Molecular Genetics & Genomic Medicine

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CLINICAL REPORT OPEN ACCESS

Functional Correlation of Two Novel Nonsense *POU4F3* Mutations Causing Late-Onset Progressive Nonsyndromic Hearing Loss in DFNA15 Families

Tianyang Zhang[1,2] | Wei Wang[3] | Luping Zhang[2] | Jingchun He[1]

[1]Department of Otorhinolaryngology Head and Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China | [2]Department of Otolaryngology-Head and Neck Surgery, Affiliated Hospital of Nantong University, Medical School of Nantong University, Nantong, China | [3]Qidong People's Hospital, Qidong Liver Cancer Institute, Affiliated Qidong Hospital of Nantong University, Nantong, China

Correspondence: Luping Zhang (zhanglp910@126.com) | Jingchun He (hejingchun@xinhuamed.com.cn)

Received: 27 December 2024 | **Revised:** 27 March 2025 | **Accepted:** 8 April 2025

Funding: This work was supported by the National Natural Science Foundation of China (82371159, 82271164, and 82071069) and the Social Development Project of the Jiangsu Provincial Key R&D Program (BE2022764).

Keywords: DFNA15 | nonsense mutation | nonsyndromic hearing loss | POU4F3

ABSTRACT

Background: *POU4F3* mutations cause DFNA15, an autosomal dominant nonsyndromic hearing loss. POU4F3 encodes a transcription factor crucial for inner ear hair cell development and maintenance.

Objective: To identify and functionally characterize novel *POU4F3* mutations in two Chinese families with late-onset progressive hearing loss.

Methods: Massively parallel DNA sequencing (MPS) was performed on affected individuals from two unrelated Chinese families. Sanger sequencing validated mutations and confirmed co-segregation. Functional analyses included protein expression analysis by Western blots and subcellular localization studies by immunofluorescence.

Results: We identified two novel nonsense mutations in *POU4F3*: c.863C > A (p.Ser288Ter) and c.172G > T (p.Glu58Ter), both co-segregating with the hearing loss phenotype. Functional studies showed p.Ser288Ter produced a stable but mislocalized protein with impaired nuclear transport, while p.Glu58Ter resulted in a severely truncated, rapidly degraded protein.

Conclusion: This study expands the DFNA15 mutation spectrum and provides new insights into POU4F3-related hearing loss pathogenesis. Our findings demonstrate that different molecular mechanisms can lead to similar DFNA15 phenotypes, supporting POU4F3 haploinsufficiency as the primary pathogenic mechanism.

1 | Introduction

Hearing loss is one of the most common sensory defects, affecting millions of people worldwide (Chadha et al. 2021). Genetic factors play a significant role in the etiology of hearing impairment. More than 50% of congenital hearing loss cases are attributed to genetic causes (Morton and Nance 2006). Genetic hearing loss can be classified as syndromic, where hearing impairment is associated with other clinical features, or nonsyndromic, where hearing loss is the sole clinical manifestation. Nonsyndromic hearing loss (NSHL) accounts for approximately 70% of genetic hearing loss cases and can be inherited in autosomal dominant, autosomal recessive, X-linked, or mitochondrial patterns (Walls et al. 2024). To date, over 150 genes have been identified as causative for NSHL, highlighting the genetic heterogeneity of this condition (Shearer et al. 2023).

Tianyang Zhang and Wei Wang contributed equally to this work.

