Cloning the zebrafish otoferlin b wildtype and a transmembrane mutant to characterize changes in cellular localization

by
Rebecca France

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Molecular Biology
(Honors Scholar)

Presented April 23, 2021
Commencement June 2021

AN ABSTRACT OF THE THESIS OF

Rebecca France for the degree of Honors Baccalaureate of Science in Biochemistry and Molecular Biology presented on April 23, 2021. Title: Cloning the zebrafish otoferlin b wildtype and a transmembrane mutant to characterize changes in cellular localization.

Abstract approved:_____

Colin P. Johnson

Otoferlin is a protein that is essential in the process of hearing. Mutations in otoferlin are associated with profound recessive deafness. The long-term goal of the Johnson lab is to understand how otoferlin contributes to hearing and why certain mutations result in deafness. This project sought to characterize the difference between wildtype and mutant otoferlin i*n vitro* to go alongside *in vivo* studies on mutant zebrafish presenting with deafness and vestibular effects. The wildtype otoferlin was cloned from zebrafish DNA, and site directed mutagenesis introduced an early stop codon in the transmembrane domain to create the mutant otoferlin. Preliminary immunofluorescence results indicate that wildtype otoferlin appears to associate with the membrane to a greater extent than mutant, the mutant otoferlin traffics differently in the cell, and is possibly degraded faster than wild type. These results and future studies will further our understanding of the specific role of otoferlin in the process of hearing and why this particular nonsense mutation results in deafness.

Key Words: otoferlin, ferlins, zebrafish, HEK293, vesicle trafficking

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APPROVED:

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Rebecca France, Author

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Introduction

Ferlins are a family of large (~200-240 kDa), eukaryotic calcium binding proteins that play an important role in membrane trafficking events (Lek et al., 2010). Members of the ferlin family have similar overall structures, with four to seven C2 domains that interact with calcium, lipids, membranes, and other proteins. C2 domains are known to bind lipids, often in a calcium dependent manner, and proteins with tandem C2 domains are rare (Marty et al, 2013). Ferlins also have a single transmembrane domain at the C-terminus. It has been established that ferlins regulate membrane fusion events in a calcium dependent manner, but much remains unknown about the mechanism (Johnson, 2017). Humans have six ferlin genes, *FER1L1/DYSF* (dysferlin), *FER1L2/OTOF* (otoferlin), *FER1L3/MYOF* (myoferlin), *FER1L4* (Fer1L4), *FER1L5* (Fer1L5) and *FER1L6* (Fer1L6) (Bulankina & Thomas, 2020). Mutations in ferlins have been associated with a number of pathologies in both humans and model organisms such as mice or zebrafish.

Dysferlin, which is enriched in skeletal and cardiac muscle tissue, appears to play an important role in the transverse tubule system and sarcolemma repair (Otto et al., 2020). Mutations in dysferlin have been associated with limb girdle muscular dystrophy, Miyoshi myopathy, and cardiomyopathy (Johnson, 2017, Bansal et al. 2003). Myoferlin is thought to play a role in muscle development (Doherty et al., 2005) and cancer cell invasion (Eisenberg et al., 2011). Fer1L4 encodes for a non-protein coding RNA and is expressed primarily in the stomach. (Redpath, 2016). Fer1L5 is expressed in myoblasts undergoing fusion and is thought to act as a mediator of endocytic recycling during myotube formation (Posey, 2011). Studies in zebrafish have shown that Fer1L6 plays an essential role in zebrafish skeletal and cardiac muscle development (Bonventre et al., 2019).

Due to the difficulty of using biophysical techniques like X-ray crystallography and Nuclear Magnetic Resonance to elucidate the structure of integral membrane proteins, much remains unknown about the structures and the precise functions of ferlins. By understanding more about the specific roles of ferlins in membrane trafficking events, it will become possible to address the human pathologies caused by mutations in ferlins. This project specifically focuses on otoferlin, in which loss of function mutations which are associated with a recessive form of profound hearing loss in humans (DFNB9) which accounts for 2–8% of all cases of congenital genetic deafness. Around 1 in 1000 children under the age of 2 are affected by deafness, many of them due to genetic nonsyndromic deafness, the majority of which are inherited in an autosomal recessive mode (Yasunaga et al., 1999). However, the size of otoferlin (~6 kb) makes it very difficult to use gene therapy as a treatment. Therefore, it is imperative to study how otoferlin contributes to the process of hearing and why certain mutations result in deafness.

Otoferlin (240 kDa) has six C2 domains and a transmembrane domain (Figure 1A), and is expressed in inner, outer and vestibular hair cells, as well as certain regions of the brain (Chatterjee et al., 2015). The cochlea, a snail shaped structure, is the part of the inner ear that is involved in hearing. The cochlea contains inner hair cells, which serve as the receptors for both the auditory and vestibular systems (Figure 1B). During the process of hearing, sound waves mechanically stimulate stereocilia, the protrusions at the apical end of inner hair cells. This results in an influx of potassium ions inside the cell, which leads to membrane depolarization and the opening of voltage gated calcium channels at the opposite, or basal, end of the hair cell. The subsequent influx of calcium ions in the cell triggers the release of vesicles containing neurotransmitters at the presynaptic membrane (Figure 1C).

