Dominant ER stress-inducing WFS1 mutations underlie a genetic syndrome of neonatal/infancy onset diabetes, congenital sensorineural deafness and congenital cataracts

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Abstract

Neonatal diabetes is frequently part of a complex syndrome with extra-pancreatic features: 18 genes causing syndromic neonatal diabetes have been identified to date. There remain patients with neonatal diabetes who have novel genetic syndromes.

We performed exome sequencing in a patient and his unrelated, unaffected parents to identify the genetic aetiology of a syndrome characterized by neonatal diabetes, sensorineural deafness and congenital cataracts. Further testing was performed in 311 patients with diabetes diagnosed before 1 year of age in whom all known genetic causes had been excluded.

We identified 5 patients, including the initial case, with 3 heterozygous missense mutations in WFS1 (4/5 confirmed de novo). They had diabetes diagnosed before 12 months (2 before 6 months) (5/5), sensorineural deafness diagnosed soon after birth (5/5), congenital cataracts (4/5) and hypotonia (4/5). In vitro studies showed that these WFS1 mutations are functionally different from known recessive Wolfram syndrome-causing mutations, as they tend to aggregate and induce robust endoplasmic reticulum stress.

Our results establish specific dominant WFS1 mutations as a cause of a novel syndrome including neonatal/infancy onset diabetes, congenital cataracts, and sensorineural deafness. This syndrome has a discrete pathophysiology and differs genetically and clinically from recessive Wolfram syndrome.
Introduction

Neonatal diabetes is diagnosed before 6 months of age and reflects a severe reduction in beta-cells number or function. A genetic diagnosis is possible in 82% of patients with 23 genetic causes identified to date (1; 2). Thirty-nine percent of patients with neonatal diabetes have a genetic aetiology that results in the development of at least one extra-pancreatic feature, in addition to diabetes (1). This subtype is phenotypically and genetically heterogeneous with mutations in 18 genes known to cause syndromic forms of neonatal diabetes (1). Fifteen percent of cases with syndromic neonatal diabetes do not have a mutation in one of the known aetiological genes, suggesting undescribed novel genetic syndromes.

The identification of novel aetiological genes in patients with syndromic neonatal diabetes has been revolutionised by the introduction of next generation sequencing. In outbred pedigrees, exome or whole genome sequencing of an affected case and their unaffected, unrelated parents is a powerful tool to identify de novo heterozygous mutations. We have successfully used this approach to identify two novel aetiological genes for syndromic neonatal diabetes in our cohort (2; 3).

Neonatal diabetes has not been described in patients with recessive loss of function mutations in \(WFS1\), the genetic cause of Wolfram syndrome, even though early-onset diabetes is a cardinal feature of this multi-system disease. In these patients, diabetes onset occurs in the first decade of life (median age at onset 6 years, range: 1-32 years (4-6)). Additional extra-pancreatic features (optic atrophy, diabetes insipidus and hearing loss) usually develop sequentially between the first and third decade of life.

Heterozygous mutations in \(WFS1\) have been found to cause less severe phenotypes than Wolfram syndrome-associated recessive mutations. In particular, dominant \(WFS1\) mutations have been reported as causing isolated adult-onset diabetes (7), isolated low frequency hearing loss (8; 9), optic atrophy and hearing impairment (10-12) and isolated congenital nuclear cataracts (13) (Figure 1).
Figure 1: Phenotypic spectrum caused by autosomal dominant and autosomal recessive WFS1 mutations. The figure includes WFS1 mutations reported as disease causing in the HGMD database which have a minor allele frequency <0.00447 in the ExAC database (maximum allele frequency possible for Wolfram-syndrome causing mutations, assuming a disease frequency of 1/100,000, as calculated by the alleleFrequencyApp https://jamesware.shinyapps.io/alleleFrequencyApp/).
We used a trio-based exome sequencing strategy to identify dominant *WFS1* mutations as the cause of a novel syndrome characterized by permanent neonatal or infancy–onset diabetes, congenital or early-onset sensorineural deafness and congenital cataracts.
Methods

Genetic analysis

We performed exome sequencing on samples from an affected proband and parents using Agilent’s SureSelect Human All Exon Kit (v4). Paired-end sequencing was performed on an Illumina HiSeq2500, using 100bp reads. The resulting reads were aligned to the hg19 reference genome with BWA. Variants were called with GATK UnifiedGenotyper and annotated using Annovar and SeattleSeq Annotation server as previously described (3).

