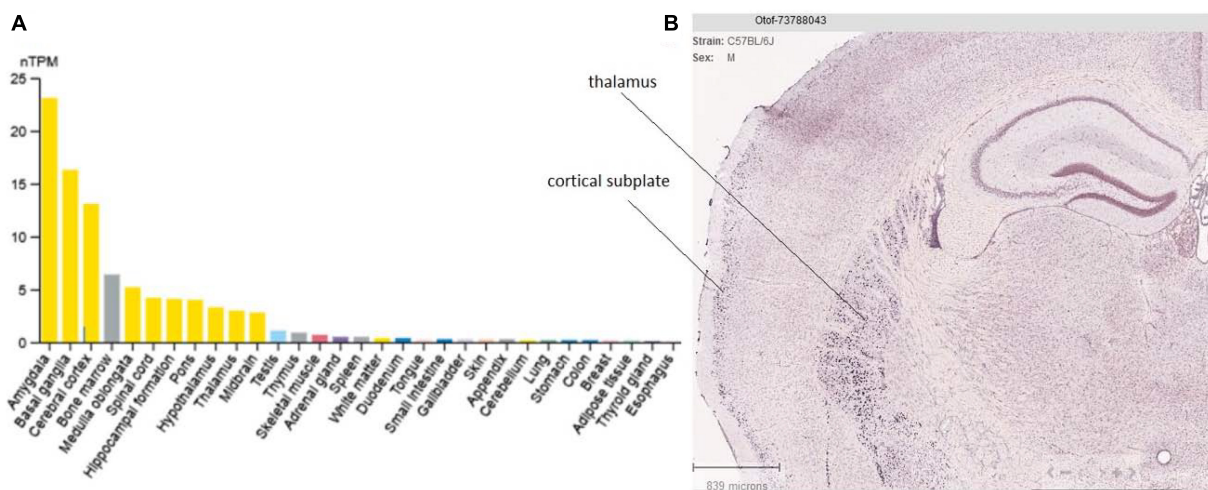
## 5.2. Otoferlin and membrane fusion

Structural analysis and prediction using Protter (Omasits et al., 2014) and AlphaFold (Jumper et al., 2021) display otoferlin as a globular protein anchored to the vesicle and/or plasma membrane through its C-terminal domain, while its N-ter  $Ca^{2+}$ -sensing C2-domains are in the cytosol (Figure 4). The structural features of its C2-domains, through their  $Ca^{2+}$ -binding and phospholipid-binding activities, allow otoferlin to play a role in membrane fusion and trafficking, in endocytic, secretory, and lysosomal pathways. By deleting exons 14 and 15 of the OTOF gene, an OTOF KO mouse model was created (Roux et al., 2006). These otoferlin-deficient mice (*Otof*<sup>-/-</sup>) are profoundly deaf. Patch-clamp recordings for

time-resolved change of membrane capacitance in IHCs from *Otof*<sup>-/-</sup> mice show a significant impairment of fast and sustained exocytotic activity (Beurg et al., 2010; Michalski et al., 2017). Another mouse model, Pachanga, carrying a missense mutation in the C2-F domain of otoferlin, was obtained by ENU (N-ethyl-N-nitrosourea) mutagenesis (Schwander et al., 2007). Pachanga mice have profound hearing loss, but unlike *Otof*<sup>-/-</sup> mice, IHCs still weakly express otoferlin and maintain rapid fusion of the pool of fusogenic vesicles, forming the RRP (for “readily releasable pool of vesicles”) (Pangrsic et al., 2010). However, IHC exocytosis cannot be sustained upon repeated stimulations in these Pachanga mutant mice, indicating impaired recruitment of vesicles to IHC fusion sites. These results suggested that



**FIGURE 5** Frequency of OTOF pathogenic mutations according to their location in the different domains of Otoferlin, showing the particular importance of the C, D region and the F domain (map established from the Human Gene Mutation Database).



**FIGURE 6** Tissue and brain expression of otoferlin. (A) Tissue specificity of otoferlin RNA expression (data from the Human Protein Atlas). (B) Otoferlin expression (mRNA *in situ* hybridization) in the mouse brain thalamus and cortex (cortical subplate) from the Allen Brain Atlas portal. This analysis appears to well corroborate previous findings showing otoferlin expression in brain tissues (Schug et al., 2006).

otoferlin is also an essential calcium sensor for synaptic vesicle replenishment at IHC active zones. It should be noted that vesicle recruitment is a calcium-dependent process in hair cells (Spasova et al., 2004; Levic et al., 2011). Gradual intracellular Ca<sup>2+</sup>-uncaging experiments in IHCs revealed that otoferlin-induced exocytosis has an intrinsically high Ca<sup>2+</sup> sensitivity (affinity) with a mean Kd of 4.0 ± 0.7 μM and responses spanning from 1 to 20 μM Ca<sup>2+</sup> (Vincent et al., 2014). As a comparison, the neuronal Ca<sup>2+</sup> sensors, synaptotagmins, have nearly a one-order of magnitude lower Ca<sup>2+</sup> affinity (higher Kd), a property in good correlation with their specific functions of being activated only by high Ca<sup>2+</sup> concentrations typical for AP-evoked Ca<sup>2+</sup>-elevations near Ca<sup>2+</sup> channels (Bornschein and Schmidt, 2018).

On the other hand, the importance of otoferlin in the maintenance or development of ribbons and the presence of corresponding synapses has been demonstrated in mice (Stalman et al., 2021). Otoferlin depletion in zebrafish hair cells also results in abnormal synaptic ribbons and altered intracellular calcium levels (Manchanda et al., 2019), suggesting Ca<sup>2+</sup> as an important factor for the maintenance and survival of the ribbons as in aging auditory synapses (Peineau et al., 2021).

