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Late-onset hearing loss case associated with a heterozygous truncating variant of *DIAPH1*

Running title: Late-onset hearing loss associated with truncation of DIAPH1

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Conflict of Interest Statement:

The authors declare no conflict of interest.

Data Availability Statement:

The data that supports the findings of this study are available in the supplementary material of this article.

Abstract

DIAPH1 is a formin homology F-actin elongating protein encoded by *DIAPH1*. Homozygous recessive variants resulting in the loss of DIAPH1 function cause seizures, cortical blindness and microcephaly syndrome (SCBMS), but hearing loss has not been reported. In contrast, dominant variants of human *DIAPH1* are associated with DFNA1 nonsyndromic sensorineural hearing loss. The deafness phenotype is due partly to abnormal F-actin elongation activity caused by disruption of the DIAPH1 autoinhibitory mechanism. We report an elderly female heterozygous for the c.3145C>T: p.R1049X variant who showed late-onset sensorineural hearing loss in her fifth decade. p.R1049X lacks F-actin elongation activity because this variant truncates one third of the FH2 domain, which is vital for DIAPH1 dimerization and processive F-actin elongation activity. Concordantly, no increase of F-actin nor processive F-actin elongation activity was observed after overexpression of p.R1049X DIAPH1 in HeLa cells or by single-molecule microscopy using *Xenopus* XTC cells. However, overexpression of the p.R1049X variant impairs formation of cell-cell junctions and mitosis. We speculate that late-onset hearing loss is a long-term consequence of heterozygosity for the recessive p.R1049X variant, a phenotype that may have been overlooked among carriers of other recessive alleles of *DIAPH1*.

Key words: cell division, cell junctions, cell toxicity, DFNA1, DIAPH1, hearing loss

Introduction

Diaphanous-related formin 1 (DIAPH1), encoded by *DIAPH1*, is a formin homology protein that can processively elongate F-actin¹. In human, *DIAPH1* variants are associated with autosomal dominant hearing loss (DFNA1) resulting from constitutive activation of F-actin elongation by disrupting the wild type autoinhibition between the DIAPH1 inhibitory domain (DID) and autoregulatory domain (DAD)². Heterozygosity for the missense variant p.A265S in the DID, and five other variants in or near the DAD (p.A1221VfsX22, p.E1184AfsX11, p.E1192_Q1220del, p.R1213X and p.A1210SfsX31) have been reported in families segregating hearing loss²⁻⁹. In contrast, three biallelic recessive truncating variants (p.F923fs, p.Q778X p.R1049X) and one splice variant (c.684+1G>A) that cause a complete loss of DIAPH1 protein were associated with seizures, cortical blindness and microcephaly syndrome (SCBMS; OMIM: #616632)¹⁰⁻¹². DIAPH1 is involved in a variety of cell functions including cytokinesis, cell migration, stress fibers, filopodia and cell-cell junctions although its physiological functions are still unclear¹³⁻¹⁵.

Here, we report a person with late-onset hearing loss who is heterozygous for the DIAPH1 truncation variant c.3145C>T: p.R1049X. This variant is missing F-actin elongation activity and impairs formation of cell-cell junctions and cell proliferation. We speculate that late-onset hearing loss is a long-term consequence of heterozygosity for the p.R1049X variant.

Materials and Methods

Patient and Sanger sequencing

Genomic DNA was sampled after obtaining written informed consent. Exome sequencing (ES) was performed followed by filtering steps (Table S1). Exon 23 of *DIAPH1* (NM_005219.4) was amplified by PCR using primers tagcctctatgagttcagtggttg and tgaactgaaaatgctctcacatct, which was Sanger-sequenced to confirm the variant. Details of other methods are in the Supplementary Information.

Results and Discussion

A 72-year-old woman (SB70-122) with a family history of hearing loss has bilateral, moderate hearing loss across all frequencies (Figure 1, A and B), while other family members declined to participate. No other abnormalities were found upon a physical examination and blood tests did not reveal a meaningful abnormality either in the platelet count nor its morphology, excluding macrothrombocytopenia. Variants from ES were filtered using bioinformatics tools, allele frequencies and relevance to hearing loss (Table S1). We finally identified two candidates, c.3145C>T:p.R1049X in *DIAPH1* and c.1703_1712dupTTGCCCTCCC:p.Ile572fs in *WFS1*. *WFS1* was excluded since variants associated with autosomal dominant nonsyndromic hearing

loss are almost always missense alleles via ‘gain-of-function’, while ‘loss-of-function’ variants including nonsense or frameshift variants are associated with Wolfram syndrome¹⁶.

Approximately 200 cases associated with autosomal dominant non-syndromic hearing loss (DFNA 6/14/38) found in ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) are missense mutations mainly in *WFS1* exon 8¹⁷. A final candidate nonsense variant, c.3145C>T, in exon 23 of *DIAPH1* (NM_005219) replaced an arginine codon with a stop codon truncating one third of the FH2 domain (Figure 1, C and D). Detailed genotypic characteristics of seventeen candidate variants before the final filtering step are summarized (Table S2).