Replication studies were performed in a cohort of 311 patients diagnosed with diabetes before 12 months of age in whom mutations in the 22 known neonatal diabetes genes had been excluded. All the patients in the cohort were analysed using our targeted next generation sequencing assay which includes baits for WFS1 as previously described (14).

The bioinformatics tools SIFT, PolyPhen-2 and Align GVGD were accessed through the ALAMUT software (Interactive Biosoftware, Rouen, France) to predict the effect of novel variants on the WFS1 protein. If the variant was deemed likely pathogenic or of unknown significance, mutation testing was performed in the patient (to confirm the mutation) and the parents (when available) in order to assess whether the mutation had arisen de novo. Microsatellite analysis of parent/proband trios using the PowerPlex kit (Promega, Southampton, UK) was used to confirm family relationships for the putative de novo mutations. In silico modelling of the WFS1 p.Glu809Lys and p.Glu830Ala mutations was carried out using the SWISS-MODEL server (15). Additionally, as templates of only limited sequence similarity could be identified, the PredictProtein server was used to predict secondary structure and functional motifs from primary sequence alone (16).

Immunostaining

COS7 cells were transiently transfected with dominant and recessive WFS1 mutations for 48 hours. The cells were fixed in 2% paraformaldehyde for 30 minutes at room temperature and then permeabilized with 0.1% Triton X-100 for 2 minutes. The fixed cells were washed with PBS/Tween
0.1%, blocked with Image-It FX signal enhancer (Invitrogen) for 30 minutes, and incubated in primary antibody overnight at 4°C. The cells were washed three times in PBS/Tween 0.1% and incubated with secondary antibody for 1 hour at room temperature. Images were obtained with an FSA 100 microscope (Olympus). Anti-Flag antibody and anti-calnexin antibody were obtained from Cell Signaling Technology (Danvers, MA) and Protein Tech Group (Chicago, IL), respectively.

INS-1 cells were plated in 8-chamber slides (Thermo Fisher Scientific) at a density of 50,000 cells per chamber and transiently transfected with dominant and recessive GFP-tagged WFS1 mutant constructs for 48 hours. The cells were fixed in 4% paraformaldehyde/PBS for 30 minutes at room temperature, washed three times with PBS and then permeabilized with 0.1% Triton X-100/PBS for 10 minutes at room temperature. Cells were blocked overnight in a 2% BSA solution of 0.1% Triton X-100/PBS and washed three times the next day with PBS. Slides were prepared with antifade mounting medium containing DAPI (Vector Laboratories) and images were obtained with an FSA 100 microscope (Olympus).

**Immunoblotting**

HeLa cells were transfected with full-length human WFS1 cDNA tagged with a FLAG epitope, as well as p.Pro724Leu, p.Glu809Lys, p.Glu830Ala and p.His313Tyr mutant WFS1 cDNA. After transfection for 48 hours, cells were lysed for 15 minutes in ice-cold buffer (10 mM Tris-HCl, 50 mM Heps pH 7.4, 1% v/v Triton X-100, 1 mM EDTA) containing protease inhibitors. Insoluble material was recovered by centrifugation at 13,000 rpm for 15 minutes and solubilized in 10mM Tris-HCl and 1% w/v SDS for 10 minutes at room temperature. After the addition of 4 volumes of lysis buffer (20 mM Heps pH 7.4, 1% v/v Triton X-100, 150 mM NaCl, 10% v/v glycerol, 1 mM EDTA), samples were sonicated for 30 seconds. Lysates were separated using 4–20% linear gradient SDS-PAGE (Bio-Rad) and then electroblotted. Primary antibodies used in this study were anti-Flag (Cell Signaling Technology) and anti-alpha tubulin (Cell Signaling Technology).
Luciferase reporter assay