Although some of the interacting proteins start to be identified (Table 1), the precise mode of action of otoferlin in membrane fusion is not fully elucidated, particularly in comparison with the neuronal calcium sensor (synaptotagmin1) which regulates exocytosis function as a multimer with oligomerization via a cluster of juxtamembrane linker (Courtney et al., 2021). Note that, in



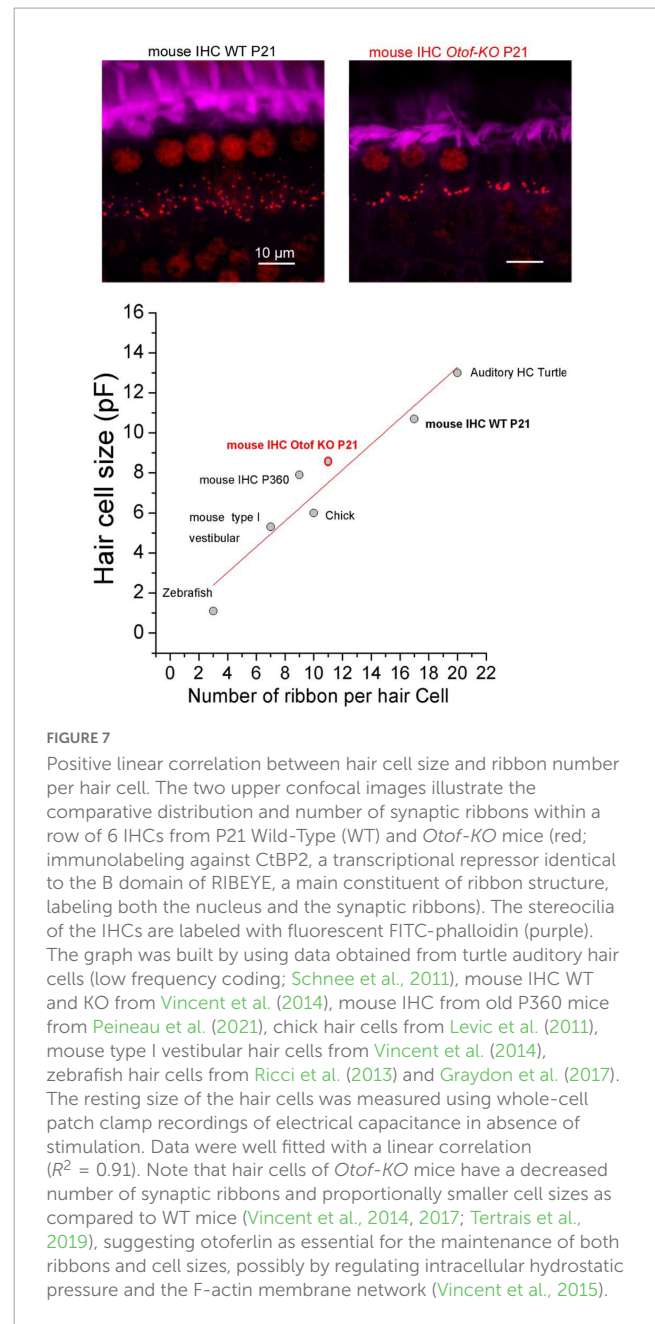
contrast to ferlin proteins, the TMD of synaptotagmins is located at the N-terminal region of the protein for unknown reasons. The homolog of otoferlin, dysferlin, has also been shown to dimerize in living cells, at its transmembrane domain and at its multiple C2 domains (except C2A), as probed by fluorescence resonance energy transfer (FRET) (Xu et al., 2011).

The C2-A domain is the only one of the six otoferlin domains that cannot bind  $\text{Ca}^{2+}$ , which probably explains the absence of pathogenic mutations in this domain (Johnson and Chapman, 2010; Helfmann et al., 2011; Padmanarayana et al., 2014). The idea of a simple repeat between each C2 domain is rejected. An *Otof* KI mouse model carrying two mutations in C2C (substitution of 2 aspartic acid by alanine, affecting  $\text{Ca}^{2+}$  binding), demonstrated that otoferlin is essential in both rapid fusion and vesicle recruitment (Michalski et al., 2017). Interestingly, the viral expression of truncated forms of otoferlin (C2-EF, C2-DEF, and C2-ACEF) can only partially rescue the fast and transient release component of exocytosis in mouse hair cells lacking otoferlin (*Otof*<sup>-/-</sup>), yet cannot sustain exocytosis after long, repeated stimulations (Tertrais et al., 2019). To note, a C2-EF otoferlin truncated form can also be produced by intracellular calpain-1 digestion in cell lines (Redpath et al., 2014) but it is unknown whether such truncation occurs in auditory IHCs.

Note that otoferlin is also essential for the fast exocytosis of type I vestibular cells. *Otof*<sup>-/-</sup> mice show altered vestibular compound action potentials, suggesting impaired vestibular hair cell function (Dulon et al., 2009). In intact utricles *ex vivo*, otoferlin was found to be critical for a highly sensitive and linear calcium-dependent exocytosis, facilitating the linear encoding of low-intensity stimuli at the vestibular hair cell synapse. Surprisingly, mice and humans lacking otoferlin do not have apparent vestibular symptoms. The lack of vestibular phenotype may be explained by some compensatory mechanisms, in particular by stimulus-evoked acidification of the synaptic cleft of vestibular hair cells (protons acting as neurotransmitters) (Highstein et al., 2014) or by the use of another type of neurotransmission via potassium accumulation in the calyx, which depolarizes it (Holt et al., 2007). Although these compensatory modes of transmission are rather slow and non-linear, they may explain the absence of a vestibular phenotype in OTOF patients and animal models.

### 5.3. A specific mechanical tuning between $\text{Ca}^{2+}$ , otoferlin, F-actin, and the ribbon could determine the specific firing frequency of each auditory nerve fiber

Mammalian auditory inner hair cell (IHC) ribbon synapses have to deal with the great challenge of encoding an extremely wide range of sound intensities, with a dynamic range of more than 100 dB. To perform this challenging task, IHCs have partitioned their synaptic output sensitivity and dynamic range. Indeed, each IHC forms synapses with a pool of 10 to 30 afferent nerve fibers, among which spontaneous activity, acoustic threshold, and dynamic range vary widely. One of the most important questions in auditory neuroscience is to elucidate the functional mechanisms that dictate the synaptic diversity of each type of sensory synapses within a single IHC. Variation in the voltage-gating and spatial



organization of  $\text{Ca}_V1.3$  calcium channels at each presynaptic active zone has been proposed to determine the firing specificity of the auditory nerve fibers (Özçete and Moser, 2021). Other factors, such as a mechanical tuning of each ribbon, could also participate in the firing frequency characteristic of each auditory fiber (Figures 7–9).

Otoferlin has been proposed to control IHC intracellular hydrostatic pressure and in turn exocytosis, presumably via interactions with a synaptic F-actin network (Vincent et al., 2015) and the  $\text{Ca}^{2+}$  channels  $\text{Ca}_V1.3$  at the ribbon (Vincent et al., 2017). Otoferlin is essential for synchronous multivesicular release at IHC active zones; this process underlying fast transient  $\text{H}^+$ -inhibition of  $\text{Ca}_V1.3$   $\text{Ca}^{2+}$  channels (Vincent et al., 2018).