Almost all reported variants associated with DFNA1 deafness affect residues in or near the autoinhibitory DID and DAD²⁻⁹ with one exception¹⁸. Thus, we considered whether or not p.R1049X can be the cause of progressive, late onset hearing loss via a novel mechanism and not constitutive activation of F-actin elongation activity. We evaluated the F-actin elongation activity of p.R1049X after overexpression in HeLa cells. Wild-type and a constitutively active variant associated with hearing loss, p.R1213X², were used as controls. Abnormal, microvilli-like structures indicating upregulated actin polymerization were observed only in cells expressing AcGFP-DIAPH1 (p.R1213X), but not in cells expressing AcGFP-fused DIAPH1 (p.R1049X) or wild-type DIAPH1 (Figure 2A), suggesting that F-actin elongation activity of the p.R1049X

variant is lost or significantly disrupted. Both AcGFP-fused DIAPH1 (p.R1049X) and DIAPH1 (p.R1213X) localized at the plasma membrane (Figure 2B) probably via exposed DIDs due to the lack of functional DADs¹⁹.

Corroborating data were obtained using single-molecule fluorescence microscopy of *Xenopus* XTC cells expressing AcGFP-fused wild-type and variant DIAPH1 (Figure 2C, Movie S1). The number of fluorescent puncta appeared in the cytoplasm was larger in cells expressing AcGFP-DIAPH1 (p.R1049X) than those expressing wild-type AcGFP-DIAPH1, indicating that p.R1049X protein is likely trapped by structures limiting diffusion. AcGFP-DIAPH1 (p.R1049X) molecules did not show directional movements and appear to lack processive F-actin elongation activity. Immunoprecipitation assays showed that truncated FH1-FH2 of p.R1049X DIAPH1 cannot dimerize with intact FH1-FH2 domains (Figure S1).

Overexpression of the p.R1049X variant impairs formation of cell-cell junctions and mitosis. MDCK cells overexpressing AcGFP-DIAPH1 (p.R1049X) have heterogenous shapes (particularly in size) and form a layer significantly thicker than cells expressing AcGFP-fused wild-type DIAPH1 (Figure 3, A and B). HeLa cells overexpressing AcGFP-DIAPH1 (p.R1049X) die more frequently and undergo fewer mitoses compared to cells overexpressing wild-type AcGFP-DIAPH1 (Figure 3C). Immunoblotting of HeLa cell lysates showed that expression

levels of AcGFP-DIAPH1 (p.R1049X) were lower than AcGFP-fused wild-type and the p.R1213X variant (Figure 3E). The p.R1049X protein may be translated only at a low efficacy, unstable in the cytoplasm or lethal for cells at high expression. DIAPH1 is involved in the maintenance and stabilization of adherens and tight junctions¹³ and in the cortical tension and spindle assembly during mitotic metaphase and anaphase^{14,15}. Overexpressed p.R1049X DIAPH1 may interfere or compete with the functions of wild-type DIAPH1, perhaps explaining how one copy of the p.R1049X *DIAPH1* allele causes late-onset hearing loss.

p.R1049X *DIAPH1* and other large truncating variants (p.F923fs, p.Q778X, c.684+1G>A) has been considered to be non-functional. Homozygosity for these variants have similar phenotypes characterized by seizures, cortical blindness and microcephaly¹⁰⁻¹². In these studies, auditory functions were reported to be normal via interviews with some patients and by screening at birth in one patient using ABR and ASSR. However, the long-term consequence of heterozygosity for the p.R1049X variant, especially a mild to moderate progressive hearing loss, may have been overlooked or could not be evaluated since sensitive auditory tests, such as pure-tone audiogram, would be difficult for patients due to the significantly delayed psychomotor development with severe intellectual disability, poor speech and lethality in the first or second decade. Hearing loss caused by the long-term consequence of p.R1049X DIAPH1 is hardly

distinguishable in an outpatient clinic from age-related hearing loss. Here, we propose that one copy of the p.R1049X allele, previously thought to be benign in carriers, is associated with a late-onset hearing loss.

Author Contributions

BJK, BYC and TU are responsible for the integrity of the data and accuracy of the data analysis.

Study conceptualization: BJK, TM, BYC and TU. Investigations: BJK, TM, TC, BYC and TU.

Formal analysis: BJK, TM, BYC and TU. Funding acquisition: BYC, TBF and TU. Validation:

BJK, TM, BYC and TU. Visualization: BJK, TM, BYC and TU. Manuscript: BJK, TM, BYC,

TBF and TU.

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Figure legends

Figure 1: Clinical data of the proband and identified DIAPH1 variant.

(A) Audiogram of the proband (72-year-old female) showing bilateral, moderate hearing loss.

Thresholds are shown in red and blue for her right and left ears, respectively.

(B) Pedigree of the family. Auditory functions of family members (asterisks) were interviewed since other family members declined to participate in this study. Filled symbols indicate individuals with hearing loss. Diagonal lines represent deceased individuals.

(C) Candidate variant validated by Sanger sequencing.

(D) Schematic *DIAPH1* gene structure and DIAPH1 domains. Previously-reported variants affect either the autoinhibitory domains DID or DAD. The p.R1049X variant causes truncation within the FH2 domain.

Figure 2: Disrupted F-actin elongation activity and autoinhibition in p.R1049X.

(A) Actin structures of HeLa cells expressing AcGFP-fused wild-type, (p.R1049X), or (p.R1213X) DIAPH1. Cells expressing DIAPH1 are indicated by asterisks. Abnormal, microvilli-like structures indicating upregulated actin polymerization are observed in cells expressing AcGFP-DIAPH1 (p.R1213X) (magenta arrows) while microvilli in cells expressing AcGFP-DIAPH1 (p.R1049X) appear normal (arrows). Stress fibers appear normal in all cells. Bars, 10

μm.