For reporter assays, HeLa cells were co-transfected with the endoplasmic reticulum stress response element (ERSE) (rat promoter GRP78)–luciferase construct (17) and various constructs as indicated. Prior to lysis at 24 hours after transfection, cells were treated with or without 100 nM of thapsigargin (TG) for 8 hours. Firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and normalized to Renilla luciferase values of the cotransfected pRL-TK vector (Promega) to control for differences in transfection efficiency. Statistical analysis of the data was performed by one-way analysis of variance followed by Dunnet’s test using SPSS 22 (IBM).
Results

Genetic analysis

We performed exome sequencing analysis on a male patient (Patient 1, Table 1) born to non-diabetic, unrelated parents. He was diagnosed with neonatal diabetes at the age of 13 weeks. He also had congenital cataracts, congenital sensorineural deafness and bilateral club feet. This patient died at the age of 2 years from sepsis.

Exome sequencing identified two de novo coding variants in Patient 1: WFS1 c.2425G>A p.Glu809Lys and ZNF513 c.1516G>A p.Ala506Thr. The variant in ZNF513 was excluded from further analysis as it was listed in the ExAC (Exome Aggregation Consortium) database (MAF 0.000008268, Cambridge, MA, October 2016). The WFS1 c.2425G>A, p.Glu809Lys variant was not listed in 59,030 exomes in ExAC and affects a highly conserved residue located in the endoplasmic reticulum domain of the WFS1 protein.

Since WFS1 recessive loss of function mutations usually cause childhood-onset diabetes and parental carriers are unaffected, we hypothesized that the heterozygous p.Gly809Lys mutation was causing neonatal diabetes through a dominant-negative mechanism rather than haploinsufficiency. Therefore we investigated the presence of heterozygous missense (but not nonsense or frameshift) WFS1 mutations in a cohort of 311 individuals with diabetes diagnosed in the first year of life in whom mutations in the known neonatal diabetes genes had been excluded using our targeted next-generation sequencing assay (14).

We identified four additional patients harbouring likely pathogenic variants in WFS1. Patients 2 and 3 were heterozygous for the same p.Glu809Lys variant identified in Patient 1. Patient 4 had a heterozygous c.2489A>C, p.Glu830Ala variant and patient 5 carried the c.937C>T, p.His313Tyr mutation. Both the p.Glu830Ala and p.His313Tyr variants affect highly conserved residues and are predicted to be pathogenic by in silico analysis. The p.Glu830Ala mutation affects a residue located in the endoplasmic reticulum domain whilst the p.His313Tyr involves an amino acid located in the first trans-membrane region of the WFS1 protein. These mutations were found to have arisen de novo in
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td><strong>Birth weight (g)/ Gestation (weeks)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1570/31 (SDS -0.23, Centile 40.58)</td>
<td>2500/40 (SDS -2.07, Centile 1.9)</td>
<td>3010/38 (SDS -0.28, Centile 39.09)</td>
<td>2400/41 (SDS -2.69, Centile 0.35)</td>