Among the genes implicated in NSHL, POU4F3 (602,460, POU domain, class 4, transcription factor 3) has emerged as a critical player in inner ear development and function (Vahava et al. 1998). *POU4F3*, also known as *BRN3C*, is a member of the POU family of transcription factors and is expressed in the hair cells of the cochlea and vestibular system (Xiang et al. 1997). POU4F3 plays a crucial role in the differentiation and survival of inner ear hair cells, which are essential for sound transduction and balance (Erkman et al. 1996). Mutations in *POU4F3* have been associated with autosomal dominant NSHL DFNA15, characterized by progressive hearing impairment typically beginning in the second to third decade of life (Collin et al. 2008). The mechanisms underlying DFNA15 pathogenesis are complex and not fully elucidated. However, studies have shown that *POU4F3* mutations can lead to impaired transcriptional activity, altered protein localization, or reduced protein stability (Weiss et al. 2003), resulting in haploinsufficiency of POU4F3 protein (Zhu et al. 2020). These molecular defects can result in progressive hair cell stereocilia and mitochondrial dysfunction and hair cell degeneration, ultimately leading to hearing loss (Hertzano et al. 2004; Zhu et al. 2020). The phenotypic variability observed in DFNA15 patients, including differences in age of onset, progression rate, and severity of hearing loss, suggests that additional genetic or environmental factors may influence the disease course (Lee et al. 2010; Zhu et al. 2020).

Despite the growing understanding of POU4F3's role in hearing loss, the full spectrum of pathogenic mutations in this gene remains to be elucidated. In this study, we report two novel nonsense mutations in the *POU4F3* gene identified in two Chinese families with late-onset progressive NSHL and provide functional characterization of these mutations on POU4F3 protein expression and localization. These findings not only expand the mutation spectrum of DFNA15 but also provide new insights into the pathogenic mechanisms of POU4F3-related hearing loss. Our results contribute to the growing body of knowledge on DFNA15 and may have implications for future diagnostic and therapeutic strategies for hereditary hearing loss.

2 | Materials and Methods

2.1 | Subjects and Clinical Evaluation

Two families, NT-107 and NT-213, with autosomal dominant hearing loss have resided in Jiangsu Province, China for more than four generations. Participants underwent comprehensive auditory evaluations, including pure tone audiometry (PTA), otoscopic examination, and temporal bone high-resolution CT scanning when necessary. Detailed medical histories were collected, including the age of onset, progression rate, hearing aid usage, noise exposure, vestibular symptoms, and other syndromic manifestations. None of the recruited individuals had syndromic diagnoses. The PTA was calculated from audiometric thresholds at four frequencies (0.5, 1, 2, and 4 kHz). Hearing loss levels were categorized into five groups on the basis of pure tone air conduction averages: mild (26–40 dB HL), moderate (41–55 dB HL), moderately severe (56–70 dB HL), severe (71–90 dB HL), and profound (> 91 dB HL). The study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (2023-L115). Written informed consent was obtained from all participating individuals or their parents.

2.2 | Massively Parallel DNA Sequencing

Genomic DNA was extracted from peripheral blood samples of probands and their family members (when available) using a Blood DNA kit (Tiangen Biotech, China). The proband (IV-2) from family NT-107 and the proband (III-5) from family NT-213 underwent targeted massively parallel DNA sequencing (MPS) covering 270 deafness-associated genes (Table S1). Variants were selected through stepwise genetic analysis as previously described (Wu et al. 2020; Zhang et al. 2024). Sequencing data were analyzed on the basis of autosomal dominant (AD) inheritance patterns. Candidate variants were confirmed through Sanger sequencing in all available family members, and their pathogenicity was interpreted using American College of Medical Genetics (ACMG) standards and guidelines. Sanger sequencing was performed using the primers: Forward 5′-TCTGATCCACACGTCTGTTCC-3′ and Reverse 5′-AGGTGGTGTGGATGGATCTG-3′ for c.172G > T mutation; Forward 5′-GAAGAACAGCAAGCCAGAGC-3′ and Reverse 5′-GATGAAGGACGTGGCTGGAT-3′ for c.863C > A mutation.

2.3 | Plasmid Constructions

Mouse Pou4f3 (NM_138945.2) wildtype, p.Glu58Ter, and p.Ser288Ter variant with Flag-tagged at their N terminus were cloned into pcDNA3.1 or pIRES-EGFP plasmids, using EcoRV (R3195V, New England Biolabs), EcoRI (R3101S, New England Biolabs), and BamhI (R3136S, New England Biolabs) sites. DH5α bacteria were used to amplify all mouse expression plasmids. Plasmid sequences were validated by Sanger sequencing.

