Identification of p.A684V Missense Mutation in the *WFS1* Gene as a Frequent Cause of Autosomal Dominant Optic Atrophy and Hearing Impairment

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Optic atrophy (OA) and sensorineural hearing loss (SNHL) are key abnormalities in several syndromes, including the recessively inherited Wolfram syndrome, caused by mutations in *WFS1*. In contrast, the association of autosomal dominant OA and SNHL without other phenotypic abnormalities is rare, and almost exclusively attributed to mutations in the Optic Atrophy-1 gene (*OPA1*), most commonly the p.R445H mutation. We present eight probands and their families from the US, Sweden, and UK with OA and SNHL, whom we analyzed for mutations in *OPA1* and *WFS1*. Among these families, we found three heterozygous missense mutations in *WFS1* segregating with OA and SNHL: p.A684V (six families), and two novel mutations, p.G780S and p.D797Y, all involving evolutionarily conserved amino acids and absent from 298 control chromosomes. Importantly, none of these families harbored the *OPA1* p.R445H mutation. No mitochondrial DNA deletions were detected in muscle from one p.A684V patient analyzed. Finally, wolframin p.A684V mutant ectopically expressed in HEK cells showed reduced protein levels compared to wild-type wolframin, strongly indicating that the mutation is disease-causing. Our data support OA and SNHL as a phenotype caused by dominant mutations in *WFS1* in these additional eight families. Importantly, our data provide the first evidence that a single, recurrent mutation in *WFS1*, p.A684V, may be a common cause of ADOA and SNHL, similar to the role played by the p.R445H mutation in *OPA1*. Our findings suggest that patients who are heterozygous for *WFS1* missense mutations should be carefully clinically examined for OA and other manifestations of Wolfram syndrome. © 2011 Wiley-Liss, Inc.

Key words: autosomal dominant; hearing loss; optic atrophy; mutation; *WFS1*; Wolfram syndrome

INTRODUCTION

Optic atrophy (OA) and hearing impairment are features of several syndromes such as X-linked Mohr–Tranebjaerg syndrome (OMIM 304700, deafness–dystonia–optic neuronopathy syndrome), X-linked Charcot–Marie–Tooth disease-5 (OMIM 311070, OA, deafness, and polyneuropathy), and Gustavson syndrome (OMIM 309555, X-linked mental retardation with OA, deafness, and seizures). In addition, optic atrophy and deafness are features of the Wolfram syndrome type 1 (OMIM 222300) and 2 (OMIM 604928). In contrast, autosomal dominant OA (ADOA) and sensorineural hearing loss (SNHL) without any other phenotypic abnormality have been described in relatively few families, with the first report referring to a Swedish family that was clinically described in a Swedish Physician's journal [Samuelson, 1940], and re-investigated as one of the families presented here.

Wolfram syndrome type 1 is a rare and severe autosomal recessive neurodegenerative disease, characterized by diabetes mellitus, optic atrophy, diabetes insipidus and deafness (DIDMOAD) and is caused by mutations in the *WFS1* gene (reviewed in Tranebjaerg et al. [2009]). Additional clinical features may include renal abnormalities, ataxia, dementia/mental retardation, and diverse psychiatric illnesses. The minimal diagnostic criteria for Wolfram syndrome are OA and diabetes mellitus of juvenile onset. Hearing impairment in Wolfram syndrome is typically progressive and mainly affects the higher frequencies [Cryns et al., 2003], but a small fraction of affected individuals have congenital deafness [Barrett et al., 1995; Hansen et al., 2005]. Mutations in *WFS1* are also a common cause of isolated autosomal dominant low-frequency nonsyndromic sensorineural hearing loss (LFSNHL) [Bespalova et al., 2001; Young et al., 2001]. In addition, a *WFS1* p.E864K missense mutation has been reported in two families with dominantly inherited deafness with some members being affected by OA and impaired glucose regulation/diabetes [Eiberg et al., 2006; Valero et al., 2008], thereby mimicking Wolfram syndrome, but in an attenuated version. Finally, very recently, a p.K836N mutation in *WFS1* has been found associated with autosomal dominant optic neuropathy and deafness in one family [Hogewind et al., 2010]. No plausible functional explanation has been found to explain the vast differences in clinical presentations and patterns of inheritance.

So far, more than 140 recessive mutations causing Wolfram syndrome have been identified. Most of these are truncating mutations, mainly located in exon 8 and unique to a particular individual or a few individuals/families. In the dominantly inherited disease, LFSNHL, the *WFS1* mutations are mainly missense mutations in exon 8; 28 different missense mutations have been identified (http://www.khri.med.umich.edu/research/lesperance_lab/low_freq.php).

Until recently, only one gene, Optic Atrophy-1 (*OPA1*), had been found to underlie isolated optic atrophy and hearing loss. *OPA1* encodes a dynamin-related GTPase involved in mitochondrial biogenesis (OMIM 605290) and is the gene most frequently underlying OA either isolated or in syndromic form [Amati-Bonneau et al., 2009; Yu-Wai-Man et al., 2010].

One recurrent *OPA1* mutation, the heterozygous p.R445H missense mutation, underlies the majority of reported cases of isolated OA and hearing loss. This mutation was first identified in a Japanese and a French patient [Amati-Bonneau et al., 2003; Shimizu et al., 2003].

In this study, we demonstrate that heterozygous *WFS1* missense mutations cause autosomal dominant isolated OA and hearing loss in eight families. Importantly, our data also suggest that a single, recurrent, non-founder *WFS1* mutation, p.A684V, which was identified in six of the eight families studied, may be a common cause of isolated autosomal dominant OA and SNHL similar to the p.R445H mutation in *OPA1*.