Remarkably, there is a good linear correlation between the size of the hair cells and their number of synaptic ribbons per cell when comparing the ears of various vertebrates (Figure 7). In

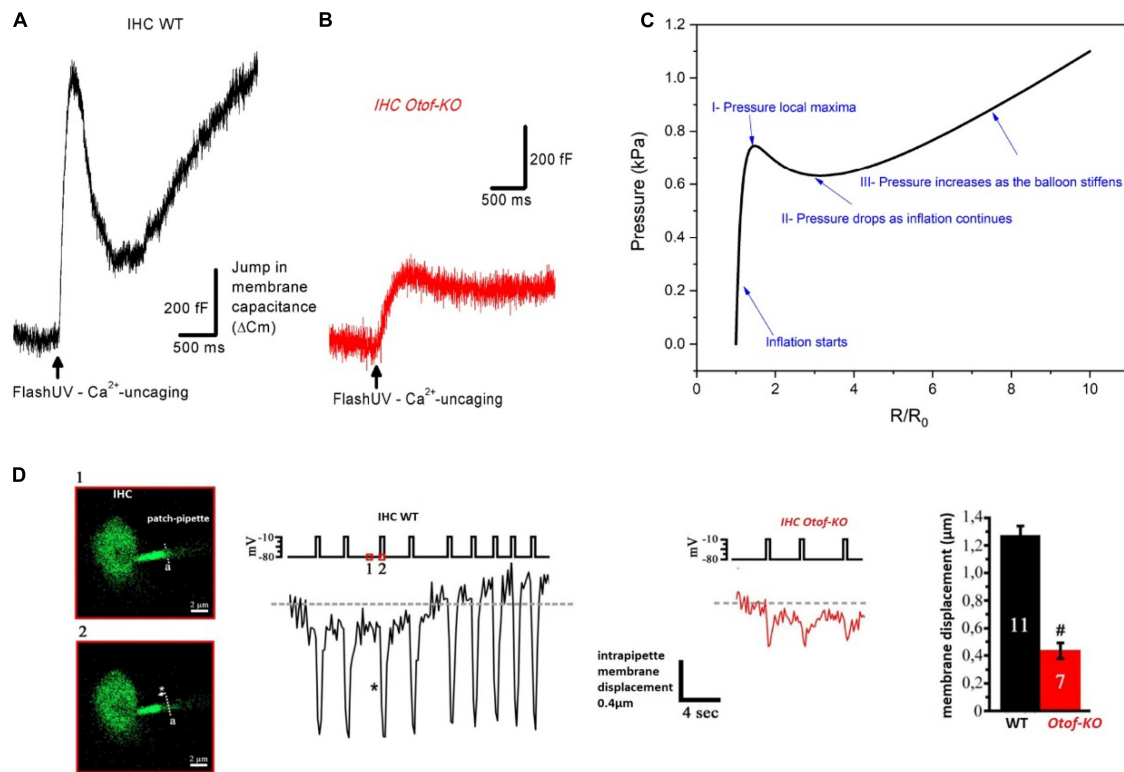


FIGURE 8

Modeling IHC as a hyper-elastic structure whose changes in membrane capacitance upon intracellular Ca<sup>2+</sup> uncaging and otoferlin action behave like the pressure change in an inflating rubber balloon. (A) Typical example of membrane capacitance change upon intracellular Ca<sup>2+</sup> uncaging in an IHC of a WT P20 mouse. (B) Similar experimental conditions in an IHC of a P20 *Otof-KO* mouse. In (A,B), IHCs were whole-cell voltage-clamped at -70 mV and loaded with the caged-Ca<sup>2+</sup> molecule DM-nitrophen [figures (A,B) are unpublished traces obtained as described in Vincent et al. (2014, 2015) and Tertrais et al. (2019)]. An increase in membrane capacitance reflects the fast fusion of synaptic vesicles at the plasma membrane. Note that the large N-shaped curve of the change in membrane capacitance in WT IHC is absent in IHC *Otof-KO*. (C) Pressure variation inside an inflating rubber balloon displays a similar N-shaped curve over time, or radius change, with the following relationship:

$$P = K \left[ \frac{R_0}{R} - \left( \frac{R_0}{R} \right)^7 \right] \left[ 1 + 0.1 \left( \frac{R}{R_0} \right)^2 \right]$$

where R is the radius, R<sub>0</sub> is the initial radius, K is a constant dependent on material properties, and P is the internal pressure of the balloon (Müller and Struchtrup, 2002; Wang et al., 2018; Ajmal et al., 2023).

(D) In whole-cell configuration, IHCs were loaded with the fluorescent Ca<sup>2+</sup> indicator molecule OGB-2 and the edge of ruptured plasma membrane inside the patch pipette could be visualized under confocal fluorescence microscopy. As evidence of intracellular pressure changes in IHCs, upon repeated depolarizing pulses, fast intrapipette membrane displacements could be visualized and measured in WT IHCs. These membrane movements are largely reduced in IHCs lacking otoferlin (figure modified from Vincent, 2015). # indicates statistical difference, unpaired t-test with p < 0.01. Overall, these elements suggest otoferlin as a mechanical protein whose interaction with Ca<sup>2+</sup> leads to the development of mechanical forces that change intracellular pressure in IHCs. The changes in intracellular pressure could result from Ca<sup>2+</sup>-induced fast conformational changes of otoferlin and/or the fast, massive, addition of vesicular membrane to the plasma membrane.

IHCs from mice lacking otoferlin, the number of synaptic ribbons is proportionally reduced with the cell size (Tertrais et al., 2019). Also in old P360 C57BL/6J mice, the reduced size of the IHCs is proportionally associated with a lower number of synaptic ribbons (Peineau et al., 2021). This correlation suggests that the number of ribbons may influence membrane exocytosis, and in turn, cell size, possibly by modifying the intracellular hydrostatic pressure. Numerous studies in various cell type have shown a reciprocal link between cell biomechanics, the submembrane actomyosin network, and exocytosis (Wang and Galli, 2018). Auditory IHCs seem to be able to sense the biomechanical properties of the environment since their exocytotic properties are sensitive to changes in intracellular hydrostatic pressure (Vincent et al., 2014). Membrane addition during hair cell synaptic exocytosis can be visualized by confocal-fluorescent microscopy (Hudspeth and Issa, 1996), indicating membrane deformation and a possible local change in hydrostatic pressure.