2.4 | Cell Culture and Transfection

Penicillin–streptomycin solution (E607011, Sangon Biotech), nonessential amino acid (NEAA, 11140050, Gibco), and 10% fetal bovine serum (FBS, 40130ES76, Yeasen) were added to DMEM (12,800,017, Gibco) in order to support the growth of HEK293T (ATCC: CRL-3216) cells. Hieff TransTM Liposomal Transfection Reagent (40802ES03, Yeasen) was used to transfect HEK293T cells in Opti-MEM (31,985,062, Thermo Fisher). Cells were harvested 24 h after transfection for further analysis.

2.5 | RNA Extraction and RT-qPCR

After two rounds of washing with 1 mL of phosphate-buffered solution (PBS), 0.5 mL of RNAiso Plus reagent (9109, Takara) was added to HEK293T cells. As directed by the manufacturer, the samples' total RNA was extracted. HiScript III RT SuperMix (+gDNA wiper) (R323-01, Vazyme) was used to perform reverse transcription (RT) on the total RNA samples. AceQ qPCR SYBR Green Master Mix (Q111-02, Vazyme) was used for quantitative PCR (qPCR) using a Roche Cycler 96 instrument. Primers used in the qPCR experiments were: for Pou4f3, 5′-TCTGGCGGCGGTGGATAT-3′

and 5′-GCTGCTCATGGTATGGTAGGT-3′ and for Gapdh, 5′-ACCACGAGAAATATGACAACTCAC-3′ and 5′-CCAAAGTTGTCATGGATGACC-3′. The internal reference gene Gapdh was used to normalize the expression of the target gene.

2.6 | Western Blot Analyses

Cultured cells were rinsed twice with PBS. Cells were extracted and homogenized with the lysis buffer, 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1% Triton X-100 (A110694-0500, Sangon Biotech), 1 mM ethylene diamine tetra-acetic acid pH 8.0, and 1 mM phenylmethylsulfonyl fluoride (ST005, Beyotime) containing the complete Protease Inhibitor Cocktail (11,697,498,001, Roche) at 4°C for 10 min. Proteins were subjected to SDS–PAGE and transferred to PVDF membrane and analyzed by following antibodies: mouse anti-Flag (1:2000, 30503ES60, Yeasen), mouse anti-Actin (1:20000, 4970, Cell Signaling Technology), and mouse anti-GAPDH (BioWorld, MN, USA) antibodies, followed by incubation in HRP anti-mouse (1:5000, BS12478, Bioworld) secondary antibody for 2 h at room temperature (RT). The signals were visualized using ECL substrate (180–5001, Tanon) on an automatic chemiluminescence/fluorescence image analysis system (Tanon 4600, Tanon).

2.7 | Wholemount Immunofluorescence

After being fixed on ice in 4% PFA for 30 min, rinsed twice with PBS, the transfected cells were blocked in 5% normal horse serum (NHS) with 0.3% Triton X-100 in PBS for 1 h at room temperature. Samples were then incubated overnight at 4°C with primary antibodies (Anti-Flag, Cell Signaling Technology, 14,793) that had been diluted in 1% NHS with 0.3% Triton X-100 in PBS. Samples were incubated with goat anti-rabbit IgG (H + L) secondary antibody (Jackson ImmunoResearch, UK) for 2 h at RT. Samples were imaged by a Leica SP5-II (Leica, Germany). ImageJ software (version 1.46r, NIH, MD) was used for image processing of confocal z-stacks.

3 | Results

3.1 | Audiological Phenotypes and Clinical Assessment

The pedigrees of families NT-107 (five-generation) and NT-213 (four-generation) demonstrated autosomal dominant inheritance (Figure 1A,B). All affected individuals exhibited nonsyndromic sensorineural hearing loss (NSHL), which was bilateral and symmetric, ranging from moderate to profound. The hearing loss typically initiated at high frequencies and developed into a down-sloping audiometric configuration. The age of onset differed between families: the third decade for NT-107 and the late second decade for NT-213. Hearing deterioration occurred across all frequencies, progressing to severe flat-type hearing loss after age 60 in NT-107 and age 50 in NT-213. Family NT-213 demonstrated more severe hearing loss compared to NT-107, correlating with the duration of hearing loss. The hearing phenotype characteristics are illustrated in Figure 1C,D.