Vesicles are structures used to transport cellular components, and this release of the contents inside the vesicles at the membrane is known as exocytosis. This neurotransmitter release at the presynaptic membrane transmits the signal to the brain via the afferent neuron and allows us to hear. Otoferlin is known to bind calcium and is therefore believed to play a role in this calcium dependent exocytosis that leads to neurotransmitter release. However, the mechanism by which this happens remains poorly understood.

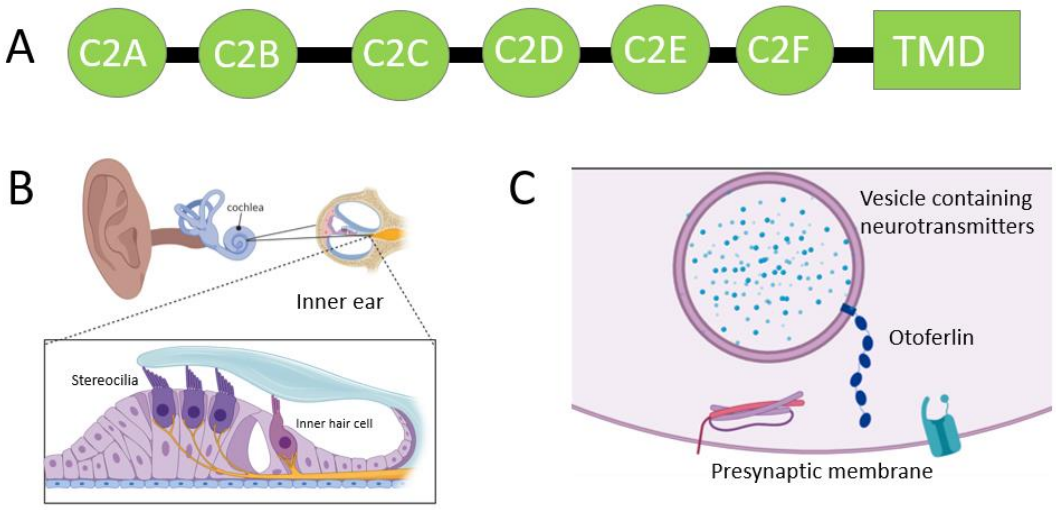


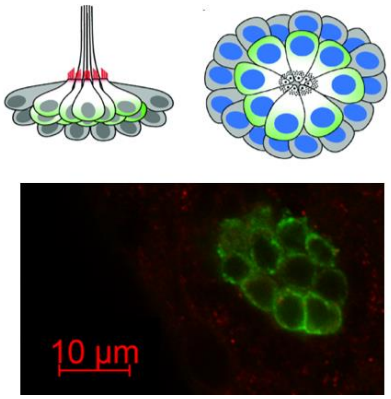
Figure 1. Otoferlin as a multi C2 domain protein. **A)** Schematic of the six C2 domains of otoferlin and the transmembrane domain. **B)** Diagram of the cochlea, inner ear, and inner hair cells, where otoferlin is expressed. **C)** Otoferlin binding to a vesicle with neurotransmitters right before it releases at the presynaptic membrane, which results in hearing.

It is very difficult to extract otoferlin from humans so our lab uses zebrafish (*Danio rerio)* as a model organism to study otoferlin *in vivo.* Traditionally, mouse is used as the model organism, but extraction of cochlea, a bony structure, makes it a challenging model for otoferlin. Zebrafish have neuromasts containing otoferlin that are conveniently located on the lateral line of the zebrafish, which is close to the outside surface. The amino acid sequence between humans and zebrafish is relatively conserved, which means studying otoferlin in zebrafish is relevant to the human version of otoferlin. Due to a genome duplication event,

zebrafish express two different otoferlin genes. Otoferlin b is encoded on chromosome 17 and has a 74% sequence identity with human otoferlin, while otoferlin a is encoded on chromosome 20 and has a 76% sequence identity with human otoferlin. Otoferlin b does not have a C2A domain. Otoferlin a and otoferlin b are both found in the otic placode, which gives rise to the inner ear. Otoferlin b is also found in the neuromasts along the lateral line of the zebrafish. Otoferlin knockdown zebrafish present with severe balance issues and uninflated swim bladders, suggesting that otoferlin plays an important role in balance as well (Chatterjee et al., 2015).

Others in the lab are characterizing zebrafish with a truncated version of otoferlin that's missing most of its transmembrane domain (Figure 2). The goal of this project is to compare the truncated otoferlin (MUT) to the wildtype (WT) in cell cultures, or *in vitro*, to see if the effects of this particular mutation on cellular localization. By exogenously expressing both WT and MUT otoferlin in human embryonic kidney (HEK) cells in culture and using immunofluorescence, a technique that can be used to visualize proteins in a sample with the use of conjugated fluorescent proteins and fluorescent stains, it will be possible to investigate changes in cellular localization. It is hypothesized that since the mutants lack most of the transmembrane domain, it will not be able to anchor to the membrane and attach to vesicles. Therefore, MUT otoferlin will be soluble and trafficked differently within the cell, compared to the wild type, and may follow a pathway in the cell that leads to protein degradation.

Figure 2. Depiction of the WT and MUT zebrafish otoferlin B constructs used for immunofluorescence studies. The WT has the C2F domain and the full transmembrane domain. The MUT has a nonsense mutation early in the transmembrane domain and is therefore missing most of it. The MUT is expected to be trafficked differently in the cell and potentially degraded.