PATIENTS AND METHODS
Clinical Data

Eight families of Caucasian origin from the US, Sweden, and UK diagnosed with OA and SNHL were included in this study (Fig. 1). Initially, we analyzed 15 probands with OA and deafness in the study, but only identified mutations in *WFS1* in 8 of these probands, and the families of these eight probands are described here in detail. The clinical data were compiled from available medical records from different hospitals. Venous blood was drawn from available patients and relatives and DNA was extracted using standard methods. The study was approved by the Danish Research Ethical Committee (reference numbers KF 01-234/02 and KF 01-108/03) and the Institutional Review Board (IRBMED) of the University of Michigan Health System. When possible, patients underwent audiometric and ophthalmological evaluations. Normal hearing was defined as a pure tone average hearing level of less than or equal to 20 dB. Mild hearing loss was defined as 21–40 dB HL, moderate loss as 41–60 dB HL, severe loss as 61–90 dB HL, and profound loss was defined as exceeding 90 dB HL. OA was evaluated based on available ophthalmological examinations. Five of the families (NSDF916, NSDF1272, NSDF1865, NSDF1793, and NSDF2032) were ascertained through a study of genetic deafness in the alumni of Gallaudet University (a university for the education of the deaf and hard-of-hearing students, located in Washington, DC, USA)
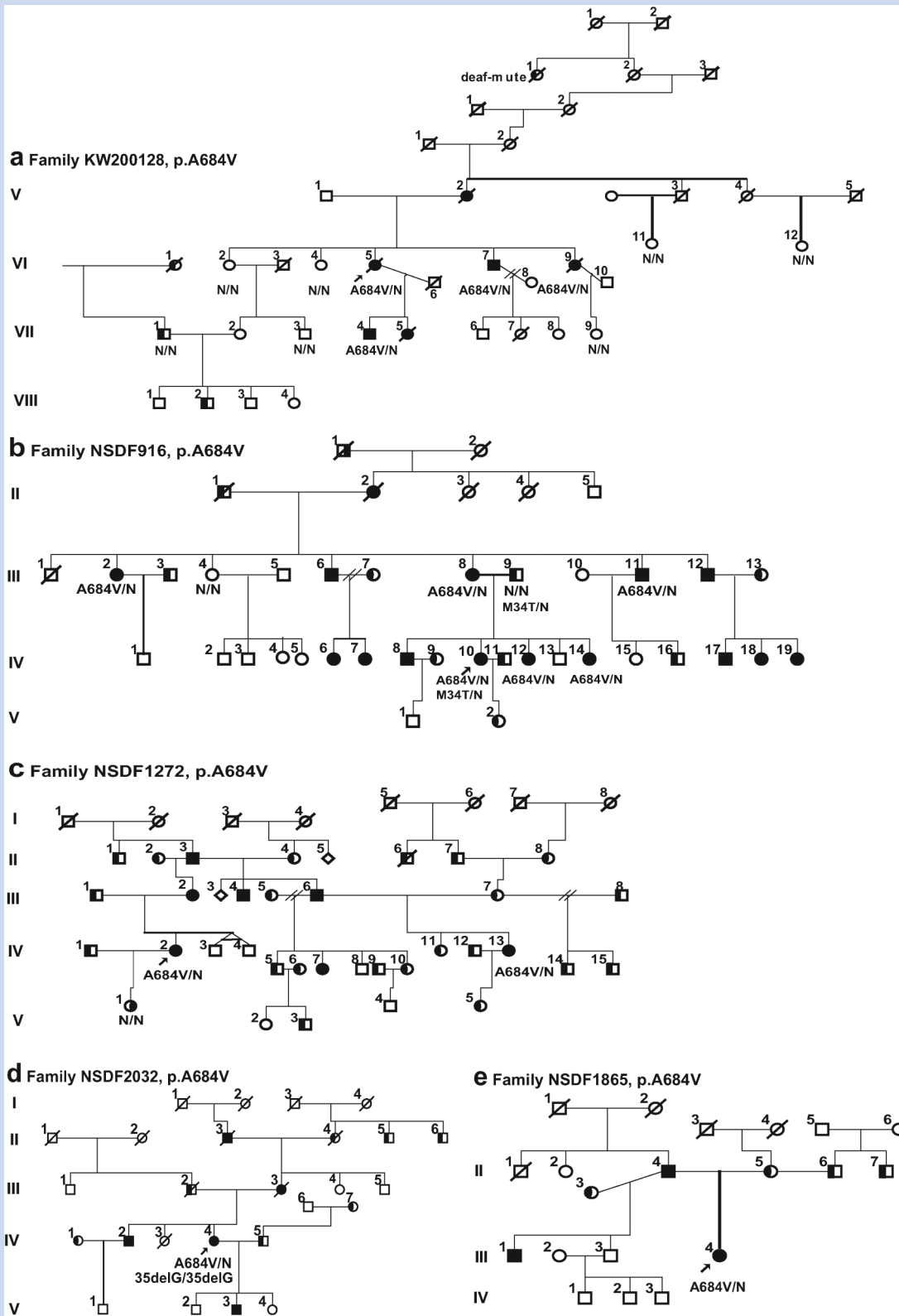
FIG. 1. Pedigrees of the eight families investigated in this study. All the families show a segregation pattern compatible with the conclusion that the indicated *WFS1* mutation causes autosomal dominant optic atrophy and hearing loss, except for family 81, where the father of two children with Wolfram syndrome is a sporadic case. Probands are indicated by arrows. Black symbols indicate optic atrophy with hearing loss patients. Black shading on the left part of symbol indicates individuals with isolated hearing loss and on the right part individuals with isolated optic atrophy. The *WFS1* molecular result is indicated below individuals from whom DNA samples were available. N = normal allele. The presence of *GJB2* mutation are indicated (c.35delG or p.M34T).
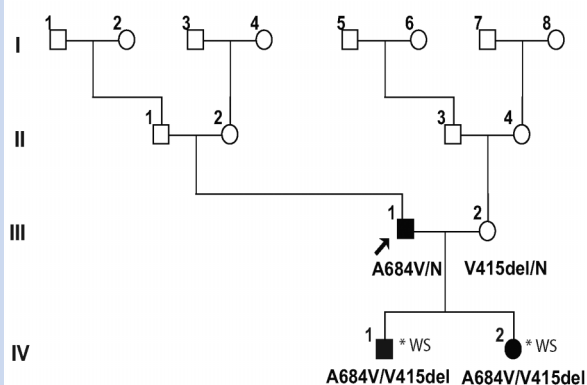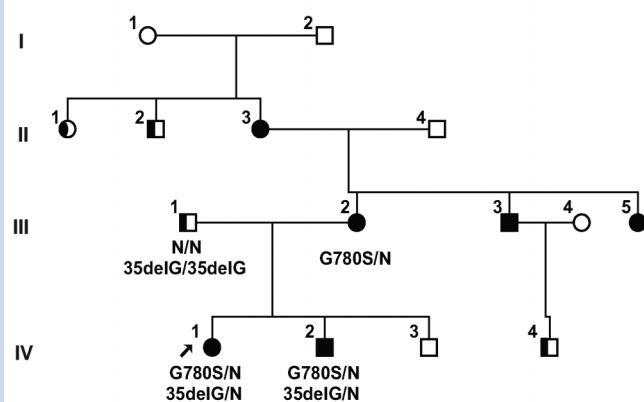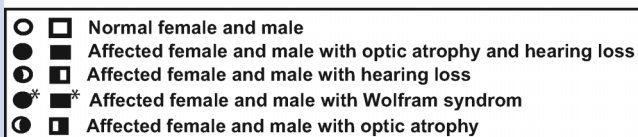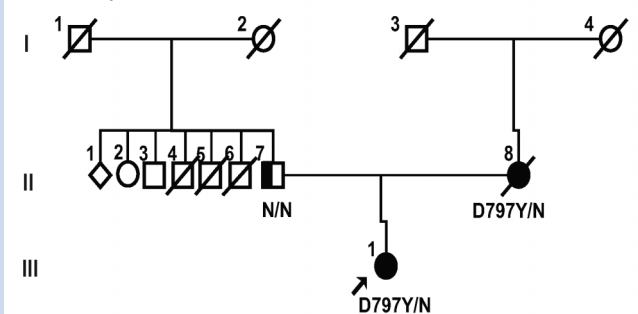
f Family 81, p.A684V

g Family KW010862, p.G780S

h Family NSDF1793, p.D797Y

○ □ Normal female and male
● ■ Affected female and male with optic atrophy and hearing loss
◖ ◧ Affected female and male with hearing loss
●* ■* Affected female and male with Wolfram syndrom
◑ ◨ Affected female and male with optic atrophy

FIG. 1. (*Continued*)

[Arnos et al., 2008] and the North American Repository of deaf individuals [Pandya et al., 2003].

Sequence Analysis

Prior to *WFS1* sequence analysis, *GJB2* sequencing was performed in all probands and mutations were ruled out as the genetic cause of deafness in all but one proband, who had a complex family history of deafness. Primers were as described in Eiberg et al. [2006] or designed to PCR amplify exons and 20–50 bp of surrounding intronic regions of *WFS1*, *OPA1*, *TIMM8A* (RefSeq NM_006005.2, NM_015560.2 (*OPA1* exons 1-28), NM_130837.2 (*OPA1* exons 4B and 5B) and NM_004085.3, respectively). *WFS1*, *OPA1* and *TIMM8A* were sequenced in DNA samples from all probands. *WFS1* exon 8 contains several polymorphisms and to avoid using primers annealing to a region with polymorphisms, which could result in PCR amplification of only one allele, *WFS1* exons 7 and 8 were amplified as one PCR product using the primers 5'-GCA-GATCATGTTCGATGGAGCGGTTGGC-3' and 5'-CCTCATGG-CAACATGCACTGGAAGCTCC-3', and AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen, Taastrup, Denmark) amplifying a fragment of 7930 bp. Furthermore, exon 8 sequencing primers were carefully positioned to avoid overlapping known polymorphisms. Primer sequences and PCR conditions are available upon request. PCR products were sequenced using BigDye Terminator chemistry (Applied Biosystems) and separated on an ABI 3130XL genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. Identified base pair changes were checked against databases of published polymorphisms and mutations (http://www.khri.med.umich.edu/research/lesperance_lab/low_freq.php; http://www.hgmd.cf.ac.uk/ac/index.php). In order to study the segregation of the mutations with the disease in each family, we used direct sequencing of PCR amplified genomic DNA. All detected mutations (Table I) were tested for their absence in 298 control chromosomes from 53 Danish and 96 UK ethnically matched Caucasian control individuals (from Sigma Aldrich).

The evolutionary conservation of wolframin amino acids among *WFS1* orthologs was investigated using the ClustalW2 multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and the Boxshade 3.21 program (hhttp://www.ch.embnet.org/software/BOX_form.html).

Haplotype Analysis

For haplotype analysis, five polymorphic dinucleotide repeat DNA markers from the 4p16.2-p15.33 *WFS1* genomic region (Tel- *D4S412*, *D4S3023*, *D4S431*, *D4S394*, *D4S403*-Cen) were used according to standard protocols (Table IS online). Information about primer sequences and physical map positions were obtained from UCSC Genome Browser Human Mar. 2006 (hg18) assembly web site (http://genome.ucsc.edu/index.html?org=Human). An 18-bp extension sequence (5'-TGACCGGCAGCAAAATTG-3') was added to the 5' end of the forward primer to allow amplification of a fluorescently labeled third primer for visualization on an ABI 3130XL genetic analyzer [Mellersh et al., 2006]. Genotypes were scored semi-automatically using Genemapper (Applied Biosystems) and the haplotypes were constructed manually.