We propose that otoferlin, by controlling membrane fusion, regulates plasma membrane rigidity and tension of its underlying actomyosin meshwork, whose mechanical properties are likely essential to maintaining active membranous synaptic ribbons. Note that upon UV-flash-photolysis allowing ultrafast intracellular Ca<sup>2+</sup> uncaging from DM-nitrophen, the change in IHC membrane capacitance (produced by massive vesicle fusion to the plasma membrane) behaves like an inflating rubber balloon (Figures 8A–C), suggesting a non-monotonic relation (non-linear stress-strain response) between the elasticity of the IHC plasma membrane and the intracellular pressure. Changes in IHC intracellular pressure can directly be visualized during exocytosis when monitoring membrane displacement during repetitive depolarizing steps (Figure 8D; Vincent, 2015). It is tempting to propose the synaptic ribbons as pulsating microspheres that are activated by Ca<sup>2+</sup>-otoferlin interactions with the membranous F-actin network; these mechanical interactions being the source generator (hydrostatic

force) triggering vesicle membrane fusion. In this model, the mechanical oscillatory frequency of the ribbons would essentially depend on their mass, similarly to a spring-block oscillator, a factor that would then determine the firing frequency of the postsynaptic afferent nerve fibers (Figure 9). In this context, it is interesting to note that amphibian hair cells have been shown to display frequency selectivity in synaptic exocytosis, presumably due to a tonotopic variation of Ca<sup>2+</sup> buffers (parvalbumin 3 and calbindin-28K) that tunes a regime of spontaneously oscillatory vesicle release (Patel et al., 2012).

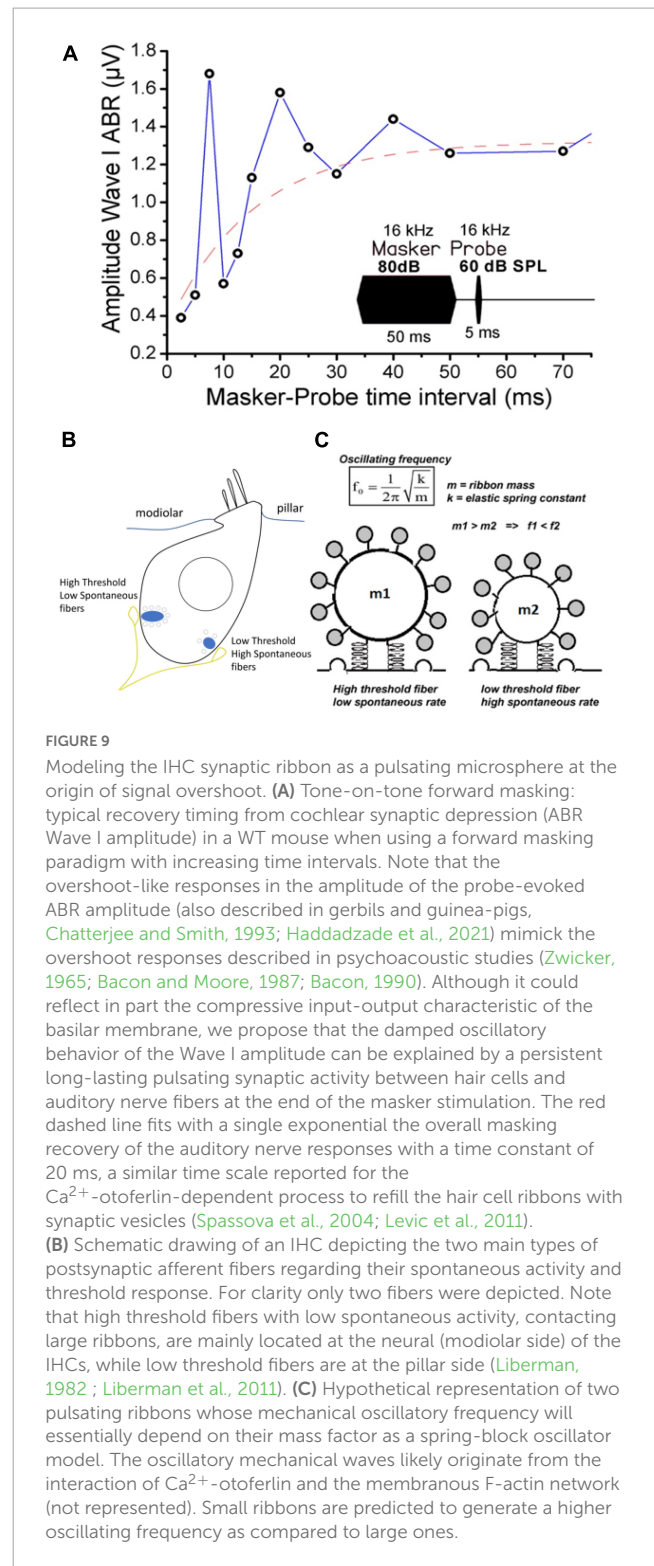
### 5.4. The ribbon synapse: an unconventional synapse

Intriguingly, some proteins usually present in glutamatergic synapses of the central nervous system, such as synaptophysin, synaptotagmin, and complexins, are absent from IHC synapses, suggesting that an unconventional mechanism of neurotransmitter release may be involved (Safieddine and Wenthold, 1999; Uthaiyah and Hudspeth, 2010; Nouvian et al., 2011). However, some classical neuronal SNARE proteins, such as SNAP-25, are essential for normal exocytotic function of the IHC ribbon synapses (Calvet et al., 2022). Also, molecular interactions of otoferlin with Soluble NSF Attachment Protein Receptor (SNARE) proteins, such as syntaxin-1 and SNAP-25, but not synaptobrevin-1 (VAMP-1) have been shown in protein-protein interaction studies *in vitro* (Ramakrishnan et al., 2014; Hams et al., 2017; Table 1). At the IHC synapse level, mutations in genes encoding proteins other than otoferlin are also implicated in auditory synaptopathies leading to congenital deafness vesicular glutamate transporter (VGLUT3) (Ruel et al., 2008), L-type voltage-dependent Ca<sup>2+</sup> channel Cav1.3 (CACNA1D) (Platzer et al., 2000; Baig et al., 2011), and Usher proteins such as harmonin (Gregory et al., 2011) and the tetraspan protein clarin-1 (Dulon et al., 2018).