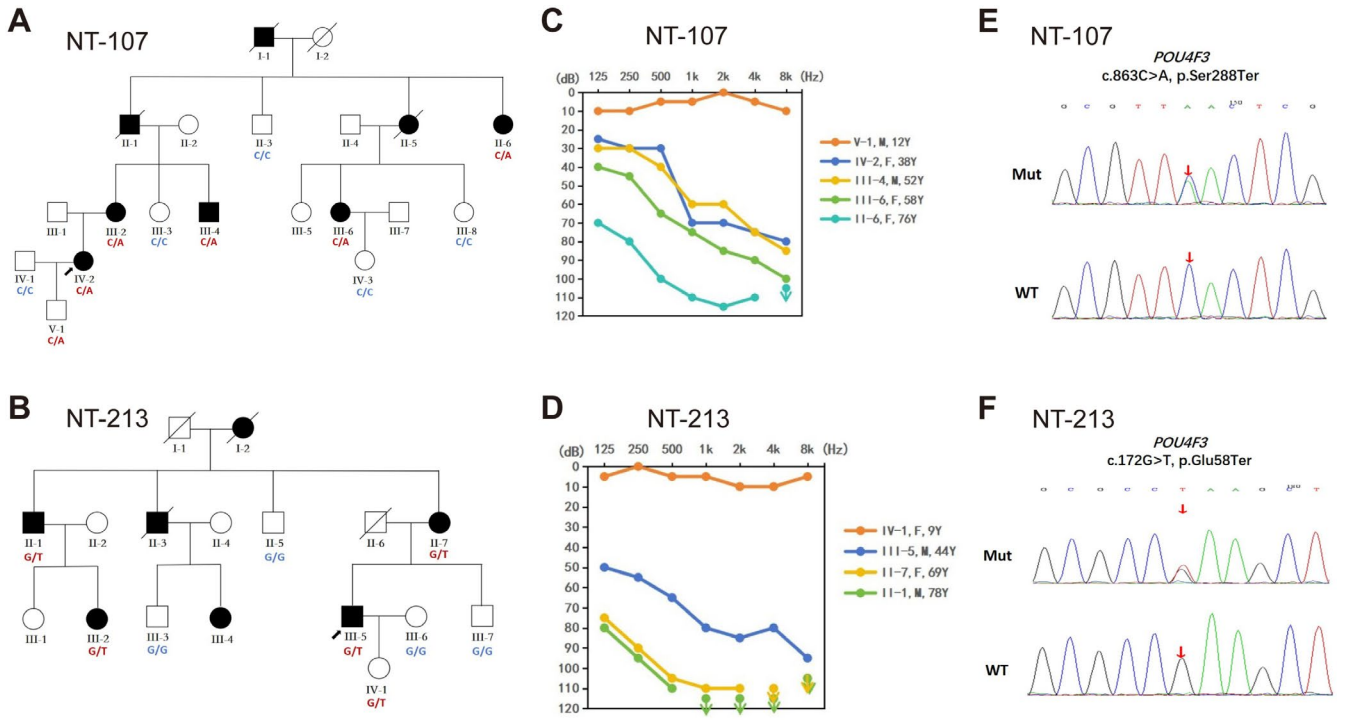

FIGURE 1 | Phenotypic and genetic analysis for the two families. (A, B) Pedigrees of two independent families are shown. (C, D) Representative audiograms of members with hearing loss of Family NT-107 and NT-213. (E, F) Sequencing results of the NT-107 and NT-213 families. Note that NT-107:V-1 and NT-213:IV-1 are 12 and 9 years old respectively, and both marked as unaffected.