Species	Otoferlin C2 domains (% identity with human)					
	C2A	C2B	C2C	C2D	C2E	C2F
Mouse	91	100	100	100	98	98
Rat	92	100	100	99	98	98
Rabbit	95	99	99	99	100	-
Guinea-Pig	94	99	98	100	99	98
Frog	-	78	91	91	94	96
Zebrafish						
1)otoferlin a	-	91	89	83	88	94
2)otoferlin b	77	91	89	83	92	95

Figure 3. Zebrafish neuromasts, where otoferlin is expressed along the lateral line. The table shows the sequence identity of otoferlin in different species. Images from Chatterjee et al., 2015.

Materials and Methods

Zebrafish and RNA Extraction

Total RNA was extracted from Tropical 5D strains of zebrafish (*Danio rerio)* by Dr. Josephine Bonventre. The strains of zebrafish used for this study were reared according to Institutional Animal Care and Use Committee protocols at the Sinnhuber Aquatic Research Laboratory, Oregon State University.

Green Fluorescent Protein Cloning into pcDNA

To create a pcDNA vector with GFP, a polymerase chain reaction using primers *Kpn1* and *HindIII* was used to amplify GFP. The PCR reaction included 1 uL of GFP F HindIII, 1 uL of GFP R Kpn 1, 2 uL of GFP template, 25 uL of DreamTaq, 21 uL water. A 1% agarose gel confirmed the expected size of 717 base pairs. The PCR product was purified using PureLink™ PCR purification kit. The PCR product and the pcDNA vector were then digested separately using 5 uL of Cutsmart buffer, 10 uL of purified PCR product/pcDNA vector, 2 uL of HindIII, 2 uL of KpnI, 31 uL of H_2O and ten incubated for 1.5 hours in 37 C water bath. PCR purified. The GFP digested product was then ligated into the linearized pcDNA vector using 2 uL of 5X Ligation Buffer, 2 uL of pcDNA, 6 uL of purified GFP PCR product, 0.5 uL of ligation enzyme, incubated at room temp for one hour. Next, 5 uL of plasmid was transformed into 70 uL of DH10B *E. coli* cells and plated on Lysogeny Broth agar plates with Ampicillin (0.1 mg/mL). A colony PCR was performed on 10 colonies, using 2.75 uL H2O, 2.75 uL Dreamtaq, 0.1 uL of GFP F HindIII, 0.1 uL of GFP R Kpn (Figure S1). There were positive results for colonies 3, 5, and 9. An overnight with 5 mL of Lysogeny Broth with ampicillin was set for colony 5, which was then miniprepped using the

PureLink™ Quick Plasmid Miniprep Kit. Genscript sequencing was performed to verify the correct GFP sequence.

Transfection into HEK293 cells

HEK 293A cells were grown using DMEM, 10% Fetal Bovine Serum, 0.2% Penicillin Streptomycin and 5% CO_2 at 37 C. The media was changed one hour before transfecting and deprived antibiotic, and then the cells were split. For transfecting a 6 well plate, per well, 300 uL Opti-MEM media, 2 mg DNA (miniprepped pcDNA-GFP), 4 uL Lipofectamine, 3 uL of P 3000 uL was used. 6 hours after transfecting, the media was changed to DMEM + FBS with no antibiotic. For imaging the cells transfected with otoferlin containing constructs, a DAPI nuclear stain was used in addition to a red membrane stain. Images of the cells were taken with a Keyance microscope (Figure S2).

Attempts to clone FL zfOtoB using Restriction Enzymes

The sequence to clone the full length otoferlin was retrieved from the National Center for Biotechnology Information. The first attempt to clone otoferlin with traditional restriction enzymes had KpnI primers in both directions because KpnI was the only cut site available (OtoB R Kpn1 and OtoB F Kpn1). The PCR product had an expected size of 5.5 kB. Attempts to clone the full length otoferlin included a 7 minute extension time at 58C with both DreamTaq and GoTaq, multiple PCR gradients with extension times varying from 7 minutes to 10 minutes, multiple different types of cDNA, and different primers using the sequence from Ensembl. Attempts were also made to clone the full length OtoA using ZF OtoA KpnI FWD and ZF OtoA BamHI REV. It was confirmed that the OtoB R Kpn1 primer worked with otoB TM-Nco1 forward primer (Figure S3). It was also confirmed that OtoB F Kpn1 worked using different sequencing primers.

cDNA Preparation

Zebrafish cDNA was prepared using the Applied Biosystems ThermoFisher Scientific, High-Capacity cDNA Reverse Transcription Kit. For a 20 uL reaction, 2 uL of 10X RT Buffer, 0.8 uL 25X dNTP Mix (100 mM), 2 uL 10X RT Random Primers, 1 uL of MultiScribeTM Reverse Transcriptase, 4.2 uL of water, and 10 uL of RNA (AB-WT 120 hpf 11-22-16) extracted from zebrafish was used.

Full length cloning of zfOtoB in parts

After several unsuccessful attempts to clone the full length otoferlin with traditional restriction enzyme cloning, it was decided to attempt to clone smaller, overlapping sections (at least 15 base pairs of overlap) of the full length protein and to try to stitch them together. All primers pairs were designed to have melting points within 5 degrees of each other, with over 50% GC content, and a melting point below 70 degrees. Section 1 was amplified with the LIC zfotoB F and Th 1A R primers, Section 2 was amplified with the Th 1A F + Th 2BR primers, and Section 3 was amplified with the Q 3F + LIC zf otoB R primers. Attempts to stick them together including trying with both 1 uL of each purified PCR product and with 5 uL of PCR product. The agarose gels showed mostly smears, but there was a band around the expected size of 5.5 kB (Figure S4), which was gel extracted using the Purelink™ Quick Gel Extraction kit. This was used to try to seed a new PCR reaction, but that just resulted in a smear. Due to the inability to get a clear band for the full length otoferlin, it was decided to proceed with the C2F-TMD constructs instead. Future plans include returning to try to clone the full length protein.