## 6. Ferlin family

Otoferlin belongs to the ferlin protein family, which in humans has five members in addition to otoferlin (fer1L2): dysferlin (fer1L1), myoferlin (fer1L3), fer1L4, fer1L5, and fer1L6 (Lek et al., 2010; Redpath et al., 2016). The first ferlin identified was Fer-1, in *Caenorhabditis elegans*. Fer-1 is a fertilization factor required for the fusion of specialized vesicles with the plasma membrane during spermatogenesis (Achanzar and Ward, 1997). The main common features of ferlins are:

- The presence of multiple C2 domains that share a high sequence similarity of about 100 amino acids, and most often allow binding to Ca<sup>2+</sup>, phosphatidylinositol 4,5-bisphosphate (PIP2) or phosphatidylserine (PS).
- The presence of "FerA" domains also allows binding to phospholipid membrane.
- Their anchoring to the membrane by a single C-terminal domain (tail-anchored proteins).



**FIGURE 9** Modeling the IHC synaptic ribbon as a pulsating microsphere at the origin of signal overshoot. **(A)** Tone-on-tone forward masking: typical recovery timing from cochlear synaptic depression (ABR Wave I amplitude) in a WT mouse when using a forward masking paradigm with increasing time intervals. Note that the overshoot-like responses in the amplitude of the probe-evoked ABR amplitude (also described in gerbils and guinea-pigs, Chatterjee and Smith, 1993; Haddadzade et al., 2021) mimic the overshoot responses described in psychoacoustic studies (Zwicker, 1965; Bacon and Moore, 1987; Bacon, 1990). Although it could reflect in part the compressive input-output characteristic of the basilar membrane, we propose that the damped oscillatory behavior of the Wave I amplitude can be explained by a persistent long-lasting pulsating synaptic activity between hair cells and auditory nerve fibers at the end of the masker stimulation. The red dashed line fits with a single exponential the overall masking recovery of the auditory nerve responses with a time constant of 20 ms, a similar time scale reported for the Ca<sup>2+</sup>-otoferlin-dependent process to refill the hair cell ribbons with synaptic vesicles (Spassova et al., 2004; Levic et al., 2011). **(B)** Schematic drawing of an IHC depicting the two main types of postsynaptic afferent fibers regarding their spontaneous activity and threshold response. For clarity only two fibers were depicted. Note that high threshold fibers with low spontaneous activity, contacting large ribbons, are mainly located at the neural (modiolar side) of the IHCs, while low threshold fibers are at the pillar side (Liberman, 1982; Liberman et al., 2011). **(C)** Hypothetical representation of two pulsating ribbons whose mechanical oscillatory frequency will essentially depend on their mass factor as a spring-block oscillator model. The oscillatory mechanical waves likely originate from the interaction of Ca<sup>2+</sup>-otoferlin and the membranous F-actin network (not represented). Small ribbons are predicted to generate a higher oscillating frequency as compared to large ones.

Their common role would therefore concern Ca<sup>2+</sup>-triggered membrane fusion and trafficking, in endocytic, secretory, and lysosomal pathways (McNeil and Kirchhausen, 2005; Jiménez and Bashir, 2007; Lek et al., 2012). Dysferlin and myoferlin mutations cause muscle diseases: limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy (Dysferlin), and muscular

dystrophy with cardiomyopathy (Myoferlin) (Bashir et al., 1998; Liu et al., 1998).

Dysferlin (Fer1L1) is ubiquitously expressed, with high expression in the brain, heart and skeletal muscle (Anderson et al., 1999). Dysferlin is composed of seven C2 domains, the DysF domain and the FerA domain preceding the FerB domain (Figure 10). Dysferlin acts as a primary emergency regulator of membrane repair in a calcium-dependent manner following membrane damage to myofibres (Bansal et al., 2003; Barthélémy et al., 2018). Dysferlin is also involved in Ca<sup>2+</sup> metabolism regulation in injured muscle fibers (Hofhuis et al., 2017). Dysferlin can be cleaved by calpain (1 and 2; calcium-dependent protease) in response to calcium influx following a membrane micro-lesion (Lek et al., 2013). Cleavage releases a “mini-dysferlin-C72” (with C2E, C2F, and TMD). Mini-dysferlin-C72 is selectively recruited and is thought to be a minimal configuration required for physiological membrane repair function (Krahn et al., 2010; Lek et al., 2013; Redpath et al., 2014). Otoferlin and myoferlin can also release a module with the last two C-terminal domains and the TMD after enzymatic cleavage of calpain *in vitro* (Redpath et al., 2014; Piper et al., 2017). This structure carries a high structural similarity to the synaptotagmin family. Moreover, a phylogenetic study reveals that these two C-terminal C2 domains are the most evolutionarily conserved domains of the ferlin family: there is 90% sequence similarity of C2-EF domains between mammals and mollusks, suggesting a key function (Lek et al., 2010).

Like otoferlin, the C2 domains of dysferlin bind to phosphatidylserine (PS), and phosphatidylinositol 4,5-bisphosphate in a Ca<sup>2+</sup>-dependent fashion (Therrien et al., 2009). Dysferlin interacts with caveolin 3 and MG53, which play an important role in maintaining dysferlin within the plasma membrane and enabling effective muscle membrane repair (Matsuda et al., 2001; Hernández-Deviez et al., 2008; Cai et al., 2009). Dysferlin also interacts with annexin A1 and A2, involved in membrane trafficking and actin organization (Lek et al., 2012), Ca<sub>v</sub>1.1 L-type calcium channels (Ampong et al., 2005), vinculin, which acts as a link between actin filament and plasma membrane (de Morrée et al., 2010), and syntaxin 4, a protein that facilitates the docking and fusion of glucose transporter type 4 (GLUT4) vesicles with the plasma membrane (Evenson et al., 2010; Codding et al., 2016; Drescher et al., 2023).

In the central nervous system, dysferlin has been observed to accumulate in endothelial cells near sclerosis lesions (Hochmeister et al., 2006), as well as within amyloid-beta plaques in people with Alzheimer's disease (Galvin et al., 2006). In addition, in humans, certain genetic variations of the Dysf gene have been associated with an increased risk of developing Alzheimer's disease, and Dysf mRNA expression has been shown to be increased in the brains of people with Alzheimer's disease (Chen et al., 2015). Interestingly, OTOF was identified as a downregulated gene in a human-mouse chimeric model of Alzheimer's disease using genome-wide expression analysis, suggesting an essential function of otoferlin in the central nervous system (Espuny-Camacho et al., 2017).