TABLE I. Clinical Manifestations in Eight Probands

Family	KW200128[a]	NSDF916	NSDF1272	NSDF2032	NSDF1865	81	KW010862	NSDF1793
Proband ID	KW200128-VI:5	916-IV:10	1272-IV:2	2032-IV:4	1865-II:4	Fam81-III:1	KW01862-IV:1	1793-III:1
Inheritance	ADOA+HI	ADOA+HI	ADOA+HI	ADOA+HI	ADOA+HI	Possibly sporadic	ADOA+HI	ADOA+HI
Age	Deceased age 63	32	38	54	46	55	14	49
Nationality	Swedish	US/Caucasian	US/Caucasian	US/Caucasian	US/Caucasian	US/Caucasian	UK	US
Phenotype	OA+HI	OA+HI	OA+HI	OA+HI	OA+HI	OA+HI	OA+HI	OA+HI
WFS1 Mutation	p.A684V	p.A684V	p.A684V	p.A684V	p.A684V	p.A684V	p.G780S	p.D797Y
Optic atrophy								
Age of onset (years)	9	[Diagnosed at age 26]	[Diagnosed at age 30]	16	11	—	12	20
Visual acuity	20/40 (R) 20/40 (L)	20/40 (R) 20/32 (L)	—	—	[Diagnosed at age 16] 20/30 (R) 20/30 (L)	[Diagnosed at age 41] 20/60 (R) 20/60 (L)	20/25 (R) 23/25 (L)	20/80 (R) 20/70 (L)
Color vision	—	Abnormal color vision test (Ishihara plates)	—	—	Normal	—	Reduced	Not reported night and color vision problems
Optic discs/ optic nerves	Pale	Pale optic discs and cupped to 0.8	—	—	Extreme pallor of optic discs with hypoplasia of the vessels and cupped to 0.7	Extreme pallor of optic nerves	Bilateral pallor of optic nerves	Some pallor of the discs and narrowing of the vessels. Pale optic nerves bilaterally
Hearing impairment								
Age of onset (years)	Early childhood	Congenital	Early childhood	Congenital	1.5y	3y	Congenital	3–4y
Severity	Severe, progressive	Severe to profound	Profound, progressive	?	Severe to profound	Profound	Profound	Severe to profound, progressive
Diabetes	—	No	—	—	No	No	No	No
Psychiatric abnormalities	Depression, hallucinations[b]	—	—	—	Depression	—	No	No
Other disorders / symptoms	—	—	—	—	Developed diabetes insipidus at age 20 following hypophysectomy for suspected pituitary tumor	—	No	No
Additional Information	Myopia in one family member	Humphrey visual fields with small enlargement of blind spot bilaterally. Retinal vessels, macula and retina periphery otherwise normal	Daughter with OA, normal hearing and bipolar illness does not have the mutation	GJB2 c.35delG homozygote	As part of evaluation for OA she had a CT scan. Mother is deaf from congenital rubella syndrome. Eye pressure 10 and 12 mm. Father is deaf, have OA and reported to have problems with color vision	Has a son and a daughter with Wolfram syndrome and two WFS1 mutations: p.V415del and p.A684V[c]	Normal p-glucose and urine osmolality. Maternal great aunt with psychosis	OA was confirmed by CT/ MRI scan. Except from deafness and OA physical examination normal. Eye pressure 10 and 12 mm. Normal macula and no evidence for retinal dystrophy. Also no diabetes in the mother of the proband who is deaf and has OA

ADOA, autosomal dominant optic atrophy; HI, hearing impairment; (—) no information; y, years; a, the symptoms of the affected individuals have previously been described in Swedish [Samuelson, 1940]; b, some affected family members have anxiety and one affected family member [VII:5] committed suicide; c, The p.V415del mutation in the children is inherited from their mother (the mutations are listed in the Krege/WFS1 database); mm, millimeters of mercury.

mtDNA Deletion Analysis

Total DNA was extracted from skeletal muscle using QIAamp® DNA Mini Kit (Qiagen). Aliquots of 0.1 µg DNA were digested with the restriction enzyme PvuII (New England Biolabs) and fractionated by electrophoresis in 0.5% agarose gels. The DNA was then transferred to Hybond-C nitrocellulose filter (GE Healthcare) by capillary blotting under standard procedures. The filter was hybridized with an equimolar mix of radiolabeled mtDNA probes corresponding to nucleotides 1-12640 and 14956-16569, as described previously [Larsson et al., 1990]. Hyperfilm MP (GE Healthcare) was exposed to the filter for 4 hr before development.

Protein Expression Analysis of Wolframin Mutants

Expression vector for wild-type, myc epitope-tagged wolframin was kindly provided by Dr. Timothy G. Barrett, UK. Mutations were introduced in wolframin using the QuickChange procedure (Stratagene) and confirmed by sequencing. Each mutant was generated three independent times and subjected to protein expression analysis. HEK293 cells were cultured and transfected as described [Doehn et al., 2009]. Briefly, 1.6×10^5 cells were seeded per 3.1 cm² dish and co-transfected the following day, using 0.5 µg of wolframin and enhanced green-fluorescent protein (EGFP) expression plasmid, respectively, complexed with 3 µl FuGENE 6 reagent (Roche), according to the manufacturer's instructions. After 16 hr, the cells were lysed with SDS-PAGE sample buffer (2% sodium dodecyl sulfate, 62 mM Tris-HCL (pH 6.8), 10% glycerol, 50 mM dithiothreitol, 0.12% bromophenol blue). Aliquots of the cell lysates were subjected to SDS-PAGE and immunoblotting analysis with antibody against the myc-tag on wolframin or EGFP, using standard immunoblotting procedures.

RESULTS
Families

The pedigrees of the eight families studied here all present autosomal dominant OA and SNHL and are shown in Figure 1. Clinical and genetic findings are summarized in Tables I–III. None of the probands contained the OPA1 mutations p.R445H or p.G439V nor any other mutations in the entire OPA1 or TIMM8A genes that might cause disease (a single silent OPA1 variant, p.R393R was identified in one proband). Below, we describe the WFS1 mutations that were identified in each family and absent from 298 ethnically matched control chromosomes, as well as the clinical manifestations. Identified polymorphisms are listed in Table IIS online. GJB2 results were negative, except in a few cases that are indicated in Figure 1. Representative audiograms are provided in Figure 3.

Family KW200128. This is a large eight-generation family (Fig. 1a) from Sweden with six affected individuals and is the family described in the first report of isolated ADOA and SNHL [Samuelson, 1940]. Four members of the family, V:2, VI:5, VI:7, and VI:9, suffered from the same complaints, but the severity of vision loss and hearing impairment varied among the family members [Samuelson, 1940]. Samuelson [1940] suggested that the cause of the reduced vision and hearing was atrophy of the optic nerve, and the cochlear nerve, respectively.

In our follow-up study, the proband, VI:5, now deceased, had OA from age 9 and severe bilateral SNHL from early childhood as well as psychiatric problems (depression and hallucinations). Furthermore, one affected family member (VII:5) committed suicide, and four affected family members have regular psychiatric treatment in order to cope with their anxiety. In one branch of the family a different and unknown dominant cause of SNHL segregated, since individuals VI:1, VII:1, and VIII:2 all had moderate sensorineural hearing loss, but no visual problems and none had the WFS1 mutation described below. GJB2 sequencing in DNA from VII:1 was normal. Cochlear implantation was performed at age 49, 57, and 77 years, respectively, in individuals VII:4, VI:7, and VI:9 with variable degree of benefit. Individual VI:7 experienced considerably improved hearing (Fig. 3), but the outcome was questionable for the other two individuals due to old age, general medical frailty and inability to perform relevant training efforts after CI treatment.

The OA in the family presented with temporal paleness of the optic nerves similar to the optic atrophy due to OPA1 mutations. The visual impairment progressed to low visual acuity after the age of 60, and a subsequent need for special visual adjustments (Table III). In four of the six individuals, glaucoma developed and responded well to standard treatment. The glaucoma treatment may have caused a relative improvement of visual capacity due to the optic atrophy, until late stages of the course where restriction in visual field occurred. These family members were followed in many instances several years ago, and details of visual acuity were not recorded in accordance with current standards.