PCR for C2F-TM

The C2F domain with the transmembrane domain (C2F - TMD) and the C2F domain alone were amplified through a PCR (Figure S5). The primers used to amplify the C2F - TMD were the LIC zf C2F primer and the LIC zf otob R primer. For 50 uL PCR reactions, 25 uL DreamTaq, 5 uL zf cDNA, 2.5 uL forward primer, 2.5 uL reverse primer, 1 uL DMSO, and 14 uL H_2O was used.

Ligation Independent Cloning

The pcDNA3-eGFP-LIC vector was purchased from Addgene. Forward and reverse primers were designed to include LIC sequence. To LIC treat the PCR product, 5 uL of the PCR product, 2 uL of T4 buffer, 0.2 uL of T4 DNA Polymerase, 0.5 uL of dCTP (0.5 uL), 11.3 uL water, and 1 uL of 100 mM DTT, was incubated at 22C for 40 minutes and 75C for 20 minutes. The *SSP* restriction enzyme for pcDNA plasmid used with 2.1 uL of pcDNA eGFP miniprepped vector, 2 uL of NEB SSPI enzyme, 5 uL of 10x NEB Buffers SSPI, 40.9 uL sterile H_2O and was incubated at 37C for 2 hours, then ran on an agarose gel and gel extracted using the PureLink^{TM} Quick Gel Extraction Kit. For LIC treating the pcDNA eGFP vector, 10 uL of the linearized vector, 2 uL of T4 buffer, 0.2 uL of T4 DNA Polymerase, 0.5 uL of dGTP, 6.3 uL water, and 1 uL of 100 mM DTT was incubated at 22C for 40 minutes and 75C for 20 minutes. To put the insert in the vector, 3 uL of digested pcDNA GFP plasmid with 3 uL of amplicon for each WT C2F-TM, WT C2F, and MUT C2F-TM and 14 uL of water was incubated for 30 minutes at room temperature.

Site Directed Mutagenesis - Quikchange

Agilent Technologies QuikChange II Site-Directed Mutagenesis Kit was used for Site Directed Mutagenesis. The reaction combined 10 uL of 10x Pfu buffer, 2 uL of miniprepped C2F, 2 uL of QC MUTotoB F + R primers (2612/2613), 1 uL of 10 mM dNTPs, 39 uL of

H_2O, and 1 uL of Pfu UltraHF polymerase. After running the thermal cycle protocol, 1 uL of DpnI was added and incubated at 37C for 1 hour

Transformation and Miniprepping

E. coli DH10β cells were transformed with the WT C2F-TMD, MUT C2F-TMD, and the C2F control and plated on Lysogeny Broth agar plates with ampicillin (0.1mg/mL). 6 uL of ligation product was transformed. Selected positive colonies and did a colony PCR. Colonies 1, 2, 3, 4, and 5 were miniprepped for the WT C2F-TMD. Colony 1 was selected for Site Directed Mutagenesis after reviewing the sequencing results. 1 uL BGA reverse primer was used to sequence, along with 10 uL of plasmid and 4 uL of H_2O.

Cell Image Analysis

Three representative images were selected for both the WT C2F-TM plasmid transfected cells and the MUT plasmid transfected cells. The total number of cells in each image was counted. Cells that were cut off in the photo were not counted. The number of cells that showed colocalization by presenting a mixture of green, red and blue were then counted for each image. The percentage of cells containing otoferlin was calculated by dividing the cells showing colocalization by the total number of cells. This process was repeated twice more. Error bars were added by calculating the standard error between the images for each category.

Discussion

The original goal of this project was to clone the full length zebrafish otoferlin b, and then to create a mutant with an early stop codon that imitates the mutant zebrafish strain that our lab uses. However, after several unsuccessful attempts to clone the full length protein, it was decided to proceed with cloning the WT C2F domain with the transmembrane domain, the C2F domain with a transmembrane mutant that has an early stop codon, and the C2F domain alone to use as a control. After successfully cloning the WT C2F-TM, through site directed mutagenesis, an otoferlin mutant was created that has a nonsense mutation leading to an early stop codon after the fourth amino acid in the transmembrane domain and will therefore be missing most of its transmembrane domain. Next, the wildtype and mutant otoferlin miniprepped plasmids were transfected in human embryonic kidney cells in culture to study changes in cellular localization using live cell fluorescence. The wildtype otoferlin was expected to localize to the plasma membrane. It was hypothesized that since the mutant lacks most of the transmembrane domain, it will not be able to anchor to the membrane and attach to vesicles. The mutant otoferlin is expected to be soluble and trafficked differently within the cell, compared to the wild type, and may follow a pathway in the cell that leads to protein degradation. However, the mutant might also localize to the membrane because it still has a small portion of the transmembrane domain. The control with just the C2F domain alone is expected to float around inside the cell.