3.2 | Identification of Candidate Mutations

Co-segregation analysis of candidate mutations identified by MPS was conducted in available family members. In family NT-107, detailed analysis of common alleles among five affected family members revealed a novel heterozygous variant *POU4F3* (NM_002700.3): c.863C > A, p.Ser288Ter. This C to A substitution at nucleotide position 863 introduced a premature stop codon at amino acid 288. In family NT-213, the novel POU4F3 mutation: c.172G > T, p.Glu58Ter (NM_002700.3) was identified in four affected individuals but was absent in normal family members over 30 years of age. This G to T transition at nucleotide position 172 also creates a premature stop codon at amino acid 58. These two heterozygous mutations in affected individuals were validated by Sanger sequencing (Figure 1E,F). Both nonsense mutations were not found in gnomAD and ClinVar databases. Following the ACMG recommendations, both *POU4F3* mutations, p.Glu58Ter and p.Ser288Ter, were classified as likely pathogenic. We also identified c.863C > A in one unaffected offspring in family NT-107 (V-1) and c.172G > T in family NT-213 (IV-1). Although both subjects (currently aged 13 and 11, respectively) were reported healthy, they may develop progressive hearing loss at a later age.

3.3 | Functional Analyses of Two Novel *POU4F3* Mutations

To explore the functional consequences of the two novel POU4F3 mutations, we constructed plasmids expressing Flag-tagged wildtype (WT), p.Ser288Ter (S288X) and p.Glu58Ter (E58X) POU4F3 proteins.

First, the S288X mutation identified from family NT-107 results in a premature stop codon that yields a truncated POU4F3