To establish a molecular diagnosis, DNA was collected from nine individuals, four of whom were affected with OA and SNHL (VI:5, VI:7, VI:9, and VII:4, Fig. 1). Since the clinical features suggested that the disease was caused by OPA1 mutation, we first sequenced this gene. However, no OPA1 mutation was identified. Next the gene for Mohr–Tranebjaerg syndrome, TIMM8A, that is also present with similar features was also sequenced, but again no mutation was identified. Since Wolfram syndrome presents with OA and deafness and sometimes with psychiatric problems, as in the proband, we speculated that mutation of WFS1 might be the cause of disease in the family. Indeed, sequencing of WFS1 in the proband identified a heterozygous sequence change c.2051C > T in exon 8, leading to a substitution of alanine for valine at position 684 of wolframin (p.A684V). A684 is located in the hydrophilic C-terminus of wolframin and is conserved in evolutionarily distant species such as mouse, rat, chicken, frog, and zebrafish (Fig. 2B). The mutation was absent in 298 control chromosomes. These facts along with the co-segregation of the p.A684V mutation with the disease strongly indicate that this mutation underlies optic atrophy and hearing loss in the family. The mutation appears to be de novo, first manifested in affected individual V:2 who had unaffected sibs and parents, who died in old age with preserved hearing and vision.

We then speculated whether the p.A684V WFS1 mutation could lead to multiple mithochondrial DNA (mtDNA) deletions since some early studies identified mtDNA deletions in Wolfram syndrome patients [Gomez-Zaera et al., 2001] and since some OPA1 mutations lead to multiple mtDNA deletions in skeletal muscle [Amati-Bonneau et al., 2008]. Therefore, skeletal muscle biopsy specimens from one p.A684V mutation carrier (VI:7) was

TABLE II. Clinical Manifestations in Additional Family Members

Family	NSDF916	KW010862	KW010862	KW010862
Patient ID	III:2	III:2	III:5	IV:2
Inheritance	ADOA + HI	ADOA + HI	ADOA + HI	ADOA + HI
Age	69	48	42	9
Nationality	US/Caucasian	UK	UK	UK
Phenotype	OA + HI	OA + HI	OA + HI	OA + HI
WFS1 Mutation	p.A684V	p.G780S	Not tested	p.G780S
Optic atrophy				
Age of onset (years)	26	Symptoms from age 20	Diagnosed at age 42 (as a consequence of diagnosis in IV:1)	Diagnosed at age 9 (as a consequence of diagnosis in IV:1)
Visual acuity	20/70 (R) 20/25 (L)	20/40+3 (R) 20/40+2 (L)	20/30 (R) 20/30 (L)	10/10 (R) 10/10 (L)
Color vision	—	—	Reduced	—
Optic discs	Pallor and shallow cup	Bilateral pallor of optic discs, particularly temporal	Bilateral pallor	Bilateral pallor
Hearing impairment				
Age of onset (years)	Congenital	Congenital	Congenital	Congenital
Severity	—	Profound	Profound	Profound
Diabetes	—	—	No	No
Psychiatric abnormalities	—	Schizophrenia. Treated for psychosis	No	Autistic
Neurological abn.	—	No	No	No
Mental retardation	—	No	No	No
Other disorders/symptoms	Late onset bilateral cataract	No	No	No
Additional Information	Eye pressure 13 mm, blurred vision	Chlorpromazine cataracts	Normal plasma glucose	Normal urine osmolality

ADOA, autosomal dominant optic atrophy; HI, hearing impairment; (—) no information; mm, millimeters of mercury.

analyzed for deletions of mtDNA by Southern blotting. However, no mtDNA deletions could be detected (data not shown).

In conclusion, these data suggested that ADOA and SNHL in the family were caused by the p.A684V mutation in *WFS1*.

Family NSDF916. This is a five-generation, large Caucasian American family of English origin with 15 affected individuals (Fig. 1b). DNA was collected from eight individuals, six of whom were affected (III:2, III:8, III:11, IV:10, IV:12, and IV:14, Fig. 1). The 32-year-old proband (IV:10) has had optic atrophy since childhood and has bilateral, congenital severe to profound hearing impairment. Sequencing of *WFS1* in the proband revealed the same heterozygous c.2051C > T mutation in exon 8 leading to the p.A684V mutation identified in the above-mentioned Swedish family. The mutation consistently co-segregated with optic atrophy and hearing loss in the family. The proband was heterozygous for the p.M34T sequence variant in *GJB2*, as is her father, who is deaf, but does not have OA.

Family NSDF1272. This is a five-generation, Caucasian American family of Polish/Russian and mixed European ancestry with seven affected family members (Fig. 1c). DNA was available from three individuals, of whom two were affected with optic atrophy

and hearing loss. The proband (IV:2) has optic atrophy which was diagnosed at approximately age 30 and bilateral profound hearing loss, which was progressive and started in early childhood.

The p.A684V mutation in *WFS1* was identified in the proband and in a remote family member (individual IV:13), who also has bilateral OA and SNHL. In contrast, the daughter (V:1) of the proband, has hydrocephalus, a complex medical history, bipolar illness, and optic atrophy, but has normal hearing and does not have the mutation. Thus, the isolated case of optic atrophy in V:1 cannot be explained by results of *WFS1* or *OPA1* sequencing, suggesting that her optic atrophy is due to another, genetic or environmental, cause.

Family NSDF2032. This is a five-generation Caucasian American family of Dutch ancestry with five affected individuals (Fig. 1d). Individual IV:4 had OA diagnosed at the age of 16 years and congenital deafness, and was found to have the p.A684V mutation in *WFS1*. In addition, this individual is also homozygous for the c.35delG mutation in *GJB2*. It is quite conceivable that she has two forms of hereditary deafness given her complex family history of deaf parents and deaf maternal grandparents.

Family NSDF1865. The proband (III:4) of this Caucasian American family (Fig. 1e) has optic atrophy, diagnosed at age

TABLE III. Ophthalmological Examination Results in Family KW200128

Patient	Year of birth	Age at diagnosis of optic nerve atrophy	BCVA 0–20 y	BCVA 21–40 y	BCVA 41–50 y	BCVA 51–60 y	BCVA 61–70 y	BCVA 71–80 y	BCVA 81–100 y	Papillae (by ophthalmoscopy)	Age at diagnosis of glaucoma	Other
V:2	1895	42	—	—	R 0.5 L 0.3	—	R 0.1 L 2/60	R 2/60 L 1/60	R 1/120 L <1/120	Bilateral pale especially temporally	68	—
VII:5	1951	8	R 0.9 L 0.9	R 0.9 L 0.9	R<0.1 L 0.2	—	—	—	—	Bilateral pale optic nerves (from age 8)	43	—
VII:4	1955	18	R 1.0 L 1.0	R 1.0 L 1.0	R 0.5 L 0.6	—	—	—	—	Bilateral pale optic nerves	No glaucoma	Myopia
VI:5 (Proband)	1928	22	R 0.5 L 0.5	R 0.5 L 0.5	—	R 0.1 L 0.1	R A L 1/120	—	—	Bilateral pale optic nerves	R 56 L 65 + CV0	—
VI:9	1925	12	—	R 0.4 L 0.5	—	R 0.1 L 0.1	R 0.1 L 0.1	—	—	Bilateral pale optic nerves	No glaucoma	Visual field reduced to 20 degrees at age 70
VI:7	1931	25	—	—	—	R 0.1 L 0.1	R 0.1 L 1/60	R 0.1 L 1/60	—	Bilateral pale optic nerves	48	—

y, age in years; BCVA, best corrected visual acuity by Snellen test; R, Right eye; L, Left eye; (—) no information; A, amaurosis; CV0, central venous occlusion.

Additional ophthalmological description: In family KW200128 there was a characteristic course of the progressive visual impairment due to optic nerve atrophy. The visual acuity is affected moderately in childhood, slowly decreasing in middle age and causing daily problems from 50–60 years of age. Four out of eight individuals affected by optic nerve atrophy, in addition, developed glaucoma, which leads to further visual decrease and one individual developed bilateral venous thrombosis leading to very low vision.