When imaging the cells, a red membrane stain was used along with a blue nuclear stain. For both the wildtype and mutant, the GFP fluoresced in the transfected HEK 293 cells, indicating successful cloning (Figures 4 and 5). The WT demonstrated higher percentage of cells (7.90%) uptake the pcDNA plasmid containing otoferlin than the MUT (6.37%) despite identical concentrations of plasmid used in the transfection. A T-test was performed to

determine the significance, which found that the results were not statistically significant, $t(4) = 0.889$, $p = 0.21$ (Figure 6). It is possible that the MUT otoB is degraded by the cell. This transfection should be repeated to ensure that there was not an error in the amount of plasmid being added, and that the differences in expression level are due to the MUT being degraded by the cell. Additional image analysis should be done in case the four images used each for the WT and MUT are not representative of the plate as a whole. Future work also needs to include transfecting the C2F domain alone. Cell fractionation could be used to determine if there is a higher amount of the MUT protein in the lysosomes of the cell than the WT, further implicating that the MUT gets degraded.

Interestingly enough, while cloning the WT C2F-TM, two of the five colonies selected for a colony PCR contained the same early stop codon mutation that was going to be attempted to create through site directed mutagenesis. The *E. coli* colonies appeared to have spontaneously mutated. Future work may include testing these colonies with the early stop codon mutation with the construct created through site directed mutagenesis and see if there are any differences.

One strategy used to try to clone the full length otoferlin was to clone smaller, overlapping sections and see if they assemble together to create the full length protein. Three overlapping sections were successfully cloned that included the entire full length of the protein, but so far attempts to get the sections to assemble *in vitro* have been unsuccessful (Figure S4). Future work on this project would include resuming attempts to clone the full length protein and get those three sections to assemble. Successfully cloning and transfecting the full length otoferlin b in HEK293 cells would provide a more accurate representation of the protein as a whole, as the other domains may play a significant role in cellular

localization as well. Studying constructs containing otoferlin in cell cultures will allow us to characterize otoferlin localization *in vitro* for both the wildtype and mutant and learn more about this particular mutation as it relates to hearing loss. For any future designed gene therapy to treat deafness, the transmembrane domain seems to be a critical component. Ultimately, the long-term goal is to be able to help inform any future treatments to recover hearing in individuals that are affected by mutations in otoferlin.

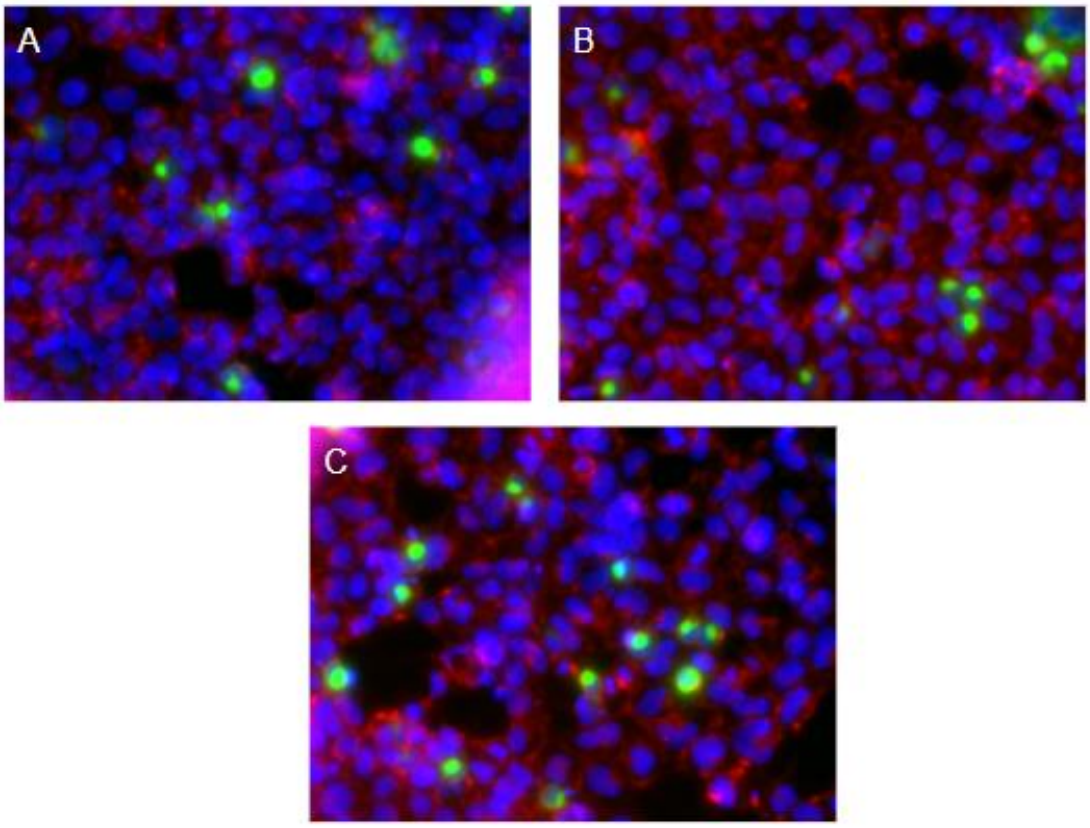


Figure 4. Overlay images of HEK293 cells transfected with WT zfotoB C2F - TM. A red membrane stain and a blue nuclear stain were used to visualize the cells. The overlay image indicates that at least some of the cells successfully took up the plasmid containing GFP and otoferlin.