<td>2670/40 (SDS -1.89, Centile 2.96)</td>
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<tr>
<td><strong>Gender</strong></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
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<tr>
<td><strong>WFS1 Mutations</strong></td>
<td>p.Glu809Lys (c.2425G&gt;A)</td>
<td>p.Glu809Lys (c.2425G&gt;A)</td>
<td>p.Glu809Lys (c.2425G&gt;A)</td>
<td>p.Glu830Ala (c.2489A&gt;C)</td>
<td>p.His313Tyr (c.937C&gt;T)</td>
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<tr>
<td><strong>De novo</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Maternal sample N/A</td>
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<td>Yes</td>
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<tr>
<td><strong>Age at last assessment (years)</strong></td>
<td>Deceased (2 y)</td>
<td>1.5</td>
<td>8</td>
<td>8</td>
<td>15 months</td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td><strong>Age at diabetes diagnosis</strong></td>
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<td>24 weeks</td>
<td>50 weeks</td>
<td>35 weeks</td>
<td>36 weeks</td>
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<td><strong>Insulin dose</strong></td>
<td>0.5-0.6 U/kg/day</td>
<td>2 U/kg/day</td>
<td>0.75-0.94 U/kg/day</td>
<td>0.5-0.6 U/kg/day</td>
<td>0.18 U/kg/day</td>
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<td><strong>HbA1c</strong></td>
<td>6.8%</td>
<td>7.2%</td>
<td>7-10.2%</td>
<td>7%</td>
<td>10.1%</td>
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<td><strong>Cataracts</strong></td>
<td>Yes, congenital</td>
<td>Yes, congenital</td>
<td>Yes (2.5y)</td>
<td>Yes, congenital</td>
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<td><strong>Glaucoma</strong></td>
<td>No</td>
<td>Yes, congenital</td>
<td>No</td>
<td>Yes, congenital</td>
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<td><strong>Other eye abnormalities</strong></td>
<td>Microphthalmia, bilateral microcornea and iris coloboma</td>
<td>Optic atrophy secondary to congenital glaucoma</td>
<td>Optic atrophy at 3.1 y</td>
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<td>Deafness</td>
<td>Yes, sensorineural (diagnosed at 1 year)</td>
<td>Yes, sensorineural (diagnosed at 18 months)</td>
<td>Yes (congenital)</td>
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<tr>
<td>Dysmorphic features</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>Yes (possibly due to facial hypotonia)</td>
<td>No</td>
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<td>Hypotonia</td>
<td>Yes (generalized)</td>
<td>Yes (limbs)</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Additional features</td>
<td>Bilateral club feet, left thumb contracture.</td>
<td>Microcephaly, widely spaced lateral ventricles on CT scan, clinodactyly.</td>
<td>Hypothyroidism (12 months). Motor and intellectual developmental delay</td>
<td>Subclinal hypothyroidism, growth hormone deficiency</td>
<td>No</td>
</tr>
</tbody>
</table>
patients 1, 2, 4 and 5. Paternal inheritance was excluded in Patient 3, but the maternal sample was not available for testing.