The optic neuropathy showed the typical picture of inherited optic nerve atrophy, i.e., early thinning of the nerve fiber layer especially in the papillomacular bundle. Defects in this part of the nerve fiber layer early influence the visual acuity and all affected family members have very low visual acuity after age 60. The optic nerve appears pale and flat, influencing the quality of vision i.e., color vision, contrast vision and mobility-orientation. Visual fields show generally increasing low sensitivity and are concentrically narrowed until about 40–50 degrees in the late stages. Individuals with glaucoma, moreover, were affected by typical Bjerrum scotoma which further narrowed the visual fields.
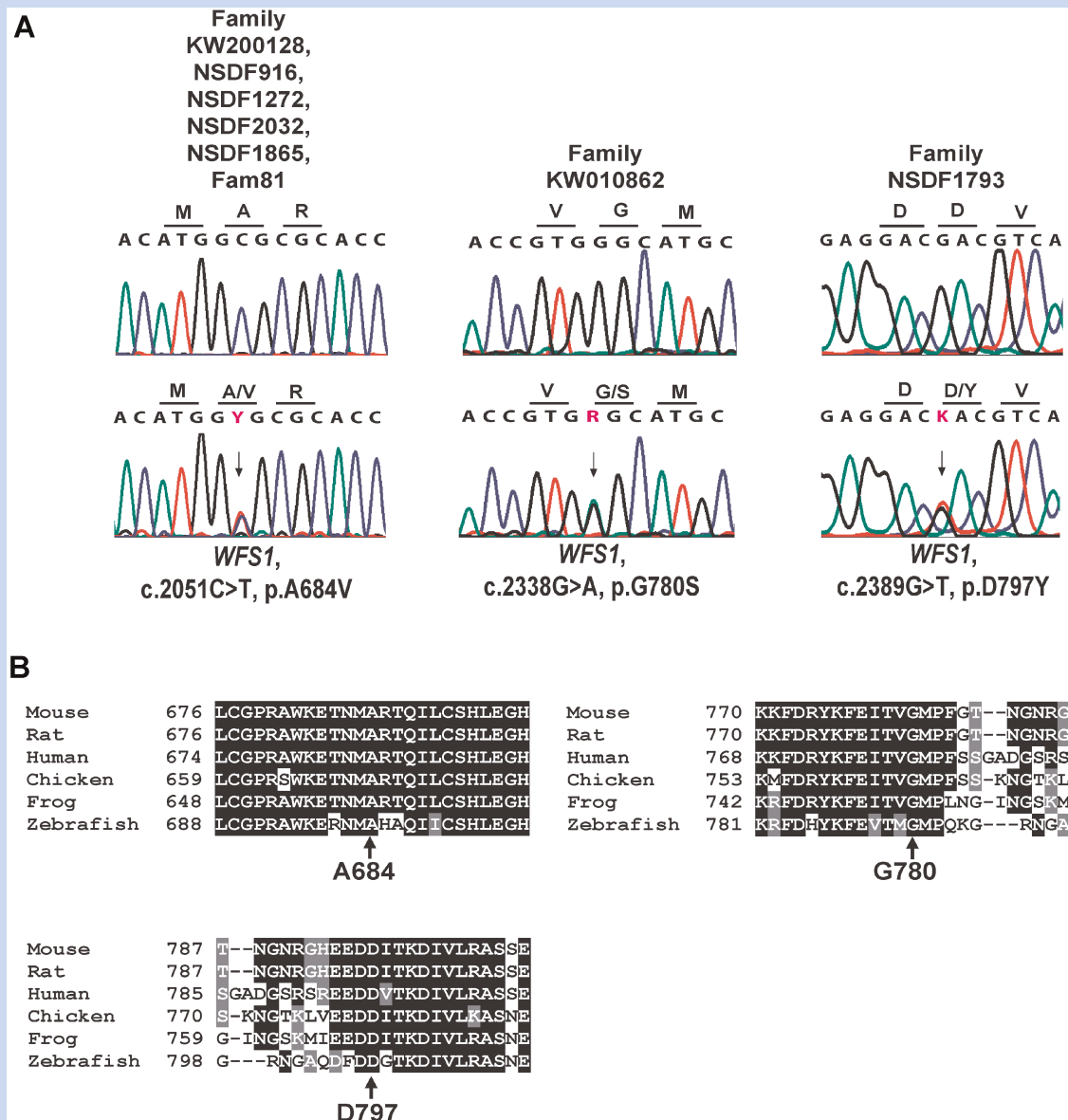
FIG. 2. **(A)** Representative sequence chromatograms for each of the *WFS1* missense mutations compared to a normal control. The arrows indicate the nucleotide changes of the heterozygous missense mutations. Nomenclature of mutations refers to the *WFS1* RefSeq NM_006005.2, with nucleotide number +1 being A of the start codon ATG. Each mutation is heterozygous in affected individuals. **(B)** Alignment in different species shows strong evolutionary conservation of the relevant amino acid mutated in the patients. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

16, and bilateral, severe to profound sensorineural hearing loss (Fig. 3). She has also been treated for depression since the age of 38. Sequencing of *WFS1* again revealed the p.A684V mutation.

Family 81. Four members, two parents and their children, of this Caucasian American family were analyzed (Fig. 1f). The father (III:1) is of Dutch and Italian descent, while the mother (III:2) is of Dutch-Polish, and German-Irish descent. III:1 is the only relative with optic atrophy and deafness without other manifestations of Wolfram syndrome. He had bilateral, profound SNHL diagnosed at age 3 years and OA diagnosed at age 41 by the ophthalmologist (M.B.M) evaluating his children. Both IV:1

and IV:2 were diagnosed with Wolfram syndrome based on juvenile onset insulin-dependent diabetes mellitus, optic atrophy, bilateral profound sensorineural hearing loss, and congenital cataracts. IV:1 had hearing loss diagnosed at birth and IV:2 had hearing loss diagnosed at age 3 months and premature birth at 30 weeks of gestation.