Myoferlin (Fer1L3) is also ubiquitously expressed in skeletal and cardiac muscle and in the placenta (Davis et al., 2000). Myoferlin is involved in the fusion of myoblasts, in repair and regeneration, and in muscle cell membrane growth (Doherty et al., 2005). Myoferlin may be involved in the maintenance of transverse tubule function (Demonbreun et al., 2014). Between the C2-C and

C2D, a domain consisting of two long  $\beta$ -sheets is called the dysferlin (DysF) domain, and a FerA domain, consisting of  $\alpha$ -helices, is located before the FerB domain (Figure 10). The exact role of the DysF domain is unknown but must be significant, as mutations in this domain cause myopathies (Sula et al., 2014). Full information about the ferlin family is available in a recent review by Peulen et al. (2019).

## 7. Otoferlin-disease management and prospects

Auditory Neuropathy Spectrum Disorder (ANSD) is most often suspected after hearing loss is detected at birth, especially if otoacoustic emissions, temporal bone MRI, and CT scans are normal. This deafness may be detected later if the otoacoustic emissions at birth are falsely reassuring. The diagnosis is confirmed by molecular genetic testing, which shows biallelic pathogenic variants in OTOF. Two phenotypes are possible: OTOF-related ANSD, with severe to profound bilateral hearing loss (>70 dB), and temperature-sensitive ANSD (TS ANSD), with normal to moderate hearing loss (0–69 dB) at normal body temperature and worsening to profound hearing loss during hyperthermia. In these patients, prevention of hyperthermia by early use of antipyretics for infection and avoidance of exposure to high temperatures is important (Azaiez et al., 2021).

As we have seen above, in OTOF-related ANSD, the defects are presynaptic at the level of the hair cells. In these situations, the cochlear nerve is intact and functional. This is a prerequisite for cochlear implantation. It is currently the only option for restoring hearing in patients with severe to profound hearing loss. Early implantation is recommended after the diagnosis of OTOF-related auditory neuropathy with severe to profound hearing loss. It involves surgery. A foreign body is implanted under the skin, and an electrode array is placed in the cochlea for life (De Seta et al., 2022). It requires the wearing of an external magnetic processor. The risk of infection or failure requiring re-intervention and the limited autonomy of the device are significant limitations of this therapy. We can also point out the potential limitations of speech recognition in noise. In OTOF-related ANSD, audiometric results are known since 2005 (Loundon et al., 2005). They can be considered of high quality since the thresholds for sound perception in silence from 500 to 2000 Hz vary on average from 25 to 45 dB SPL, depending on the series (Loundon et al., 2005; Rouillon et al., 2006; Zhang et al., 2016; Chen et al., 2018; Zheng and Liu, 2020). Regarding speech recognition, which is the main goal of cochlear implantation, discrimination scores are mostly >90%. There is no evidence that cochlear implantation outcomes are correlated with distinct OTOF genotypes (Zheng and Liu, 2020).

Gene therapy for congenital deafness has been expanding in recent years, with the aim of achieving a therapy with better hearing outcomes without the limitations of cochlear implant. A truncated form of the OTOF gene was shown to rescue hearing and balance in zebrafish (Chatterjee et al., 2015). Since then, several studies have been interested in genetic therapy using recombinant dual AAV vectors (adeno-associated viruses) to encode full-length otoferlin (Akil et al., 2019; Al-Moyed et al., 2019; Rankovic et al., 2020). These studies gave very encouraging results by showing significant

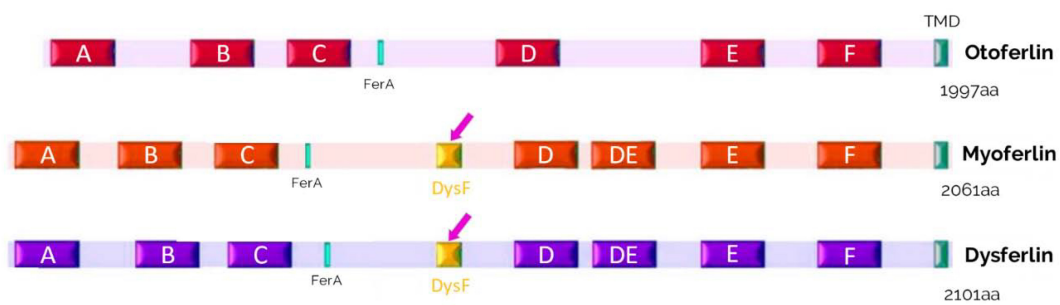


FIGURE 10

Schematic representation of the structures of otoferlin, myoferlin and dysferlin. These proteins of the ferlin family contain 6 to 7 C2 domains, a FerA domain, and a single C-terminal transmembrane domain (TMD). A domain called dysferlin (DysF) is located between the C2-C and C2-D domains in myoferlin and dysferlin (pink arrows).

restoration of hearing in mice. Recently, recombinant dual AAV vectors encoding for the whole human cochlear otoferlin were used to restore hearing in mice to near wild-type levels for 6 months (Tang et al., 2022). This raises the prospect of treatment by gene therapy in humans for DFNB9. The main limitations are the early age of AAV treatment used in most of these studies, which would correspond to an *in utero* AAV injection in humans, and the weak restoration of the ABR wave-1 amplitude, which could limit speech intelligibility and discrimination, particularly in noise, as the main current defect known for cochlear implants. The weak restoration of wave-1 amplitude during AAV gene therapy is likely due to an uncomplete rescue of the loss of ribbon synapses in the absence of functional otoferlin (Stalman et al., 2021). Note that a reduction in spiral ganglion neurons with apoptosis is also a secondary defect caused by a lack of otoferlin in IHCs (Tsuzuki et al., 2023). Currently, several biotech companies (SENSORION, NCT05402813 and AKOUOS–NCT05572073) are in the pre-clinical phase for the development of gene therapy for otoferlin mutations in humans.

## 8. Otoferlin and cancer

### 8.1. Ferlins

As we have seen, ferlins are a group of proteins that play an important role in several membrane-related processes that are crucial for cell survival and signaling. These processes include endocytosis, exocytosis, recycling, and membrane repair (Bansal et al., 2003; Roux et al., 2006; Bernatchez et al., 2009; Redpath et al., 2016). The link between these processes and cancer is through their effect on cell  $Ca^{2+}$  signaling and development, which allows tumors to form and cancer cells to adapt to a hostile environment. Despite their importance, ferlins have not been extensively studied in the context of cancer. However, recent research has shown that all ferlin genes are modulated in different types of cancer (Peulen et al., 2019). Myoferlin and fer114 genes are more commonly upregulated (Barnhouse et al., 2018; You et al., 2020). Myoferlin has been found to be highly expressed in several types of cancer, including kidney, liver, pancreatic, breast, and head and neck squamous cell carcinomas (Wang et al., 2013; Kumar et al., 2016; Blomme et al., 2017; Hermanns et al., 2017; Song et al., 2017).