(B) Localization of AcGFP-fused DIAPH1 (p.R1049X) and DIAPH1 (p.R1213X) at the plasma membrane (arrows). Bars, 10 μm.

(C) Single-molecule fluorescent microscopy of XTC cells expressing AcGFP-fused DIAPH1 (p.R1049X, wild-type or p.R1213X). Fluorescent puncta appear in cells expressing p.R1049X without showing directional movements (indicated by circles). Directional movements observed in cells expressing wild-type DIAPH1 and the p.R1213X variant indicating intact F-actin elongation activities. Directionally-moving molecules are more frequent in cells expressing constitutively-active p.R1213X variant compared with cells expressing wild-type DIAPH1 (circles and trajectories indicate molecules showing directional movements for more than two frames; crosses indicate disappearance). Time-lapse, every 200 ms. Bars, 5 μm. Movies are available in Movie S1.

Figure 3: Impaired formation of cell-cell junctions and mitosis caused by p.R1049X.

(A) Increased height of MDCK cells expressing AcGFP-DIAPH1 (p.R1049X). Average height of cells expressing AcGFP-DIAPH1 (p.R1049X) were significantly higher than those expressing wild-type AcGFP-DIAPH1. *** $P = 0.0002$ by Student's t -test, $n = 5$ each. Bars, 10 μm.

(B) Impaired formation of cell-cell junctions (Stacked images of the same samples shown in **A**).

MDCK cells expressing AcGFP-DIAPH1 (p.R1049X) have heterogenous shapes compared with cells expressing wild-type AcGFP-DIAPH1. Cell-cell junctions are visualized using anti-ZO1 antibodies. Bars, 20 μm .

(C) Impaired mitosis caused by DIAPH1 (p.R1049X). HeLa cells died during mitosis and floating are indicated by arrows. The number of undergoing mitosis was significantly fewer among cells overexpressing AcGFP-DIAPH1 (p.R1049X) than cells overexpressing wild-type AcGFP-DIAPH1 ($***P = 0.0006$) while the number of cells that died during mitosis was significantly larger among cells overexpressing AcGFP-DIAPH1 (p.R1049X) than cells overexpressing wild-type AcGFP-DIAPH1 ($**P = 0.0019$). Student's *t*-test, $n = 5$ each.

(D) Immunoblotting showing low expression level of AcGFP-DIAPH1 (p.R1049X). The amount of GFP was compared between lysates of HeLa cells expressing AcGFP-fused wild-type DIAPH1, DIAPH1 (p.R1213X) and DIAPH1 (p.R1049X) using an anti-GFP antibody.