III:1 is heterozygous for p.A684V, and III:2 is apparently asymptomatic and heterozygous for p.V415del mutation. Their children, IV:1 and IV:2, are compound heterozygotes for the *WFS1* mutations p.A684V and p.V415del (c.1243_1245delGTC). Unfortunately, neither medical data nor DNA were available
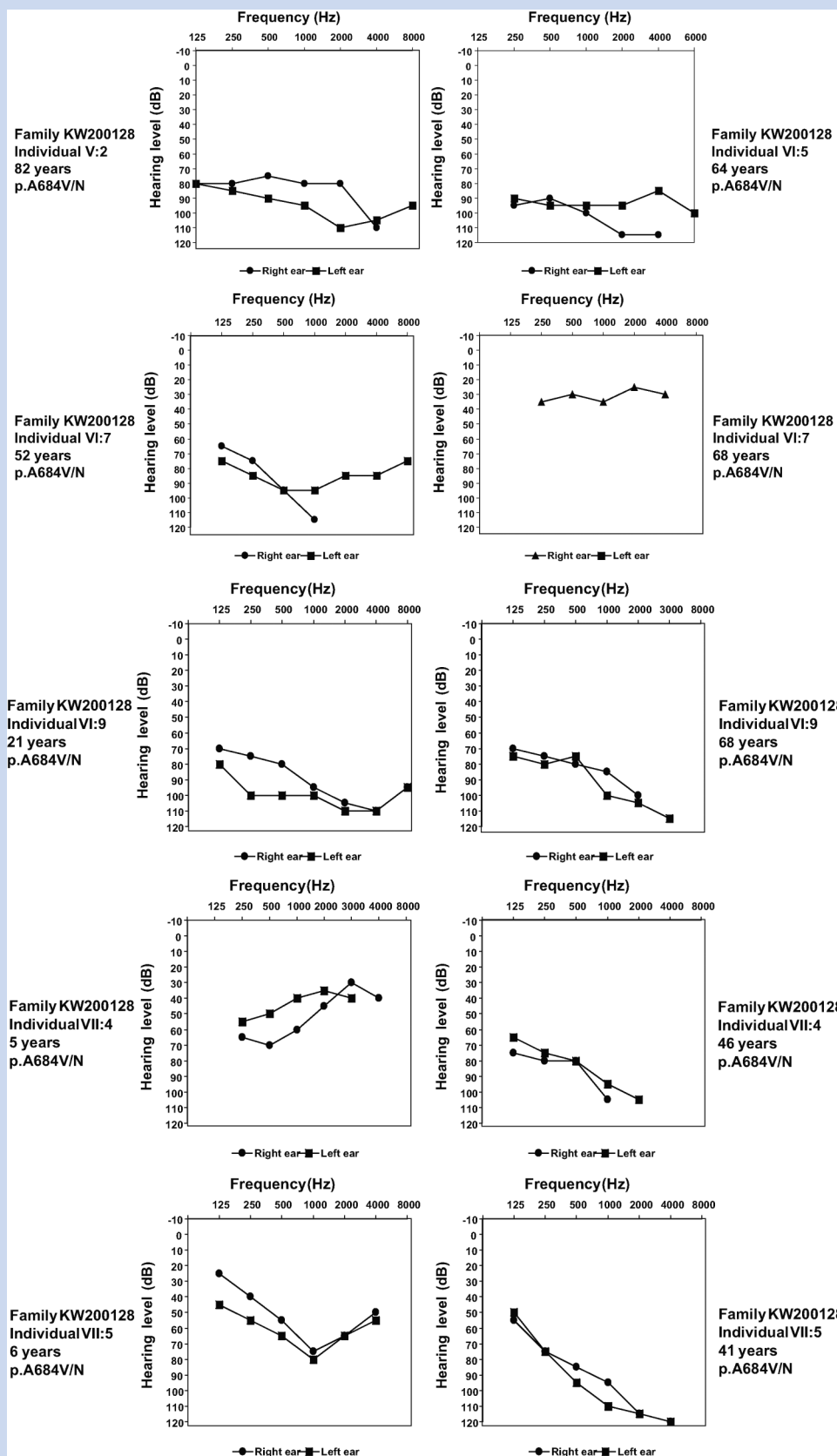
FIG. 3. Pure-tone audiograms of left and right ear of representative, affected family members heterozygous for *WFS1* mutation from six of the families' studies (KW2001128 (audiograms from six individuals), NSDF916, NSDF1865, NSDF1272, and NSDF1793). For some individuals two audiograms are shown to illustrate the hearing loss over time. Individual VI:7 (family KW200128) had cochlear implant at age 57 years, resulting in an improved hearing between age 52 and age 68.
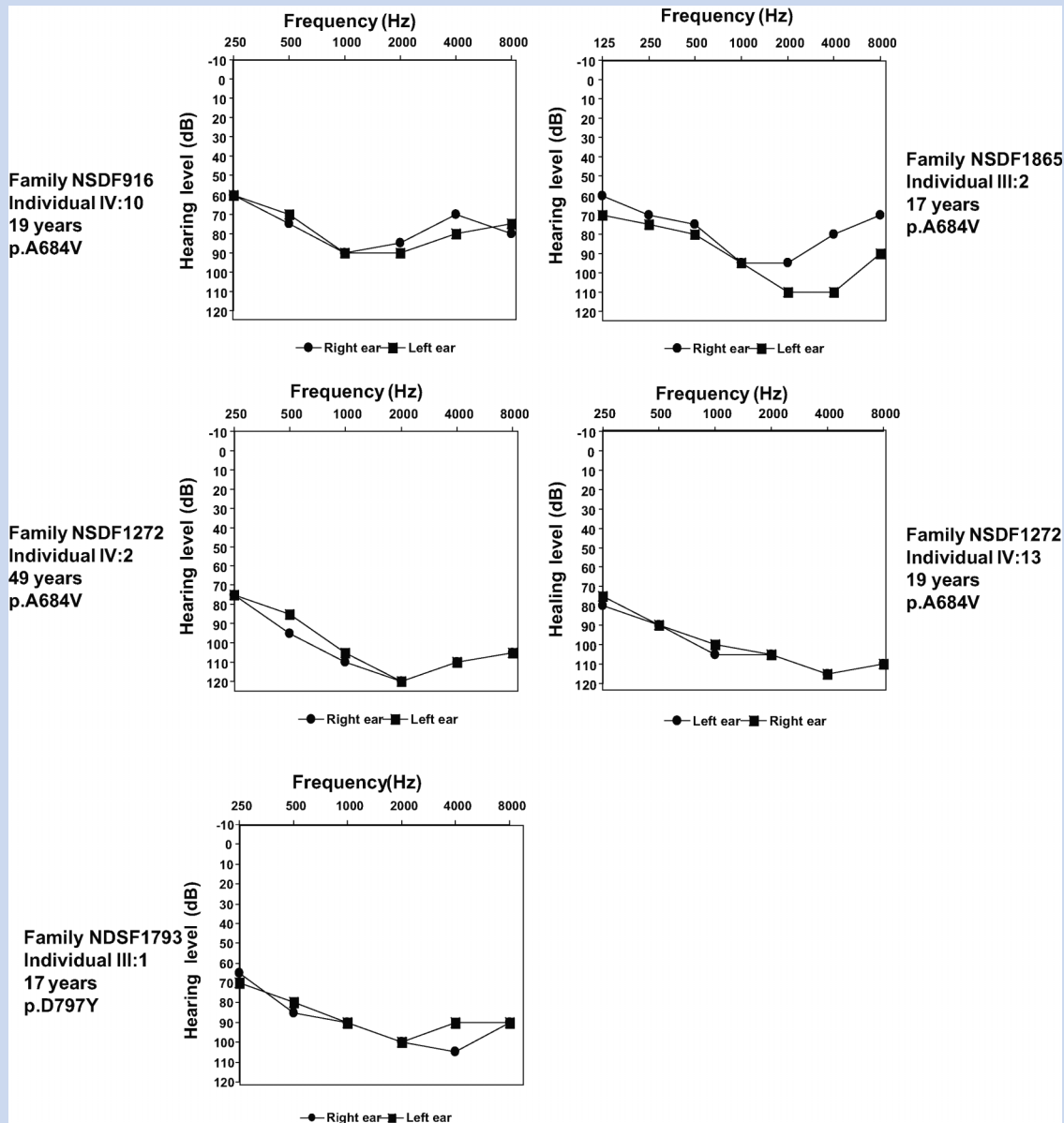
FIG. 3. (*Continued*)

from the parents of the father, III:1, and therefore it can not be determined whether the p.A684V mutation is a de novo event or transmitted from one of his parents. In any case, the phenotype of the children suggest that the p.V415del mutation aggravates the effect of the p.A684V mutation, resulting in Wolfram syndrome.

Family KW010862. This is a Caucasian UK family with six affected individuals (Fig. 1g). DNA was available from four individuals (three affected and one unaffected). The proband (IV:1) is reported to have bilateral, prelingual, profound hearing loss in addition to optic atrophy. The proband has normal plasma glucose and urine osmolality.

Sequencing of *WFS1* revealed a novel heterozygous mutation, c.2338G > A, in exon 8, which results in the substitution of a

glycine codon for a serine codon at position 780 (p.G780S). p.G780S segregates with disease in the available family members. Furthermore, the mutation was absent from 298 control chromosomes. Amino acid p.G780 is located in the hydrophilic C-terminus of wolframin and is conserved in wolframin from species such as mouse, rat, chicken, frog, and zebrafish (Fig. 2B). Upon testing for the c.35delG mutation in *GJB2*, the deaf father (Fig. 1g, III:1) of the proband was homozygous, and individuals IV:1 and IV:2 were heterozygous.

Family NSDF1793. This is a small Caucasian American family with two affected individuals (Fig. 1h). The father of the proband is of Polish descent and her mother of Scottish descent. The proband (III:1) had hearing loss first suspected at the age of 3–4 years, which progressed to a severe to profound, sensorineural hearing loss.

Optic atrophy was recognized at approximately age 20 years. The proband had normal mental and intellectual status.

Sequencing of *WFS1* in the proband revealed a novel heterozygous mutation c.2389G > T in exon 8. The mutation results in substitution of an aspartic acid codon for a tyrosine codon at position 797 (p.D797Y). The affected mother of the proband is also heterozygous for this mutation. The mutation was absent from 298 control chromosomes. Amino acid p.D797 is located in the hydrophilic C-terminus of wolframin and is conserved in wolframin from species such as mouse, rat, chicken, frog, and zebrafish (Fig. 2B).

Haplotype Analysis

Haplotype analysis was performed in family NSDF916, with the recurrent p.A684V mutation, by using five polymorphic markers (*D4S412*, *D4S3023*, *D4S431*, *D4S394*, and *D4S403*) flanking *WFS1*. A common haplotype for all affected individuals was found (Fig. 4A). Marker analysis with the same five markers in probands from the other five p.A684V families showed that one of the probands (NSDF1272-IV:2) had allele sizes for two markers (*D4S412* and *D4S3032*) in common with family NSDF916, and three probands (NSDF-2032:IV:4, NSDF1865-III:4, and Fam81-III:1) had allele size for one marker (*D4S3023*) in common with family NSDF916 (Fig. 4B). However, there is no indication of one ancient shared haplotype between these families that originate from very different parts of the world, and it is most likely that the mutation has arisen independently several times.

Protein Expression Analysis of Identified Wolframin Mutations

We next performed functional expression analysis of certain of the indentified mutations to further validate that they are pathogenic. Thus, we transiently transfected HEK cells with plasmid expressing myc epitope-tagged wild-type wolframin or wolframin harboring selected mutations and analyzed the cells for the expression level of the exogenous wolframin by immunoblotting for the myc tag. This analysis revealed that the p.A684V and the p.V415del mutants showed greatly and the p.G780S mutant mildly decreased protein expression compared to wild-type wolframin (Fig. 5, upper panel). The p.R629W mutation previously shown to confer instability to wolframin protein, resulting in decreased protein expression and Wolfram syndrome, was included as a positive control [Hofmann et al., 2003]. To control for equal transfection efficiencies across the cell populations, we immunoblotted for enhanced green-fluorescent protein (EGFP) that we had co-transfected together with wolframin. As shown in Figure 5 lower panel, all samples showed equal expression of EGFP, confirming equal transfection across all the cell populations analyzed.