### 8.2. Myoferlin

The literature on ferlins in cancer only started about 10 years ago, although the proteins have been described for over 20 years. Most of the work has focused on myoferlin, which appears to be the ferlin with the most important role in cancer. The fact that ferlin expression can be either a good prognostic factor (breast cancer) or a poor prognostic factor (kidney, head and neck cancer) is one of the most surprising elements.

In the study of Yadav et al. (2017) which shows that when cells are treated with IL-6, myoferlin dissociates from EHD2 and binds to activated STAT3, a protein involved in cell signaling. The study found that depletion of myoferlin did not affect STAT3 phosphorylation, but blocked its translocation to the nucleus. The study also found that myoferlin knockdown significantly reduced IL-6-mediated tumor cell migration, tumor sphere formation, and the population of cancer stem cells *in vitro*. In addition, myoferlin knockdown significantly reduced IL-6-mediated tumor growth and metastasis. In 211 patients with head and neck squamous cell carcinoma (HNSCC), Kumar et al. (2016) investigated the association of myoferlin with disease progression and patient outcome. The results showed that nuclear myoferlin expression is associated with poor overall survival and an increased risk of death, as well as tumor recurrence, perineural invasion, extracapsular spread, a higher T stage, and distant metastasis. The study also found a direct association between nuclear myoferlin expression and IL-6 and an inverse association with HPV status. Patients with both nuclear myoferlin expression and high levels of IL-6 and those with HPV-negative/myoferlin-positive tumors had the worst survival. These results suggest that nuclear myoferlin expression independently predicts poor clinical outcomes in these patients.

By modeling the effect of myoferlin on tumor cell invasion through altered regulation of metalloproteinase production, Eisenberg et al. (2011) identified a role for myoferlin in promoting invasive behavior in breast cancer cells. The identification of myoferlin as a key regulator of EGFR (Epidermal Growth Factor Receptor) activity through inhibition of non-clathrin endocytosis in breast cancer cells was important in understanding the molecular mechanisms involved in cancer growth (Turtoi et al., 2013). The production of myoferlin-silenced tumor cells has provided another element that may help explain the role of ferlin: these tumors lacked functional blood vessels, an effect that may be due to a reduction in

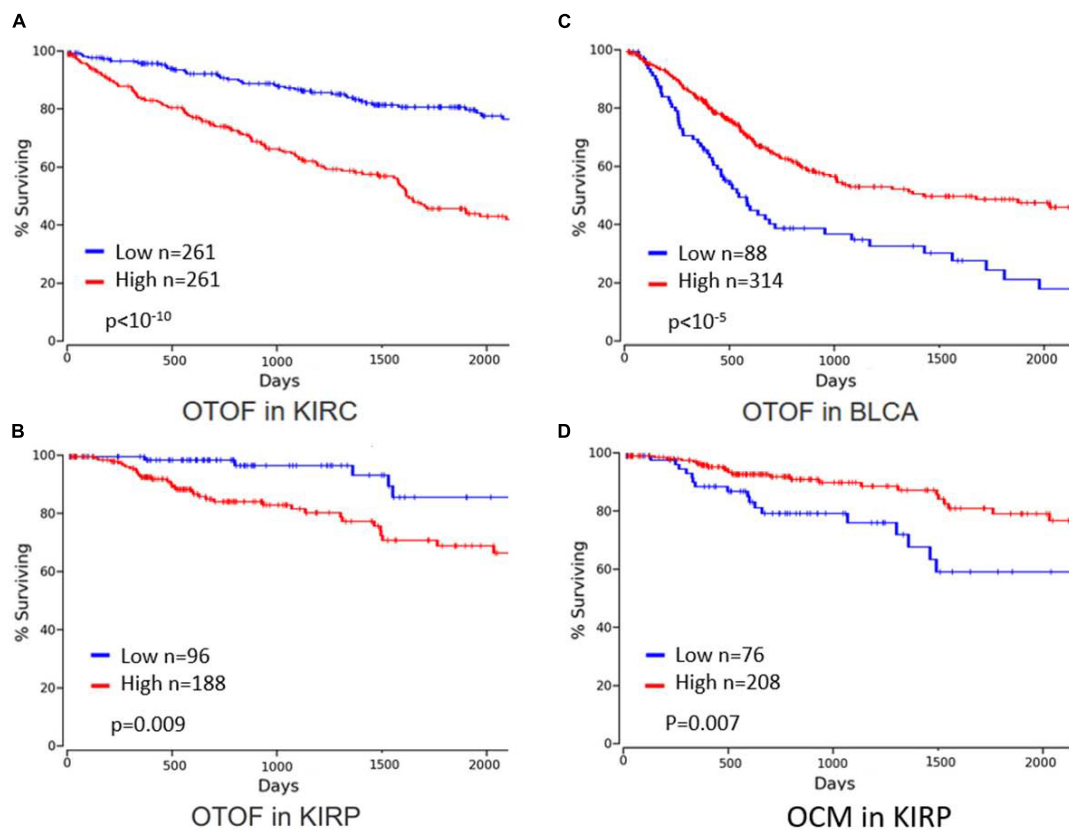


FIGURE 11

Survival data linked to otoferlin and oncomodulin expression levels (graphs were built after bioinformatics analysis using [OncoLnc, 2023](#)). Year overall survival was better for patients with lower otoferlin gene expression in (A) kidney renal clear cell carcinoma (KIRC) and (B) kidney renal papillary cell carcinoma (KIRP), whereas survival was better for patients with higher otoferlin gene expression in (C) bladder urothelial carcinoma (BLCA). High levels of oncomodulin (OCM), a parvalbumin-family calcium-binding protein highly expressed in hair cells, in KIRP (D) is associated with better 5-year overall survival.

VEGFA exocytosis ([Fahmy et al., 2016](#)). This may explain its strong association with cancer prognosis, as an independent prognosis factor in kidney ([Song et al., 2017](#)).