**Clinical features**

The clinical features of the 5 patients are summarized in Table 1. All 5 individuals had congenital sensorineural deafness and early-onset diabetes diagnosed before 12 months of age (range 13-51 weeks) which was treated with a full replacement dose of insulin. In all 5 cases the birth weight was low (median SDS -1.89 [IQR -2.07—-0.28], median centile 2.96) consistent with reduced insulin secretion *in utero*, a feature not described in patients with “classical” Wolfram syndrome.

Congenital cataracts and additional eye abnormalities were present in 4/5 patients (Patients 1, 2, 3 and 4). Two of them also had congenital glaucoma (Patients 2 and 4) which had progressed to optic atrophy in Patient 2. Patient 1 did not have congenital glaucoma, but was diagnosed with microphthalmia, bilateral microcornea and iris coloboma. Patient 3 was diagnosed with optic atrophy at the age of 3.1 years.

Additional common clinical features included hypotonia (present in Patients 1, 2, 4 and 5), hypothyroidism (diagnosed in Patients 3 and 4) and structural neurological defects identified by CT scanning (in Patients 1 and 2).

**In silico protein analysis**

Since the *WFS1* p.His313Tyr mutation had been reported to have arisen *de novo* in 2 patients before (18), we focused our *in silico* investigations on the p.Gly809Lys and p.Glu830Ala variants. There are currently no 3-dimensional molecular structures for any part of the WFS1 protein, therefore to assess the potential effect of the p.Glu809Lys and p.Glu830Ala substitutions on protein structure, the sequence of the ER luminal domain containing these residues (amino acids 653-869) was submitted to the SWISS-MODEL server to identify templates for comparative modelling, with models constructed on the best-scoring template. Predicted molecular structures suggested that both variants have significant effects on the surface properties of the molecule in terms of charge and
Figure 2: Comparative protein modeling of WFSI. The C-terminal lumenal domain of WFSI (residues 653-869) was submitted to SWISS-MODEL for template identification; models were then constructed for residues 798-838 using the top-scoring template (PDB identifier 1ltl; MCM protein from Methanothermobacter thermautotrophicus; 27% sequence similarity over region modelled). Upper figures show the protein backbone with selected side chains as indicated in stick format for wild-type and variant WFSI; the predicted molecular surface is shown as a transparent layer; broken green lines indicate predicted hydrogen bonds. Lower figures show the same structures, but rotated ~45 degree away from the viewer around a horizontal axis and with the predicted molecular surface shown as a solid layer. In both figures amino acids are coloured by type (red, acidic; blue, basic; yellow, uncharged polar; grey, non-polar/hydrophobic); regions of significantly altered surface properties are indicated by red ovals in the lower figures.
hydrophobicity (Figure 2). Given the lack of underlying secondary structure in these models, such changes might be expected to have significant effects on folding of this region in vivo which are not apparent from in silico modelling. However, as the sequence similarity between WFS1 and the template was low (27%), these models may be of limited reliability, prompting further sequence analysis using the PredictProtein server. This predicted the presence of a number of short secondary structural motifs (mostly beta strand) as well as regions of intrinsic disorder spanning ~30% of the lumenal domain (data not shown). As with 3D comparative modelling, the p.Glu809Lys and p.Glu830Ala substitutions were predicted to have local effects on the extent of these regions as well as on solvent accessibility. Again, such changes might affect protein folding, and the presence of intrinsically disordered regions is particularly interesting as misfolding of such regions has been implicated in other diseases such as synucleinopathies (19).

**Functional studies**

To study the subcellular localization of the dominant Wolfram syndrome-causing variants, we performed immunostaining of COS7 cells transiently expressing 1) the dominant mutations, p.Glu809Lys, p.Glu830Ala, and p.His313Tyr, 2) a dominant mutation causing isolated adult-onset diabetes, p.Trp314Arg (7), 3) two recessive Wolfram syndrome-causing mutations, p.Pro724Leu and p.Gly695Val (20), or 4) wild-type WFS1 tagged at its C terminus with a FLAG epitope. Immunostaining of cells expressing wild-type WFS1 showed a diffuse reticular pattern that co-localized with the ER marker calnexin (Figure 3). In contrast, both dominant and recessive WFS1 mutants showed a punctate staining pattern in the ER, suggesting a tendency for these WFS1 mutants to misfold and aggregate. We also carried out similar experiments in insulin-secreting rat INS-1 cells using GFP-tagged WFS1 constructs. These studies were suboptimal due low transfection efficiency (<10%) and expression levels. Interestingly, in these circumstances the distribution of wild-type WFS1 and the dominant variants p.Glu809Lys, p.Glu830Ala, and p.His313Tyr was similar to that observed in COS7 cells, but the recessive mutation p.Pro724Leu showed a diffuse reticular staining pattern similar to wild type WFS1 rather than a punctate appearance as seen for the dominant mutations (Supplementary Figure 1). This suggests that, although all these mutants may have a
Figure 3: Double immunofluorescence staining of COS7 cells transiently transfected with dominant and recessive variants of WFS1. COS7 cells were transiently transfected with 1) control (pcDNA3), 2) wild-type WFS1 tagged at its C terminus with a FLAG epitope, 3) the dominant mutations p.His313Tyr, p.Glu809Lys, and p.Glu830Ala, 4) a dominant variant causing isolated adult-onset diabetes, p.Trp314Arg (7), and 5) two recessive Wolfram syndrome-causing mutations, p.Pro724Leu and p.Gly695Val (20). The cells were stained with anti-flag (red fluorescence, left) and anti-calnexin (green fluorescence, center). (Right) A merged image is shown. n=4; magnification is 10X.
tendency to misfold, there might be a spectrum of severity for different mutations. Such differences are likely to only become apparent at relatively low levels of expression at which variants carrying less severe mutations can still be folded efficiently. Since WFS1 is highly expressed in beta cells, it is also possible that INS-1 cells have a greater capacity to efficiently fold WFS1 proteins carrying less severe (i.e. recessive) mutations compared to that of COS7 cells.