DISCUSSION

We have identified heterozygous missense mutations in *WFS1* as the cause of ADOA and SNHL in eight families. Previously, isolated ADOA and SNHL were reported only in association with *OPA1* mutations, but our present report of multiple families, supported by the very recent report of one family [Hogewind et al., 2010], shows that *WFS1* mutations can also underlie isolated ADOA and SNHL. Furthermore, we have discovered that a p.A684V *WFS1* mutation underlies this dual sensory impairment in the original Swedish family [Samuelson, 1940] and in five of seven additional families suggesting that the p.A684V *WFS1* mutation may in fact be a frequent (maybe even primary) cause of isolated ADOA and SNHL. Finally, we demonstrate that ectopic expression in HEK cells of wolframin with the p.A684V, p.V415del, and p.G.780S *WFS1* mutations, respectively, results in reduced levels of mutant wolframin as compared to wild-type wolframin, supporting the conclusion that these mutations are disease-causing.

Wolfram syndrome is an autosomal recessive condition with minimal diagnostic criteria of juvenile OA and juvenile onset diabetes mellitus. Importantly, these clinical criteria were developed prior to identification of the responsible gene, *WFS1*. Several studies on families with Wolfram syndrome have been performed. In these studies, Wolfram syndrome is in agreement with an autosomal recessive mode of inheritance and mutations have been identified on both alleles in Wolfram syndrome patients and carriers of one mutation are healthy. Thus, isolated optic atrophy and hearing loss has not to our knowledge been reported as a feature in heterozygous carriers of these *WFS1* mutations. However interestingly, heterozygous carriers of *WFS1* mutations may have an increased incidence of psychiatric disorders including suicide attempts, endogenous depression, short-term memory loss, and anxiety (reviewed in Cryns et al. [2003]).

Our study supports recent data that a dominant form of partial Wolfram syndrome exists with the key features of optic atrophy and sensorineural hearing loss, not necessarily of juvenile onset. Furthermore, psychiatric illness was present in family KW200128 and family NSDF1865. A *WFS1* p.E864K missense mutation has been reported in two families with dominantly inherited deafness and some members affected by OA and impaired glucose regulation/diabetes [Eiberg et al., 2006; Valero et al., 2008]. In two Japanese families, however, the p.E864K mutation only caused isolated LFSNHL [Fukuoka et al., 2007]. Finally, most recently, a p.K836N mutation in *WFS1* has been found associated with autosomal dominant optic neuropathy and deafness in a family with three affected members [Hogewind et al., 2010]. Interestingly, simultaneously with the family described by Hogewind et al. [2010], Fujikawa et al. [2010] published a family with 14 affected members segregating SNHL associated with mutation of the same *WFS1* amino acid, K836, but here mutated to threonine instead of an asparagine. However, in this family, the deafness-affected individuals had no OA, nor any other *WFS1*-associated abnormalities, such as diabetes insipidus or mellitus (impaired glucose tolerance was reported in an individual with no deafness and severe depression in another with no deafness in the family, but genetic studies could not be performed in these individuals). These findings suggest that there may be a spectrum of phenotypes associated with *WFS1* based on differences in functional implications of the mutations, as suggested in the review by Tranebjaerg [2008].

We found three different wolframin missense mutations (p.A684V, p.G780S, and p.D797Y) to be associated with a dominant phenotype of OA and SNHL in eight families. The causative changes were all identified in exon 8 of *WFS1*, similar to the many
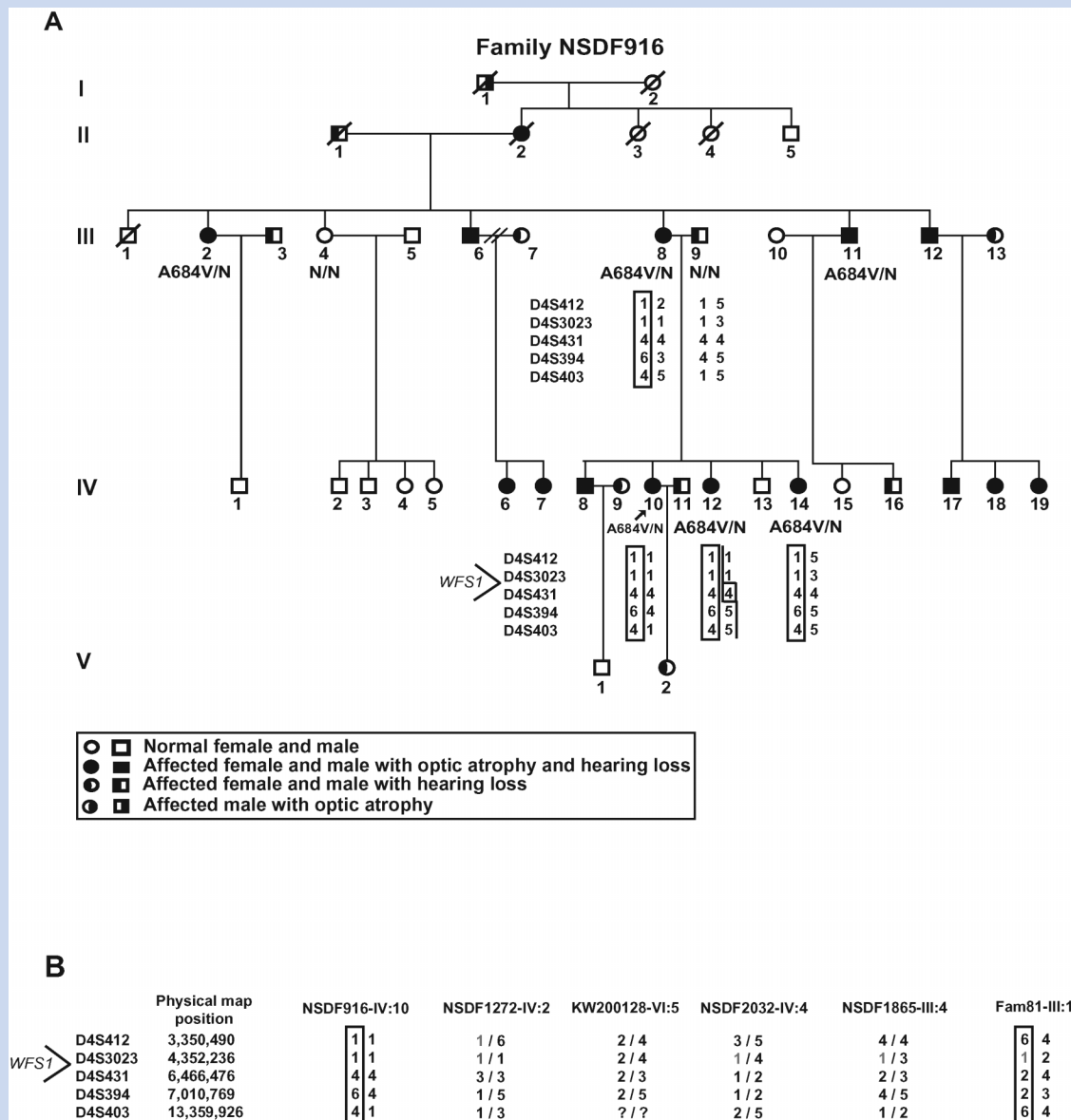
FIG. 4. Haplotype analysis. (A) Haplotype analysis in Family NSDF916, harboring the p.A684V mutation, using five polymorphic markers flanking *WFS1*. **(B)** Marker analysis with the same five markers in probands from the other five p.A684V families. A bar indicates the haplotype carrying the mutation in those families where this could be deduced.

known *WFS1* mutations causing Wolfram syndrome or LFSNHL. Several lines of evidence support the conclusion that they are pathogenic, rather than rare polymorphisms. Firstly, the three mutations were heterozygous and in perfect dominant co-segregation with optic atrophy and hearing loss. Secondly, they were absent from 298 ethnically matched control chromosomes and from the NCBI SNP database. Thirdly, all three mutations affect residues evolutionary conserved in human, rat, mouse, chicken, frog, and zebrafish wolframin (Fig. 2B), suggesting that altering these residues have deleterious consequences for the wolframin protein. Finally, we obtained experimental evidence for this later hypothesis by showing that the p.A684V, and to a smaller extent the p.G780S mutation, decreased wolframin protein expression levels. It may be noted that although the expression analysis provide strong evidence for a deleterious effect of the mutation, it does not provide a straight forward relationship between wolframin expression levels and severity of disease, since the p.V415del mutation caused the greatest decrease in wolframin expression, yet was associated with a phenotype only in compound heterozygosity with the p.A684V mutation. Furthermore, it may be noted that although the expression data suggest that the mutations cause disease, they do not provide evidence that they can cause dominant disease (as opposed to recessive disease). Rather, combined with the absence of mutations in any other known OA genes in any of our probands, these data collectively suggest that the identified mutations act dominantly to cause OA and SNHL. It can be speculated that the mutations cause a
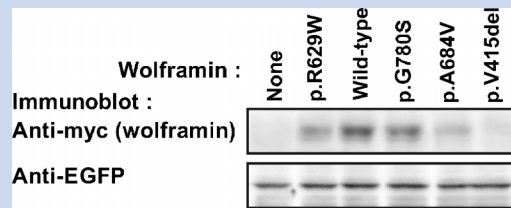
FIG. 5. Functional protein expression analysis of *WFS1* mutations. HEK293 cells were co-transfected with plasmid expressing myc-tagged wild-type or mutant wolframin together with plasmid expressing EGFP. The cells were lysed 16 hr post-transfection and the cell extracts were subjected to SDS-PAGE and immunoblotting for the myc tag and EGFP. The experiment was repeated three times with independently generated mutants with similar results.

misfolded wolframin protein. Part of the misfolded wolframin pool is degraded, but the remaining pool act as dominant negative mutants in the wolframin pathway to cause dominant disease. In conclusion, our findings support the involvement of missense mutations in *WFS1* in an isolated autosomal dominant monogenic form of hearing loss and optic atrophy.