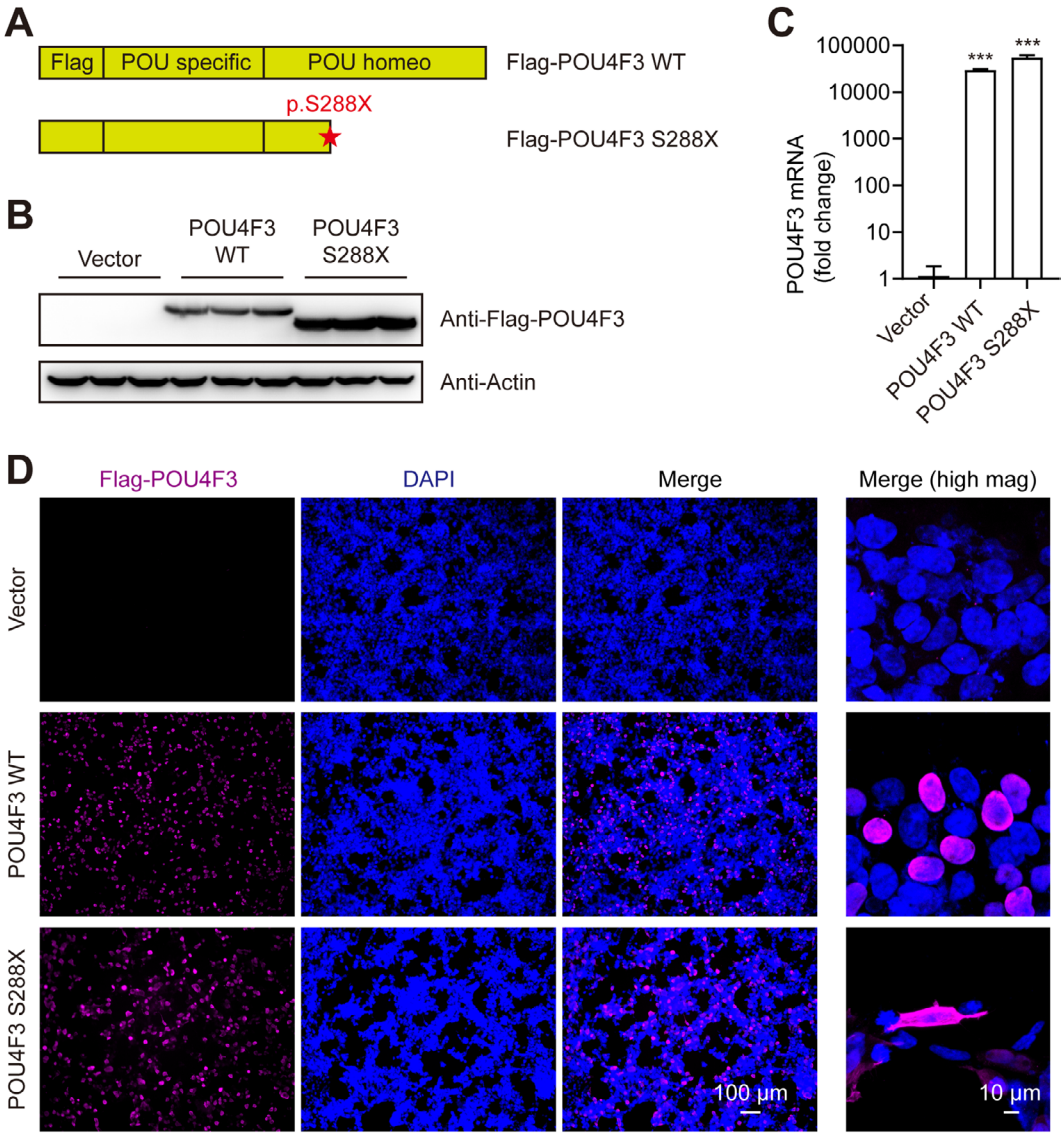

FIGURE 2 | POU4F3 S288X mutant proteins mislocalize to the cytoplasm in HEK293T cells. (A) Schematic illustration of the Flag-tagged wide type (WT) and mutant (S288X) POU4F3 protein structures. Star indicates the mutation site. (B) Expression of Flag-POU4F3 WT or Flag-POU4F3 S288X proteins in transfected HEK293T cells by western blotting. (C) mRNA of both Flag-POU4F3 WT and Flag-POU4F3 S288X constructs were detected by RT-qPCR in transfected HEK293T cells. ***$p < 0.001$ by unpaired Student's *t*-test. (D) Immunofluorescent images of Flag-POU4F3 in HEK293T cells transfected with empty vector, Flag-POU4F3 WT, or Flag-POU4F3 S288X plasmids.

protein within the POU homeo domain (Figure 2A). Western blot analyses of transfected HEK293 cells showed that while the S288X mutant POU4F3 was readily expressed, it was at a smaller size than the wildtype controls (Figure 2B). Both WT and S288X *POU4F3* mRNA transcripts were expressed at equivalent levels (Figure 2C). Interestingly, while the WT POU4F3 protein was localized to the nucleus as expected, the S288X mutant POU4F3 protein was distributed in both the nucleus and cytosol (Figure 2D). These results indicate that the S288X mutant may have impaired the nuclear localization of POU4F3, consistent with a putative nuclear localization signal at the C terminus of POU4F3 (Weiss et al. 2003).

Second, the E58X mutation identified from family NT-213 results in a premature termination within the N-terminal POU specific domain (Figure 3A). As expected, the E58X mutant protein failed to be stably expressed in the transfected HEK293 cells (Figure 3B), although the mRNA of the mutant was expressed (Figure 3C). Consistently, immunofluorescent results also demonstrated a lack of stable POU4F3 E58X mutant protein expression, while the EGFP protein from the pIRES plasmid was readily detectable (Figure 3D). These results suggest that the E58X mutation results in significant degradation of the truncated POU4F3 protein.

Together, E58X and S288X mutations result in degradation and mislocalization of the POU4F3 protein, respectively, likely impairing the transcriptional activity of POU4F3.

4 | Discussion

In this study, we identified and functionally characterized two novel nonsense mutations in the POU4F3 gene (c.863C > A, p.Ser288Ter and c.172G > T, p.Glu58Ter) in two Chinese families with late-onset progressive NSHL. These findings not only