Figure 1

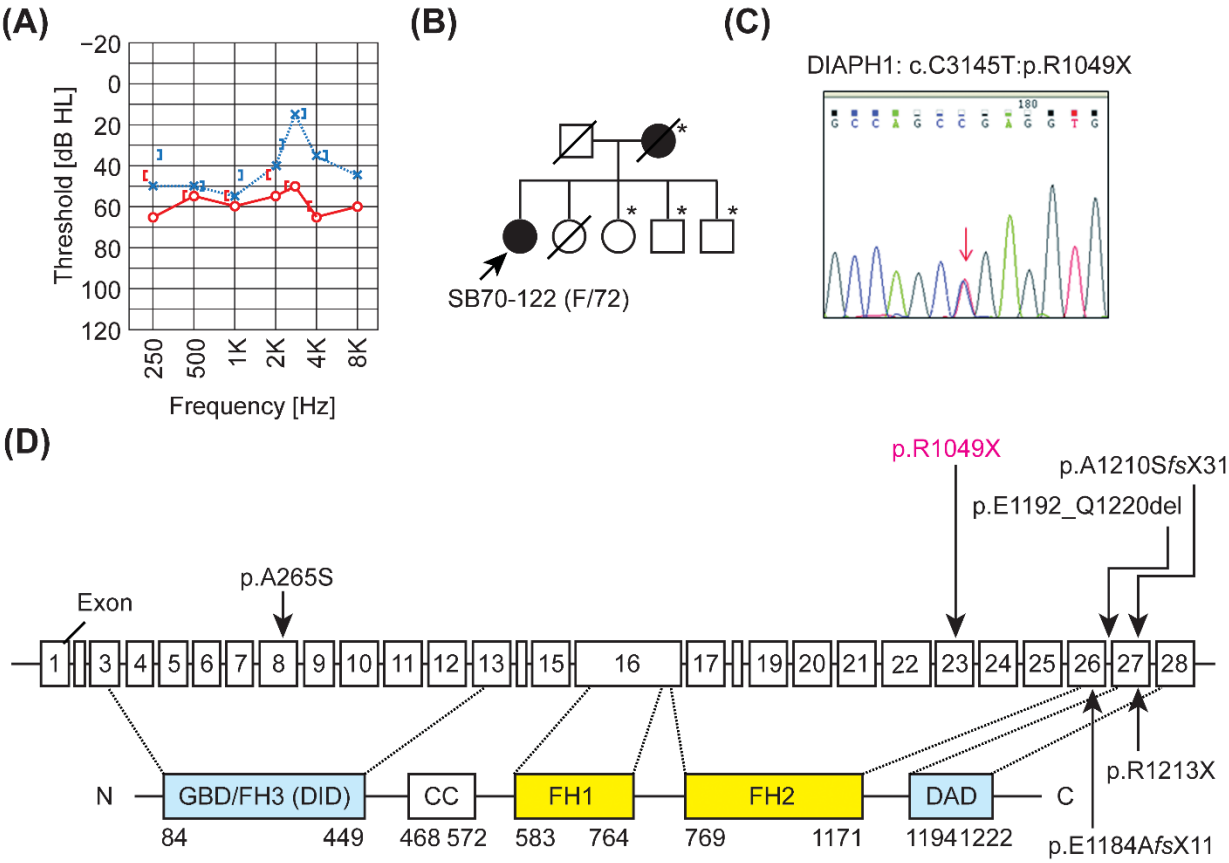


Figure 2

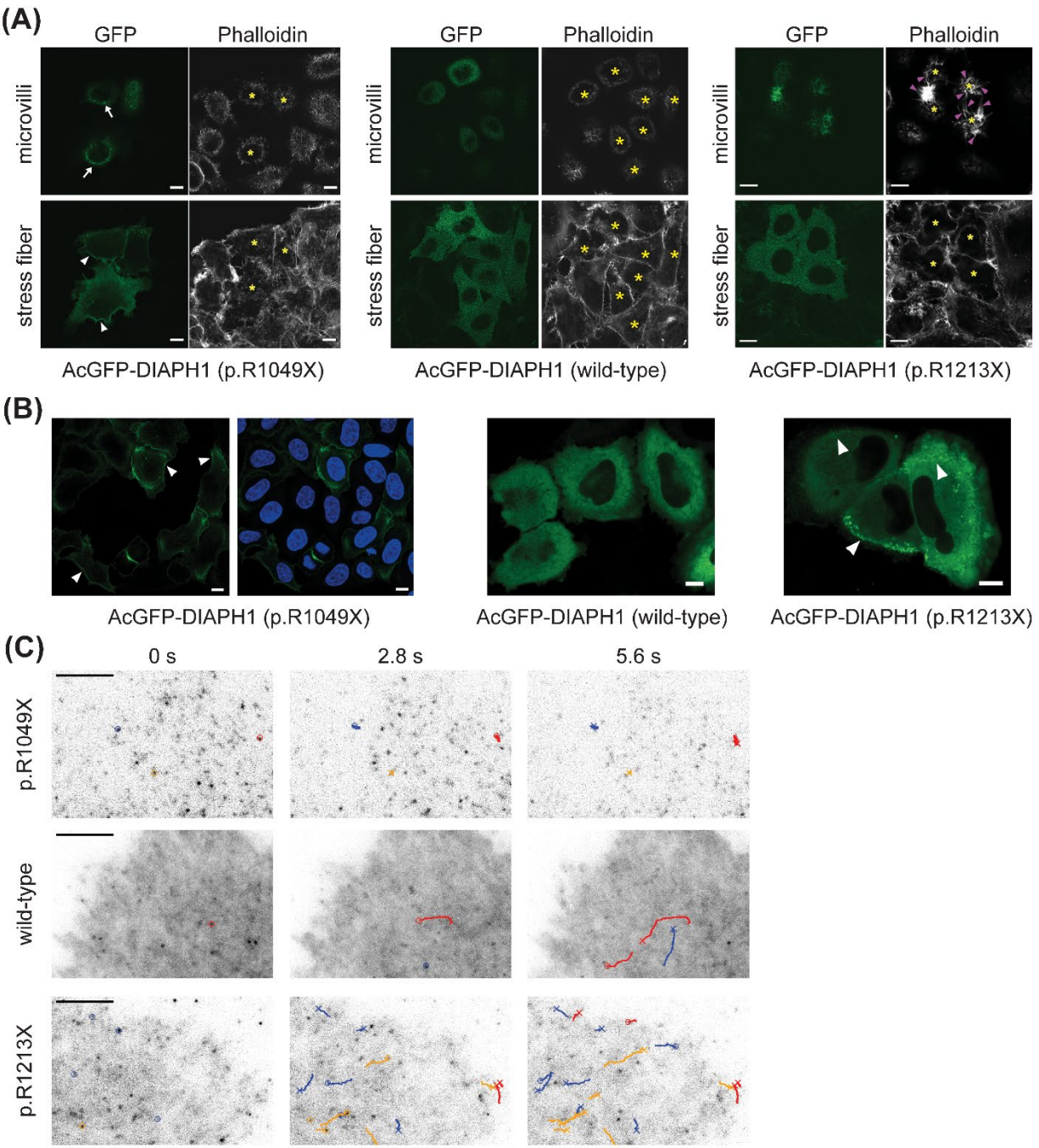
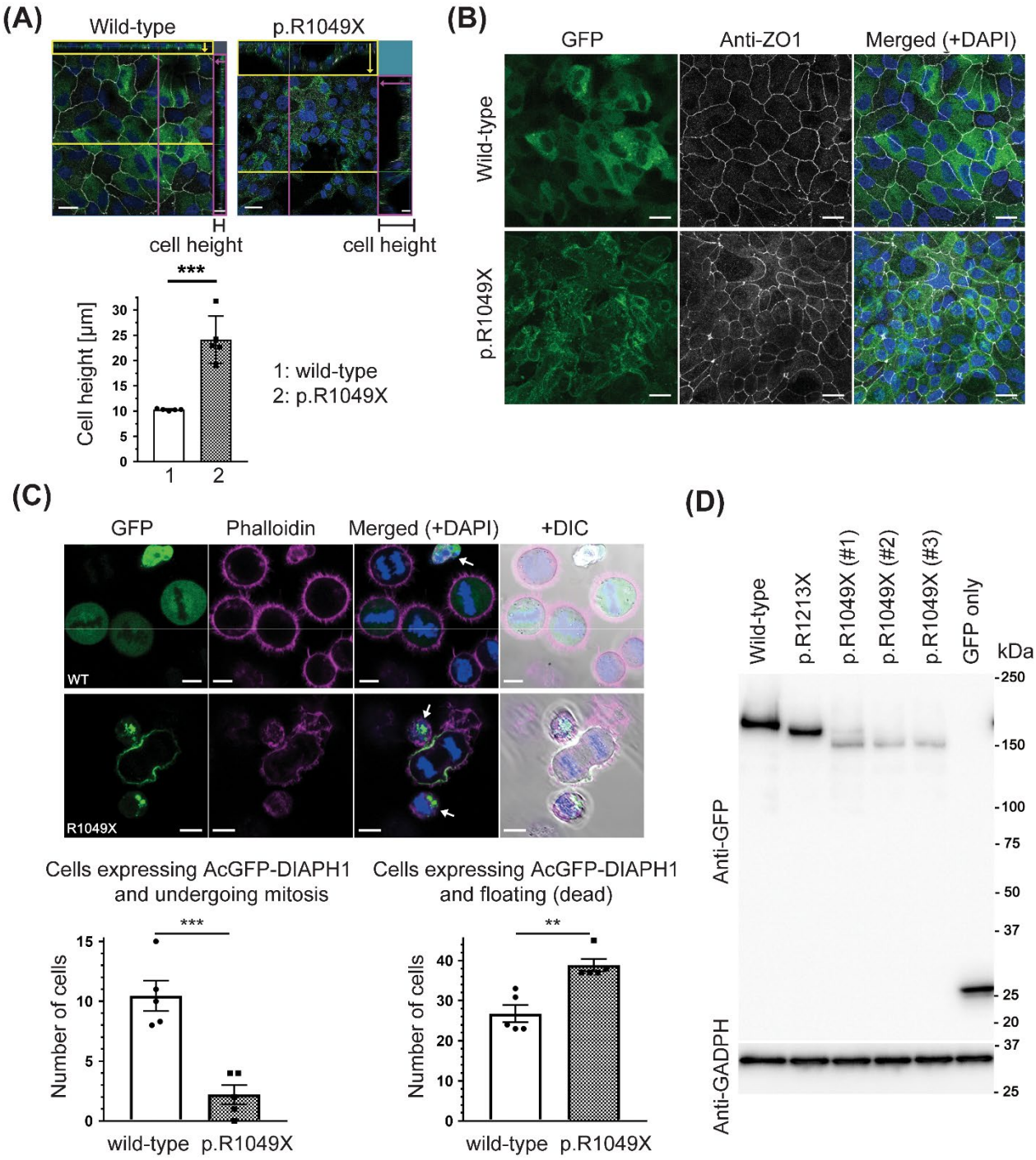