### 8.3. Otoferlin

Currently, there is limited information available on otoferlin. Involvement of IL-6 in inner ear damage during noise trauma or cisplatin treatment has been reported ([So et al., 2007](#); [Wakabayashi et al., 2010](#)), but a possible link with otoferlin function during disease progression has not yet been explored. Using OncoLnc, a tool for exploring correlations between the expression of mRNAs, mRNAs, and lncRNAs, we analyzed 5-year overall survival in 21 cancer types ([Anaya, 2016](#)). In three cancers, we found a significant impact of otoferlin expression on survival: in renal clear cell, papillary cell carcinoma, and in bladder urothelial carcinoma ([Figure 11](#)). Interestingly, otoferlin expression was either a protective or a risk factor depending on the histology of the cancer. Given the differences in 5-year survival, it seems appropriate to consider using otoferlin expression as a prognostic biomarker to guide therapeutic decisions.

In clear cell renal cell carcinoma, [Cox et al. \(2021\)](#) showed in a study of 79 patients that otoferlin was an indicator of tumor

staging and a prognostic biomarker for cancer-specific survival. In 25 patients with oral squamous cell carcinoma, Kraus showed complete suppression of otoferlin expression in malignant tissue ([Kraus et al., 2022](#)). Taken together, these results suggest a role for otoferlin in the carcinogenesis of these tumors, which deserves investigation to confirm and understand its exact role, especially as it appears to vary according to tumor site. Studies are needed to ensure that otoferlin is an independent prognostic factor for overall survival. This could lead to new cancer therapies targeting this protein, which are already underway for myoferlin ([Zhang et al., 2018](#); [Li et al., 2019](#); [He et al., 2021](#)).

### 8.4. Oncomodulin

Hair cells, notably OHCs, highly express another protein, which has also been used as a prognostic marker in cancer: oncomodulin (OCM). OCM is a small EF-hand  $\text{Ca}^{2+}$ -binding protein (CaBP) of approximately 12 kDa. It belongs to the parvalbumin family. It is the  $\beta$ -isoform of parvalbumin. It shares at least 53% sequence identity with alpha-parvalbumin (PVALB) ([Climer et al., 2019](#)). OCM has an unusually restrictive post-embryonic expression pattern in mammals, mostly restricted to subsets of sensory hair cells in the inner ear and more recently found in certain subtypes

of immune cells. Oncomodulin is essential for normal hearing function since its deletion in mice leads to progressive deafness due to alterations in the mechanical amplification function of OHCs (Tong et al., 2016). We recall that OHC lateral mechanical stiffness and motility have long been recognized as regulated by calcium acting on the actin-based cortical cytoskeleton (Dulon et al., 1990). Oncomodulin, acting as a CaBP, may be essential in sculpting calcium signals important for F-actin remodeling and membrane mechanics in OHCs, a role somewhat similar to that of otoferlin in IHCs, as proposed in our review.

The initial discovery of OCM as an oncoprotein in cancer tissue and its similarity to calmodulin as a CaBP led to the term "oncomodulin" (Durkin et al., 1983). OCM was considered oncogenic because no expression was detected in normal post-embryonic tissue. However, after its initial discovery, OCM was identified as a major protein in sensory cells of the cochlea. Studies have suggested that OCM may play a role in cell proliferation, and recently, Yin et al. (2006) identified oncomodulin as a potent macrophage-derived growth factor for retinal ganglion cells (RGCs) and other neurons. Oncomodulin also stimulates the outgrowth of peripheral sensory neurons.

## 9. Discussion-conclusion

Otoferlin, a protein mainly produced in the brain and inner ear, plays a key role in hearing. More than 220 mutations causing DFNB9-type deafness have been identified so far. Many of these mutations alter the folding, stability, or function of the protein, resulting in severe to profound hearing loss associated with a defect in synaptic transmission between the IHC and the nerve fibers of the auditory nerve.

An intriguing question is: why have auditory and vestibular hair cells selected otoferlin as a key  $\text{Ca}^{2+}$ -sensor to control exocytosis of synaptic vesicles instead of the classical synaptotagmins found in central synapses? The need to encode tiny graded microphonic potentials spanning over a large range of amplitude (several dozens of dB) by graded multivesicular release at each ribbon synapses and the requirement to sustain an extremely high rate of vesicle release by an efficient vesicular replenishment are probably the two main factors explaining the use of a large multi-C2  $\text{Ca}^{2+}$  sensing protein such as otoferlin. Another important property of otoferlin is its capability to function as a high affinity large-range  $\text{Ca}^{2+}$  sensor as compared to synaptotagmins. In our review, we propose otoferlin as a  $\text{Ca}^{2+}$ -dependent mechanical interactor between the membranous F-actin network and the synaptic vesicles at the hair cell ribbon. This protein would confer  $\text{Ca}^{2+}$ -dependent oscillatory movement of the ribbon, whose resonant frequency would depend on their size (mass), tuning the firing frequency of the postsynaptic fibers.

In this context, it is important to note that otoferlin belongs to the ferlin protein family in humans: dysferlin, myoferlin, fer1L4, fer1L5, and fer1L6, all of which being expressed in mechanically active or contracting cells. These proteins share common features such as the presence of multiple C2 domains, FerA domains, and membrane anchoring through their C-terminal domain, and are thought to play a role in calcium-triggered membrane fusion and trafficking. Although otoferlin is expressed in the central

nervous system, in particular in the cortex and amygdala, its role in brain tissues remains unknown. Interestingly, a genome-wide expression analysis of a human-mouse chimeric model of Alzheimer's disease identified OTOF as a downregulated gene, suggesting a critical function of otoferlin in the central nervous system. Dysferlin has been observed to accumulate in endothelial cells adjacent to sclerotic lesions and within amyloid-beta plaques in individuals with Alzheimer's disease. These findings highlight the need for further research into the role of ferlin proteins in the central nervous system, particularly their potential involvement in neurodegenerative diseases such as Alzheimer's disease.

Also, otoferlin, like many of the other ferlins, plays a crucial role in membrane-related processes that are important for cell survival and signaling. Studies have suggested a link between ferlins and cancer through their effect on cell signaling and development, allowing tumors to form and cancer cells to adapt to a hostile environment. Despite their importance, ferlins have not been extensively studied in the context of cancer. Data show that otoferlin expression is significantly associated with survival in specific cancer types, including clear cell and papillary cell renal carcinoma, and urothelial bladder cancer. These results suggest a role for otoferlin in the carcinogenesis of these tumors, which needs further investigation to confirm and understand its exact role, especially as it appears to vary according to tumor site. This could lead to new cancer therapies targeting this protein.

## Author contributions

J-CL and DD wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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