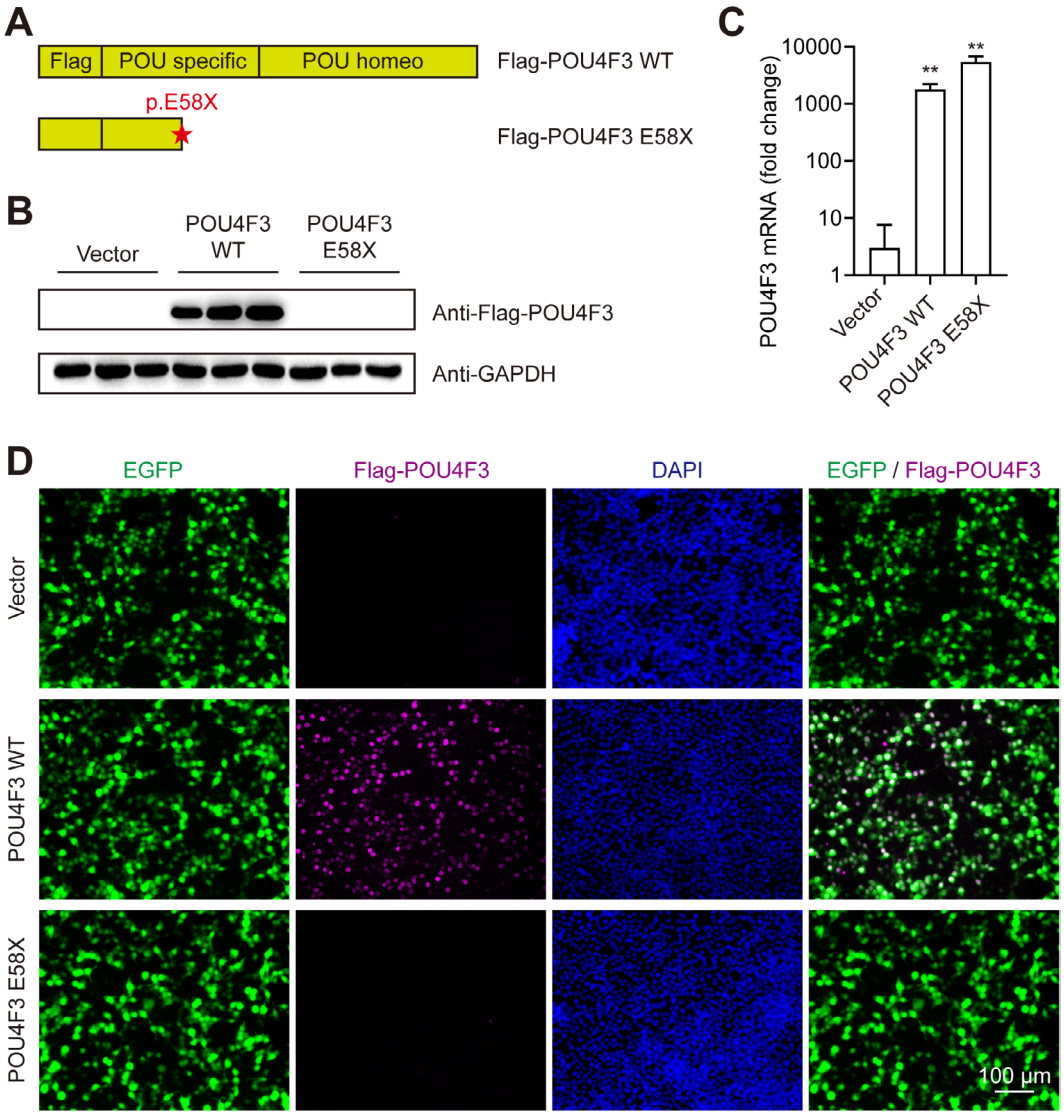

FIGURE 3 | POU4F3 E58X mutant proteins fail to express in HEK293T cells. (A) Schematic illustration of the FLAG-tagged wide type (WT) and mutant (E58X) POU4F3 protein structures. Star indicates the mutation site. (B) Expression of Flag-POU4F3 WT or Flag-POU4F3 E58X proteins in transfected HEK293T cells by western blotting. (C) mRNA of both Flag-POU4F3 WT and Flag-POU4F3 E58X constructs were detected by RT-qPCR in transfected HEK293T cells. $^{**}p < 0.01$ by unpaired Student's *t*-test. (D) Immunofluorescent images of Flag-POU4F3 in HEK293T cells transfected with empty vector, Flag-POU4F3 WT, or Flag-POU4F3 E58X plasmids. EGFP proteins were co-expressed from the vector pIRES-ERFP.

expand the mutation spectrum of DFNA15 but also provide new insights into the pathogenic mechanisms of POU4F3-related hearing loss.