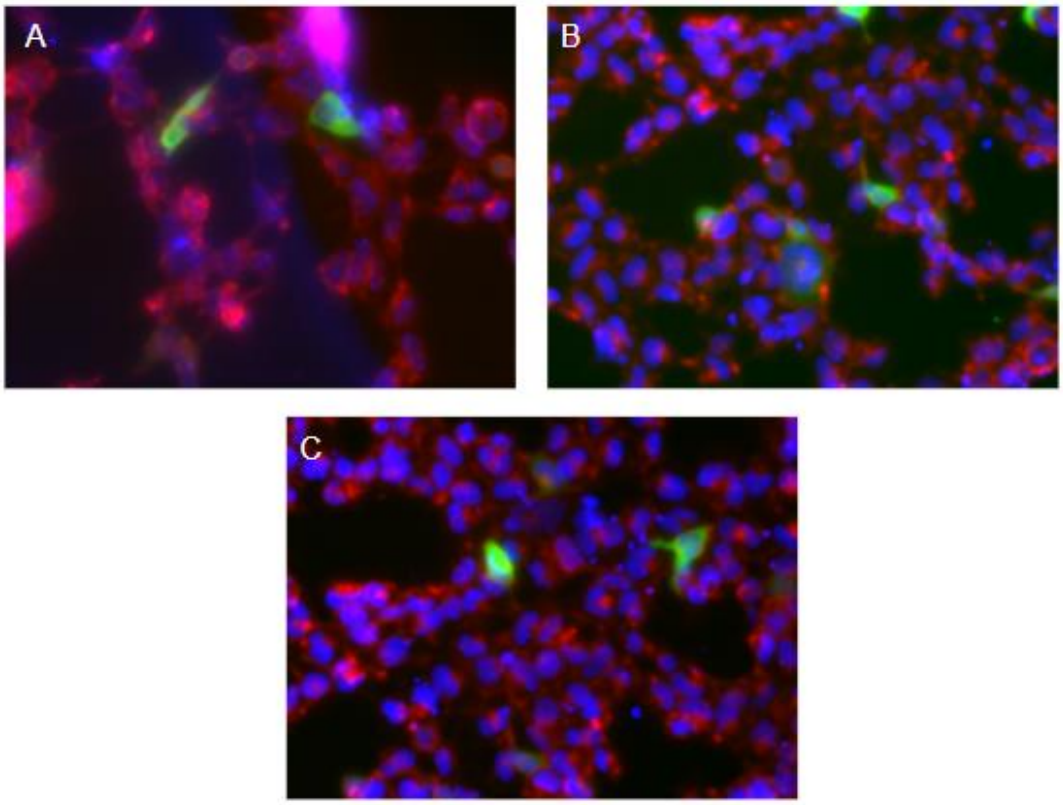


Figure 5. Overlay images of HEK293 cells transfected with MUT zfotoB C2F plasmid that is missing most of its transmembrane domain. Similarly to the WT, the overlay image indicates that at least some of the cells successfully took up the plasmid containing GFP and otoferlin.

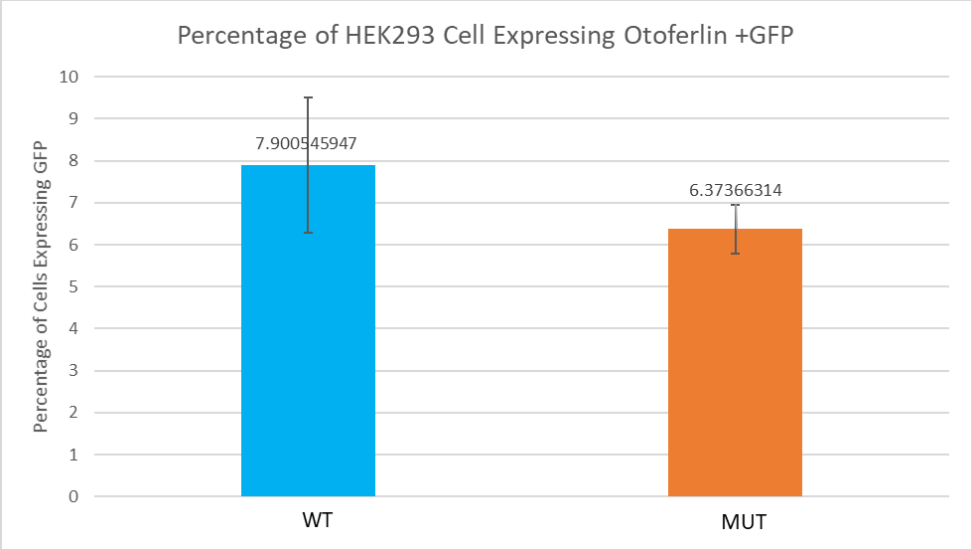


Figure 6. The percentage of imaged cells that showed green fluorescence localization with the red and blue fluorescence. The WT (M = 7.90, SD = 2.80) demonstrated a higher percentage of cells uptake the pcDNA plasmid containing otoferlin than the MUT (M = 6.37, SD = 1.00), but the results are not statistically significant, $t(4) = 0.889$, $p = 0.212$. Error bars represent the differences in counting the total number of cells in the three replicates.