Figure 3



Supplementary information

Tables

Table S1: Filtering of variants found in exome sequencing

Step	Number of variants
Total variants	66,754
Variant depth quality filter (minimum read coverage: 20)	43,595
Variant class filter (nonsynonymous, splice region, frameshift, nonsense variant, etc.)	9,322
Frequency filter: ≤ 0.0005 (1000G, ESP6500, ExAC, etc.)	363
Variant impact prediction (SIFT, Polyphen-2, Mutationtaster, etc.)	155
Omitting genes not related to clinical phenotype (manual curation of genes using the ClinVar DB)	53
Korean frequency filter: ≤ 0.0005 (KRGDB, Korea1K, etc.)	17
Genes with variants associated with nonsyndromic hearing loss (HHL, OMIM, LOVD3, etc.)	2 (<i>DIAPH1</i> and <i>WFS1</i>)

Table S2: Detailed genotype of potential causative variant before the final curation in the present study

Gene	Genomic Position [GRCh37/hg19]	dbSNP 151	HGVS Nucleotide Change	HGVS Protein Change	Zygosity	GERP++	CADD phred	Global MAF (gnomAD, 1000 Genomes, ExAC)	KRGDB MAF (1722 individuals)	Classification (ACMG/AMP) : Rule	Reference (PMID)
<i>DIAPH1*</i>	Chr5:140908023	rs863225243	c.3145C>T	p.Arg1049* (p.R1049X)	Het	3.63	40	Absent	Absent	VUS : PM2	26463574
<i>WFS1</i>	Chr4:6303224	rs745734152	c.1703_1712dupTTG CCCTCCC	p.Ile572fs	Het	NA	NA	dupTTGCCCTCCC=0.000004 (1/251006, GnomAD_exome)	Absent	VUS : PM2	Absent
<i>XPNPEP3</i>	Chr22:41282514	rs772726035	c.787T>A	p.Ser263Thr	Het	5.74	24.4	A=0.000008 (2/250584, GnomAD_exome) A=0.000017 (2/120258, ExAC)	A=0.000291 (1/3444, KRGDB)	VUS : PM2	Absent
<i>CARD10</i>	Chr22:37900729	Absent	c.1431G>T	p.Trp477Cys	Het	5.47	27.6	Absent	Absent	VUS : PM2	Absent
<i>DCLK2</i>	Chr4:151000480	Absent	c.301G>A	p.Ala101Thr	Het	4.22	26.1	Absent	Absent	VUS : PM2	Absent
<i>DNMT3B</i>	Chr20:31372582	rs751017290	c.223G>A	p.Asp75Asn	Het	3.38	22.6	A=0.000012 (3/251430, GnomAD_exome) A=0.000025 (3/120908, ExAC)	Absent	VUS : PM2	Absent
<i>E2F7</i>	Chr12:77436967	Absent	c.1001G>A	p.Arg334His	Het	5.95	32	Absent	Absent	VUS : PM2	Absent
<i>ERVV-2</i>	Chr19:53553784	rs989105714	c.1280C>T	p.Ala427Val	Het	NA	8.512	T=0.000057 (8/140196, GnomAD) T=0.000097 (13/134550, GnomAD_exome)	Absent	VUS	Absent