Expanding the mutation spectrum of POU4F3 is crucial for several reasons. First, it enhances our understanding of genotype–phenotype correlations in DFNA15, potentially explaining the variability in age of onset and progression rates observed among patients (Lee et al. 2023). Second, identifying novel mutations contributes to improving genetic diagnosis and counseling for families affected by hereditary hearing loss (Usami et al. 2002). Third, characterizing new mutations and their functional consequences can provide insights into the molecular mechanisms underlying hair cell dysfunction and loss, potentially leading to new therapeutic targets (Bowl and Brown 2018).

The POU4F3 protein is a critical transcription factor for the development and maintenance of inner ear hair cell (Xiang et al. 1997). Our functional analyses of the two novel mutations reveal distinct molecular mechanisms leading to POU4F3 dysfunction. The p.Ser288Ter mutation results in a truncated protein that, while stable, shows impaired nuclear localization. This observation is consistent with previous studies suggesting the presence of a nuclear localization signal at the C-terminus of POU4F3 (Weiss et al. 2003). The mislocalization of the mutant protein likely compromises its function as a transcription factor. In contrast, the p.Glu58Ter mutation leads to a severely truncated protein that is rapidly degraded, as demonstrated by the lack of detectable protein expression in our Western blot and immunofluorescence analyses. This finding suggests that this mutation likely results in complete loss of POU4F3 function, potentially through nonsense-mediated decay of the mutant mRNA or rapid degradation of the truncated protein (Mort et al. 2008). Consistent with this notion, other POU4F3 mutations have shown to compromise its stability after translation (Weiss et al. 2003).

Interestingly, despite the different molecular consequences of these two mutations, both families exhibited similar phenotypes of late-onset progressive hearing loss, albeit with some variations in severity and age of onset. This phenotypic similarity, despite different underlying molecular mechanisms, supports the hypothesis that haploinsufficiency of POU4F3 is the primary cause of DFNA15 (Collin et al. 2008; Zhu et al. 2020). The variability in age of onset and progression rate observed between our two families and in comparison with previously reported DFNA15 cases suggests that additional genetic or environmental factors may modulate the disease course, as previously demonstrated in DFNA15 mouse models (Zhu et al. 2020).

The impairment of POU4F3 expression and localization due to these mutations is likely to affect the expression of numerous genes crucial for hair cell development and maintenance. Previous studies have identified several POU4F3 target genes, including *Gfi1* and *Lhx3*, which are essential for hair cell differentiation and survival (Hertzano et al. 2007, 2004). POU4F3 mutations may lead to dysregulation of these target genes, ultimately resulting in progressive hair cell degeneration and hearing loss.

In conclusion, our study identifies two novel nonsense mutations in *POU4F3* and elucidates their distinct molecular consequences, providing new insights into the pathogenic mechanisms of DFNA15. These findings contribute to the growing body of knowledge on DFNA15 hearing loss and may inform future efforts in developing targeted therapies for genetic hearing impairment. Further studies investigating the long-term effects of these mutations on hair cell function and survival in animal models would be valuable in understanding the progressive nature of DFNA15 and in developing potential interventions to slow or prevent hearing loss progression.

Author Contributions

Concept and design: Luping Zhang, Jingchun He; Clinical studies: Tianyang Zhang, Wei Wang; Experimental studies: Tianyang Zhang, Wei Wang, Jingchun He; Data curation: Tianyang Zhang, Wei Wang; Funding acquisition: Luping Zhang, Jingchun He; Writing – original draft: Tianyang Zhang, Wei Wang; Writing – review and editing: Luping Zhang, Jingchun He.

Acknowledgments

The authors would like to thank the patients and their families for their participation in this study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data relating to the findings of this study are available from the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.