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Supplemental Figures

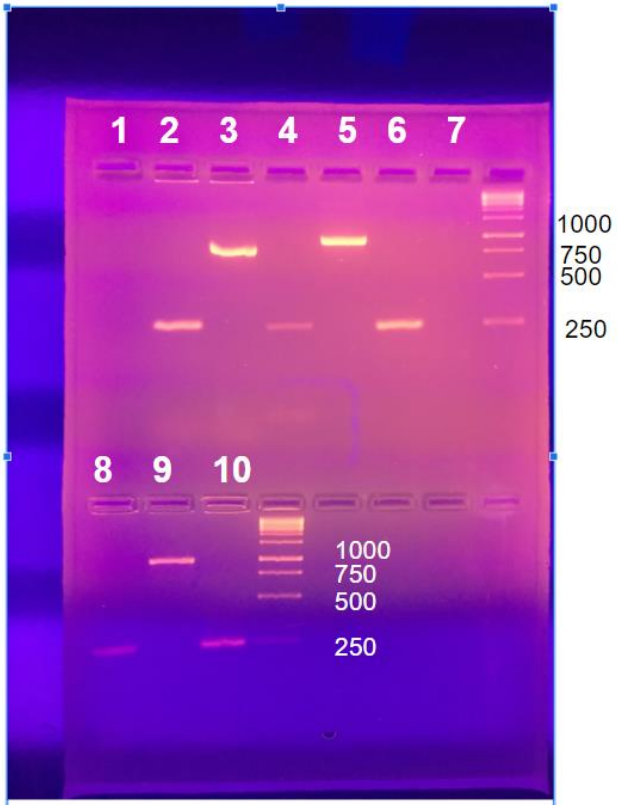

Figure S1. Colony PCR of GFP (717 bp). Colonies 3, 5 and 9 indicated positive clones.

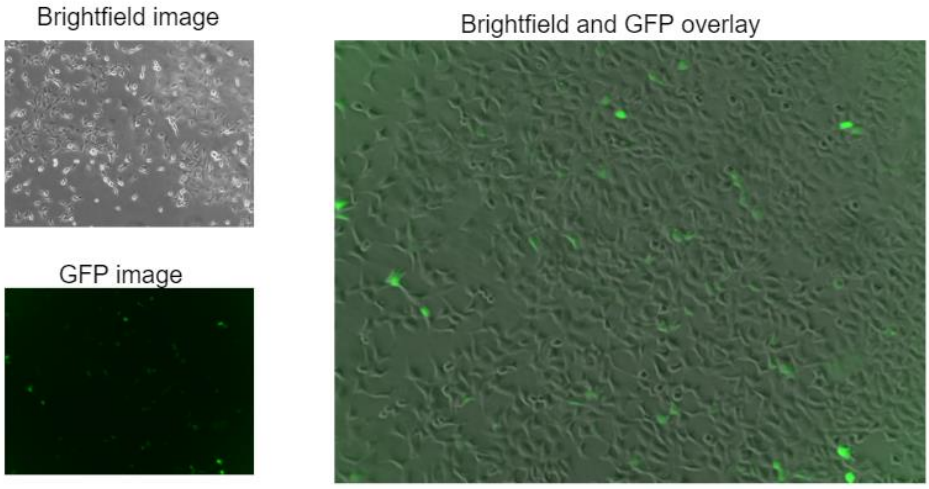

Figure S2. Brightfield and GFP images of HEK293 cells after being transfected with the plasmid pcDNA containing N-terminal GFP. The overlay image indicates that at least some of the cells uptook GFP and that the cloning and transfection of GFP and pcDNA was successful.

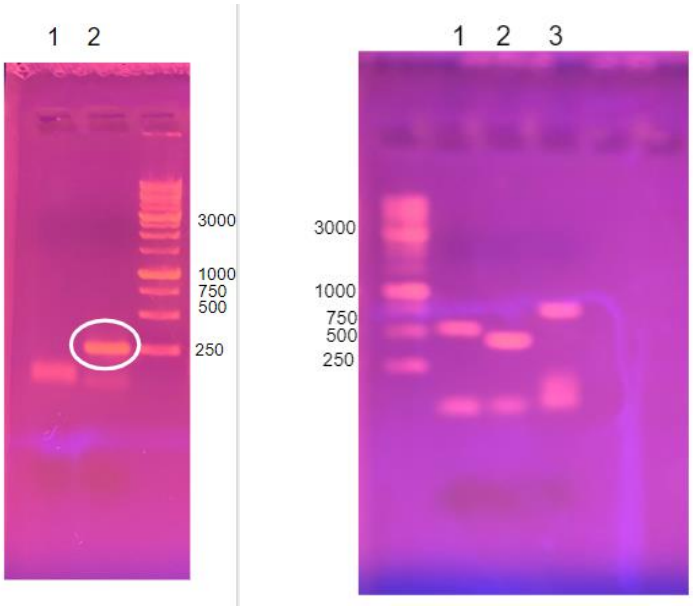

Figure S3. Agarose gels to confirm that the primers to amplify the full length zf otoB were effective. The gel on the left was for the OtoB R Kpn1 primer with otoB TM-Nco1 forward primer and showed the expected band size around 250 bp. The gel on the right was for OtoB F Kpn1 with different sequencing primers. The expected band sizes were 550 bp for lane one, 441 bp for lane 2, and 769 bp for lane three.