<i>MFAP4</i>	Chr17:19287969	Absent	c.646C>T	p.Gln216*	Het	3.94	36	Absent	Absent	VUS : PM2	Absent
<i>NES</i>	Chr1:156640656	Absent	c.3322_3323delCT	p.Leu1108fs	Het	NA	22.4	Absent	Absent	VUS : PM2	Absent
<i>OASI</i>	Chr12:113354429	rs766223435	c.770C>T	p.Thr257Met	Het	4.84	22.5	T=0.000064 (16/251432, GnomAD_exome) T=0.000043 (6/140188, GnomAD) T=0.000033 (4/121392, ExAC)	Absent	VUS	Absent
<i>RBBP9</i>	Chr20:18470565	Absent	c.404C>T	p.Ser135Phe	Het	4.98	24.8	Absent	Absent	VUS : PM2	Absent
<i>RNF157</i>	Chr17:74169822	rs776109025	c.257G>C	p.Ser86Thr	Het	5.2	27.1	G=0.000004 (1/245426, GnomAD_exome) G=0.00001 (1/88034, ExAC)	Absent	VUS : PM2	Absent
<i>RNF180</i>	Chr5:63509788	rs1463914513	c.635C>A	p.Pro212His	Het	6.08	26.1	A=0.000004 (1/251038, GnomAD_exome)	Absent	VUS : PM2	Absent
<i>SHBG</i>	Chr17:7536626	rs752629317	c.1165A>G	p.Ser389Gly	Het	5	25.9	G=0.000004 (1/251414, GnomAD_exome) G=0.000008 (1/120896, ExAC)	G=0.000291 (1/3444, KRGDB)	VUS : PM2	Absent
<i>TAGAP</i>	Chr6:159461825	rs373518592	c.517G>A	p.Asp173Asn	Het	5.78	25.1	T=0.000056 (14/251468, GnomAD_exome) T=0.000121 (17/140194, GnomAD) T=0.000082 (10/121398, ExAC)	T=0.000294 (1/3444, KRGDB)	VUS	Absent
<i>THEMIS2</i>	Chr1:28206397	rs751391949	c.478C>T	p.Pro160Ser	Het	2.78	23.7	Absent	Absent	VUS : PM2	Absent

Abbreviations : HGVS, Human Genome Variation Society; Het, heterozygote; NA, not ascertained; VUS, Variant of Uncertain Significance

Combined Annotation Dependent Depletion (CADD) <https://cadd.gs.washington.edu/>

Korean Reference Genomic Database (KRGDB) <http://152.99.75.168:9090/KRGDB/menuPages/intro.jsp>

Global Minor Allele Frequency (GMAF; <https://www.ncbi.nlm.nih.gov/snp/>)

*, final candidate variant in the study

Table S3: Combinations of DIAPH1 variants used in the immunoprecipitation assay

Lane	Combination of expressed DIAPH1 variants				Loaded sample volumes [†]	
	HA-DIAPH1ΔN3 (bait)		GFP-DIAPH1ΔN3 (prey)		Bait (anti-HA)	Prey (anti-GFP)
1	Wild-type	3 μg	Wild-type	1 μg	6 μl	6 μl
2	Wild-type	3 μg	p.R1213X	1 μg	6 μl	6 μl
3	Wild-type	10 μg	p.R1049X	10 μg	18 μl	18 μl
4	Wild-type	3 μg	GFP only [‡]	1 μg	6 μl	6 μl
5	p.R1049X	10 μg	Wild-type	3 μg	6 μl	6 μl
6	p.R1049X	10 μg	p.R1213X	3 μg	6 μl	6 μl
7	p.R1049X	10 μg	p.R1049X	10 μg	18 μl	18 μl
8	p.R1049X	10 μg	GFP only [‡]	1 μg	6 μl	6 μl