Six of the eight families with ADOA and SNHL reported here harbored the same heterozygous *WFS1* mutation, p.A684V (c.2051C > T). Haplotype analysis in p.A684V individuals suggested that the c.2051C > T mutation arose independently in the families studied, and may represent a mutational hotspot. Importantly, these data open the possibility that a single, recurrent mutation in *WFS1* i.e., p.A684V, may be a common cause of isolated ADOA and SNHL, similar to the role played by the *OPA1* p.R445H mutation in autosomal dominant optic atrophy.

Prior to our study, the p.A684V mutation had not been reported in association with an isolated OA and hearing loss phenotype. However, this mutation had been identified in a patient from Italy with Wolfram syndrome [Tessa et al., 2001] (Family 3 at the Gene Mutation and Polymorphism Database at http://www.khri.med.umich.edu/research/lesperance_lab/wfs_delete.php). The Italian patient had diabetes mellitus, optic atrophy, hearing loss, diabetes insipidus, ataxic gait and psychiatric abnormalities. In addition to the p.A684V mutation, the patient also harbored a c.1387delCTCT mutation. The p.A684V mutation was absent in 100 Italian control chromosomes. Unfortunately, it is not known whether one of the parents had the p.A684V mutation *in trans* and associated with impaired vision and hearing (personal feed back from the corresponding author A. Tessa, who reported that the parents were unavailable for follow-up). By contrast, in the present study, DNA was available from the parents of two children (Family 81) with Wolfram syndrome, who are compound heterozygous for p.A684V and p.V415del in *WFS1*. The children both have juvenile onset of insulin-dependent diabetes mellitus, OA, bilateral profound sensorineural hearing loss and congenital cataracts [Mets et al., 2010]. Interestingly, we identified the p.A684V mutation in heterozygous state in the father, who has OA and hearing loss only, while the mother, heterozygous for the p.V415del mutation, had no symptoms. These data support that heterozygosity for p.A684V alone causes isolated OA and hearing loss. Since the mother had no

obvious disease symptoms, we went on to demonstrate that the p.V415del mutation greatly decreased wolframin protein expression. This demonstrates that the p.V415del alteration is indeed the responsible mutation that aggravates the effect of the p.A684V mutation, causing Wolfram syndrome in the children. We believe that a similar scenario also underlies the Wolfram syndrome phenotype of the Italian patient reported by Tessa et al. [2001], although his parents would have to be mutation analyzed to substantiate this conclusion.

The current lack of knowledge about the physiological and cellular and biochemical functions of wolframin makes it difficult to explain the disease-causing mechanism of the mutations. To this end, we addressed whether *WFS1* mutations could lead to multiple mitochondrial DNA deletions, by analyzing one p.A684V patient for deletions of mtDNA in skeletal muscle. However, no deletions were detected suggesting that the p.A684V mutation does not cause ADOA and hearing loss by increasing mtDNA instability. Unfortunately, muscle DNA was not available from the other patients presented here.

Till date, only mutations in *OPA1* have been repeatedly linked to the rare phenotype featuring isolated OA with sensorineural deafness. One recurrent *OPA-1* mutation, the heterozygous p.R445H missense mutation, underlies the large majority of reported cases of isolated OA and hearing loss. This mutation was first identified in a Japanese and a French patient [Amati-Bonneau et al., 2003; Shimizu et al., 2003] and subsequently in families from the US, and Belgium [Payne et al. 2004] and finally in five patients/families from France, Spain, and of Caucasian origin [Amati-Bonneau et al., 2005; Li et al., 2005]. In a few of these families, some patients also developed ptosis, ophthalmoplegia, ataxia, and/or nonspecific myopathy at middle age [Payne et al., 2004; Yu-Wai-Man et al., 2010]. Only two other *OPA1* mutations (c.970delCGTTCTCCA and p.G401D) have so far, to our knowledge been associated with isolated OA and hearing loss [Puomila et al., 2005; Ke et al., 2006; Amati-Bonneau et al., 2009]. Phenotypic characteristics of most of these patients include onset of hearing loss at the age of 6–30 years [Amati-Bonneau et al., 2005]. In contrast, all probands with *WFS1* mutation ascertained in the present study were congenitally deaf or had hearing loss onset in early childhood (Table I). Further patients need to be studied to evaluate whether *WFS1* mutation, and in particular the p.A684V mutation, is a predominant cause of dominant OA and SNHL, and whether there is a correlation between early onset of hearing loss and *WFS1* mutation versus *OPA1* mutation, to assist in molecular diagnosis of this disease.

The fact that carriers of *WFS1* mutations associated with Wolfram syndrome do not have hearing impairment and usually not significant if present, and the previous reports of heterozygosity for other *WFS1* mutations resulting in nonsyndromic autosomal dominant LFSNHL emphasize the difficulties in predicting the consequences of the different types of *WFS1* mutations [Eiberg et al., 2006; Tranebjaerg, 2008]. Our report supports this notion, since our study is the first study of several families with OA and hearing loss without other major clinical features (two families did have psychiatric abnormalities when carefully followed) found to be associated with mutations in *WFS1*. The first family segregating optic atrophy and hearing loss in a dominant pattern with a *WFS1* mutation (p.E864K) was described by Eiberg et al. [2006]. However,

in this family, careful metabolic evaluations of three out of four mutation carriers, revealed that they had undiagnosed diabetes, impaired glucose tolerance, and/or impaired insulinogenic index indicating that the p.E864K mutation may affect pancreatic beta-cell function as well [Eiberg et al., 2006]. It is entirely possible that additional abnormalities are actually present in a higher fraction of the families with *WFS1* related disorder, as illustrated by psychiatric disease in the two families in the present report (KW200128 and NSDF1865) and reported by Eiberg et al. [2006] and Valero et al. [2008]. A similar modification of clinical presentation became evident when *OPA1* patients in larger numbers were carefully clinically examined, showing that about 20% actually turned out to have neurological abnormalities [Yu-Wai-Man et al., 2010].

In several of our families there are several spouses with SNHL, who may introduce one or more additional SNHL genes. It can, therefore, be speculated that these genes may contribute to the phenotype or are acting digenically with the identified *WFS1* mutation in the respective families. In all cases, where we have both phenotypic and molecular genetic results and found a heterozygous missense mutation in *WFS1*, the OA and SNHL phenotype was present, pointing to that the respective *WFS1* mutations alone are capable of causing the disease phenotype. However, in one family (NSDF2032) the proband is congenital deaf, and in addition to being heterozygous for the p.A864V mutation in *WFS1*, this individual is homozygous for the c.35delG mutation in *GJB2*. Thus, in this case, it cannot be solved which of the two genes is causing her deafness.

In conclusion, we fully support the recent suggestion by Valero et al. [2008] of systematic sequencing analysis of *WFS1* in patients with diabetes and deafness (+/− optic atrophy), in particular when mtDNA mutations have been excluded, and a broad clinical examination in such patients. We also recommend *WFS1* sequencing in patients with optic atrophy and early onset hearing loss, especially when *OPA1* mutations have been excluded [Tranebjaerg, 2008]. Hopefully, future *WFS1* screening of larger patient cohorts with hearing loss and optic atrophy will determine the extent to which *WFS1* mutations underlies this phenotype. Finally, supplementary efforts are essential to determine the precise functions of wolframin in the inner ear and eye and the role of genetic or environmental modifying factors. Studies focusing on the functional consequences of *WFS1* mutations identified in patients are needed and may help to resolve why different *WFS1* mutations may cause either recessive Wolfram syndrome, dominant LFSNHL or dominant optic atrophy with deafness, respectively, which would have major implications for the involved families in terms of appropriate genetic counseling and relevant clinical long-term follow-up.

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