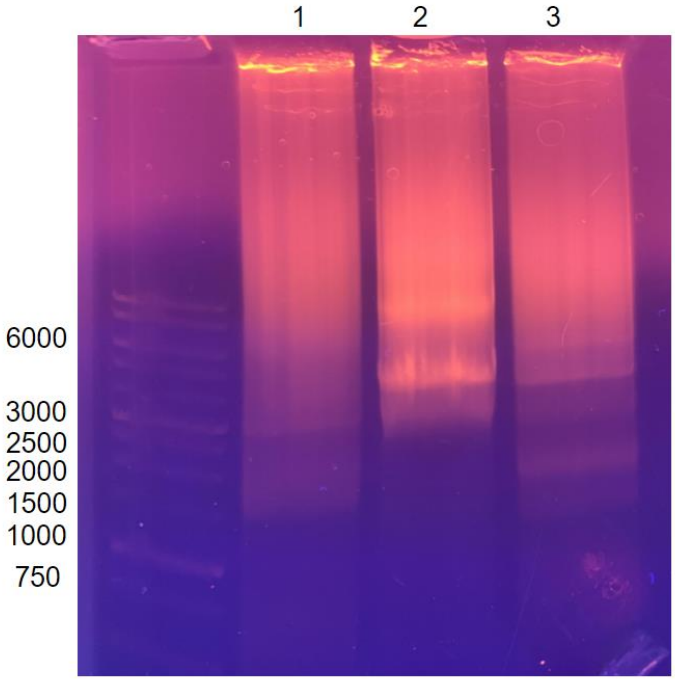

Figure S4. Agarose gel for full length otoferlin. Lane 2 (lower concentration of DNA) did show a band around the expected size of otoferlin (5.5 kB) but the band was not well resolved. The band was gel extracted.

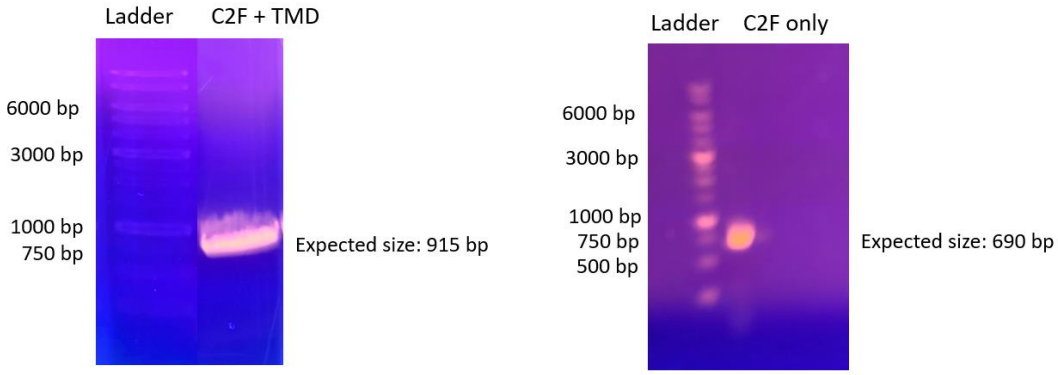

Figure S5. Agarose gels for C2F-TM otoB and the C2F domain alone. The gel on the left shows the expected band size of 915 bp for the C2F with the TMD, indicating a successful PCR. The gel on the right shows the expected band size for the C2F domain alone, which is 690 bp.

Table 1. Primer Names and Sequences

Primer Name	Sequence
GFP HindIII F	GACCCAAGCTTGCCACCATGGTGAGCAAGGGCGAGGAG
GFP KpnI R	AACGGGTACCCTTGTACAGCTCGTCCATGCCGAGA
OtoB KpnI F	GGCCGGTACCATGAAGCGTAATAAGCATCGCTCCT
OtoB KpnI R C term	CCCGCGGTACCTTAGCTCCCAGAAGCTTTTTCACCATG
OtoB KpnI R N term	CGCAGGTACCTCAAGCTCCCAGAAGCTTTTTCACCATG
Zf OtoA_BamHI REV	CACAGGATCCTTAGGCCCCGAGCAGCTT
Zf OtoA_KpnI FWD	CACAGGTACCATGGCTTTAGTGGTGCA
ZFotofbTM_NCO1_ FWD	ATAGCCCATGGGTCACCACCACCACCACCACGTCGGTGAAG GACGCAATGA
zfOtoB-seq Primer1	GGCAGTGATGTCTTCGGGATCA
zfOtoB-seq Primer2	CCGCTTCGCAGAAGATTCTTCG
zfOtoB-seq Primer3	GACATTTGCCATGATGCTGGTG

LIC zf OtoB F	TACTTCCAATCCAATGCCATGAAGCGTAATAAGCATCGCTCCT
zf otoB HiFi Th 1A R	CAGGTGGAAATAATTCCTGTCGGTGATGACTGGCTTCATTGGA
zf otoB HiFi Th 1A F	CACCGACAGGAATTATTTCCACCTGCCGTACTTTGAGAGGAAG
zf otoB HiFi Th 2B R	GTTTTTCTTGTAGTTCTGGATCAGCGACGACTGAACGCCTTTC
Zf OtoB HiFi 3 F	CTGTCCTGAGTAAATACAGGATTGAGGTTCTCTTTTGGGGTCT
LIC zf OtoB R	GTTATCCACTTCCAATTCAAGCTCCCAGTAGCTTTTTCACC
LIC zf otoB C2F F	TACTTCCAATCCAATGCCCCAGATAAACCTGGGATTGAACAGG
LIC zf OtoB C2F R	TTATCCACTTCCAATGGGACTTTTCTCAGCCTCCTCACCT
QC_mutBFw1	CAAGTGGCTGGTCATCTAAATAGTGGTGGCTC
QC_mutBRv1	GAGCCACCACTATTTAGATGACCAGCCACTTG