[†] Total ~60 μl volumes were obtained for each combination

[‡] Negative controls

Supplemental figure and legends

Figure S1

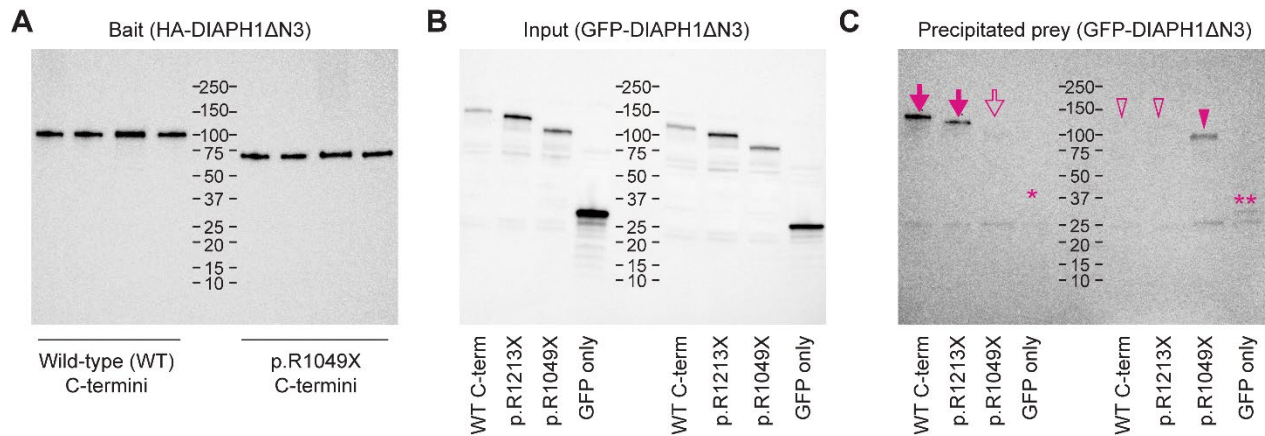


Figure S1: Immunoprecipitation showing lacked dimerization activity of the DIAPH1 FH1-FH2 domains truncated by the p.R1049X mutation.

HA-tagged DIAPH1ΔN3 baits and GFP-fused DIAPH1ΔN3 preys, which are to exclude the effect of autoinhibitory DID-DAD interaction and to suppress degradation, were co-expressed in HEK293F cells and immunoprecipitated using anti-HA tag antibody beads. (A) Western blot of immunoprecipitated HA-DIAPH1ΔN3 baits detected using anti-HA tag antibody. Each sample contains almost equal amount of HA-DIAPH1ΔN3 with either wild-type or p.R1049X C-termini. (B) Western blot of input GFP-DIAPH1ΔN3 preys detected using anti-GFP tag antibody. The amount of GFP-DIAPH1ΔN3 with truncated C-termini (p.R1213X or p.R1049X) or a negative control, GFP, in each sample was almost equal to or larger than the amount of GFP-DIAPH1ΔN3 with wild-type C-terminus. (C) Western blot of immunoprecipitated GFP-DIAPH1ΔN3 preys detected using anti-GFP tag antibody. GFP-DIAPH1ΔN3 with wild-type and p.R1213X C-terminus were precipitated by HA-DIAPH1ΔN3 with wild-type C-terminus (arrows), while

neither GFP-DIAPH1 Δ N3 with p.R1049X C-terminus nor unfused GFP was not precipitated (open arrow and asterisk). Consistently with these results, HA-DIAPH1 Δ N3 with p.R1049X C-terminus did not precipitate GFP-DIAPH1 Δ N3 with wild-type or p.R1213X C-termini (open arrowheads). HA-DIAPH1 Δ N3 with p.R1049X C-terminus unexpectedly precipitated GFP-DIAPH1 Δ N3 with p.R1049X C-terminus (arrowheads). DIAPH1 Δ N3 with p.R1049X may have non-specific binding to other proteins considering that GFP negative control was also weakly precipitated (double asterisks).

Movie S1: Single-molecule fluorescence microscopy of DIAPH1 (wild-type and variants)

Time-lapse images of XTC cells expressing AcGFP-fused DIAPH1 (p.R1049X, wild-type and p.R1213X). Many fluorescent puncta appear in cells expressing AcGFP-DIAPH1 (p.R1049X) without showing directional movements (representative puncta indicated by circles). This variant is likely to be anchored to the plasma membrane via the exposed DID and to lack processive F-actin elongation activity. Directional movements driven by processive F-actin elongation activity are observed in cells expressing AcGFP-fused wild-type DIAPH1 and DIAPH1 (p.R1213X) while the number of “activated” molecules are much larger in cells expressing the p.R1213X variant (circles indicate molecules moving directionally for more than two frames). Time-lapse, every 200 ms. Bar, 5 μ m.

Materials and Methods

Filtering of variants found in exome sequencing

Variants were filtered using bioinformatic tools¹. After applying variant depth quality filter (minimum read coverage of 20), variant class filter (to exclude synonymous variants), allele frequency (≤ 0.0005 using 1000G, ESP6500 and ExAC DB) and *in silico* prediction tools (SIFT, Polyphen-2, Mutation taster, CADD), genes not related to hearing loss were excluded using ClinVar DB (<https://www.ncbi.nlm.nih.gov/clinvar/>) and variants were filtered again by Korean ethnicity-specific allele frequency (≤ 0.0005 using KRGDB). Manual curation using literature DB and clinical phenotypes including inheritance pattern and audiogram profile was performed by clinical geneticist/bioinformatician to draw the final candidate variant list (Table S1). Variants were classified according to the 2015 American College of Medical Genetics and Genomics (ACMG) guidelines and ACMG/AMP hearing loss criteria².

Plasmids

Human wild-type DIAPH1 and DIAPH1 (p.R1213X) in the pAcGFP-C1 vector (Takara Bio), which produces N-terminally AcGFP-tagged protein, have been described previously³. DIAPH1 (p.R1049X) was cloned into pAcGFP-C1 using an In-Fusion HD cloning kit (Takara). For immunoprecipitation experiments, the EGFP sequence of pEGFP-C1 (Takara Bio) was replaced with the HA tag sequence to prepare the pHA-C1 vector. DIAPH1 Δ N3, DIAPH1 Δ N3 (p.R1049X), and DIAPH1 Δ N3 (p.R1213X), which lack the N-terminal 551 residues corresponding to 542 residues in mDia1 Δ N3⁴, were cloned into pEGFP-C1 and pHA-C1. DIAPH1 Δ N3 (p.R1049X) and DIAPH1 Δ N3 (p.R1213X) were used to exclude the effect of the autoinhibitory DID-DAD interaction and to suppress the degradation of DIAPH1.

Fluorescence immunocytochemistry

HeLa and MDCK cells (ATCC) were cultured at 37°C and 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher) containing 10% fetal bovine serum. Cells were transfected using Lipofectamine 3000 (Thermo Fisher). Stable MDCK cell lines expressing inducible AcGFP-fused DIAPH1 were prepared by transfecting the pRetroX-TetOne-Puro vector (Takara) (wild-type and p.R1049X) using electroporation (NEPA21; NEPAGENE Co., Ltd) and by selection using 0.3 µg/mL puromycin (Fujifilm Wako). Expression of the transgenes was induced using 100 ng/mL doxycycline. Cells were fixed using 4% paraformaldehyde and permeabilized using PBS containing 0.3% Triton X-100. ZO1 was stained using 1 µg/mL anti-ZO1 rabbit polyclonal antibody (Proteintech, RRID: AB 10733242) and 1 µg/mL Alexa568-conjugated goat anti-rabbit antibody (Thermo Fisher, RRID: AB_10563566). F-actin and nuclei were visualized using 1:200 Alexa568-conjugated phalloidin (A12380, Thermo Fisher) and 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were acquired using an LSM700 confocal microscope (Carl Zeiss).

Single-molecule fluorescence microscopy of DIAPH1

Single-molecule fluorescence microscopy using *Xenopus* XTC cells was performed as previously described³. Briefly, XTC cells were transfected using Lipofectamine 2000 (Thermo Fisher) to express AcGFP-fused DIAPH1 and spread on poly-L-Lysine-coated glass coverslips in 70% Leibovitz's L15 medium without serum. The coverslips were attached to flow cells and placed on the stage of an inverted total internal reflection fluorescence (TIRF) microscope (Axio Observer.Z1; Zeiss) equipped with a CMOS Camera (ORCA Flash-4.0 C11440, Hamamatsu) and

an oil immersion objective lens (alpha Plan-Apochromat 100× NA 1.46). Time-lapse images were acquired at 21–23°C using the ZEN software (Zeiss) up to 120 min after cells were seeded illuminating the cell edges using a 488-nm laser (Zeiss). Images were magnified using a 1.6× tube lens.

Immunoblotting

For the experiments in Figure 3D, 0.6×10^5 HeLa cells were seeded in 6-well plate (Corning) at 48 h prior to transfection using Lipofectamin 3000 (Thermo Fisher). Forty-eight hours after transfection, cells lysates were subjected to SDS-PAGE⁵ followed by immunoblotting for 2 h at 23°C using GFP and GAPDH antibodies. The bound antibody was detected using an ECL detection system (FUSION-SOLO.7S.EDGE, Vilber, Collegien, France).

Protein expression in the MDCK stable cell lines was confirmed by immunoblotting using GFP (recognizing AcGFP, Takara; RRID:AB_2336883), mDia1(66-77) (DP4471, ECM Biosciences, Versailles, KY, USA), GAPDH (Abcam, RRID:AB_1209515), and tubulin α ⁶ antibodies.

Immunoprecipitation

HEK293 cells seeded in 10-cm cell-culture dishes were transfected using Lipofectamine 2000 (Thermo Fisher) to co-express DIAPH1 fused with EGFP and HA-tag and allowed for 24 h expression. Cells were lysed in 1 mL IP lysis buffer containing Halt Protease Inhibitor Cocktail (Thermo Fisher) and clarified by centrifugation at $13\,000 \times g$ for 30 min. After incubation with 10 μ L of Pierce anti-HA agarose beads (Thermo Fisher) for 2 h at 4°C, precipitated proteins were eluted using 50 μ L of 3 × Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) and detected by western blotting. Anti-HA tag rabbit monoclonal antibody (1:1000;

MilliporeSigma; RRID: AB_2747373), anti-GFP rabbit polyclonal antibody (1:1000; Thermo Fisher; RRID: AB_221569) and goat anti-rabbit IgG (whole molecule)-peroxidase antibody (1:8000; MilliporeSigma; RRID: AB_257896) were applied using an iBind Flex Western System (Thermo Fisher). Chemiluminescence images were acquired using ECL substrate and a ChemiDoc imaging system (Bio-Rad). The amount of plasmid and the sample volumes subjected to SDS-PAGE were adjusted to equalize the amounts of baits and preys between combinations (Table S3).

Statistical Analysis

Z-axis heights and cell numbers (Figures 3A and 3C) were plotted as means with standard errors and analyzed with Student's *t*-tests (Prism 6.0 software, GraphPad Software Inc.); $P < 0.05$ was considered significant. Each value in Figures 3A and 3C was an average of 3–5 and 5 microscopic fields ($\times 40$ oil) per 35 mm glass-bottom dish with 14 mm microwell, respectively.

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