To further assess misfolding and subsequent aggregation of the dominant mutations, p.Glu809Lys, p.Glu830Ala, and p.His313Tyr, as well as the recessive Wolfram syndrome-causing mutation, p.Pro724Leu, we performed immunoblot analysis of detergent-soluble and detergent-insoluble lysates from HeLa cells transiently expressing these disease-causing mutations or wild-type WFS1. We found that the formation of insoluble and high molecular weight complexes was much more prominent in cells expressing disease-causing variants than in cells expressing wild-type WFS1 (Figure 4). These results suggest that folding defects could be a common feature of WFS1 missense mutations. We did not observe any clear differences between the recessive missense mutation and the dominant missense mutations in these two experiments; this may be a consequence of the high levels of protein expression in Cos7 and HeLa cells, potentially overwhelming the cell’s ability to efficiently fold even WFS1 variants with minor folding defects.

WFS1 is known to negatively regulate the cellular response to ER stress, thereby raising the possibility that dominant WFS1 mutations could alter WFS1 function by causing or enhancing a dysregulated ER stress response (17; 21). To test this hypothesis, we investigated the effects of the p.Glu809Lys, p.Glu830Ala, and p.His313Tyr variants, as well as the recessive p.Pro724Leu mutation, on the ER stress response. We co-transfected HeLa cells with a luciferase reporter construct containing an ER stress response element (ERSE) and either 1) control (pcDNA), 2) wild-type WFS1 (WT), 3) the dominant mutations p.Glu809Lys, p.Glu830Ala, and p.His313Tyr, or 4) the recessive mutation, Pro724Leu, expression vector. The ERSE reporter reflects activation levels of the ER stress response. In the absence of the ER stress inducer, thapsigargin (TG), all of the dominant disease-causing variants activated the ERSE reporter significantly more than wild-type WFS1 (Figure 5). The dominant p.Glu809Lys mutation results in significantly more ERSE reporter activation than the
Figure 4: High molecular weight complexes of WFS1 mutants in detergent-insoluble fractions. HeLa cells were transfected with control (pcDNA), Flag-tagged wild-type WFS1 (WT), mutant WFS1 p.Pro724Leu (p.P724L), p.Glu809Lys (p.E809K), p.Glu830Ala (p.E830A) or mutant WFS1p.His313Tyr (p.H313Y) expression plasmid and separated into detergent-soluble and detergent-insoluble fractions with anti-FLAG antibody.
Figure 5: Luciferase reporter assays in HeLa cells transfected with the ERSE reporter together with control (pcDNA), wild-type WFS1 (WT), mutant c.2171C>T p.Pro724Leu, mutant c.937C>T p.His313Tyr WFS1 (p.H313Y), mutant c.2425G>A p.Glu809Lys WFS1 (p.E809K), or mutant c.2489A>C p.Glu830Ala WFS1 (p.E830A) expression plasmid. Cells were untreated (UT) or treated with TG (100 nM) for 8 h. Relative intensity of luciferase (Promega Dual-Luciferase Reporter Assay System) was then measured (n = 3; dashed grey lines represent mean). Transfections were normalized with the pRL-TK vector (Promega) as an internal control. Asterisks indicate a significant difference analyzed by one-way ANOVA followed by Dunnett’s test (*: p<0.01, **: p<0.001, ***: p<0.0001).
recessive p.Pro724Leu mutation (4.40 v 3.41 p=0.0003) but the other mutations were not significantly different (mutation p.His313Tyr 3.70 v p.Pro724Leu 3.41 p=0.35, mutation p.Gly830Ala 3.84 v p.Pro724Leu 3.41 p=0.09). After inducing ER stress with TG, all the disease-causing dominant mutations exhibited significantly higher ERSE activity in TG-treated conditions than wild-type WFS1 (Figure 5). Again the dominant p.Glu809Lys mutation showed the highest ERSE activity which was significantly different from the recessive p.Pro724Leu mutation (21.32 v 13.44 p<0.0001) (Figure 5). Collectively, these results suggest that the dominant WFS1 mutations p.Glu809Lys, p.Glu830Ala, and p.His313Tyr, strongly induce ER stress, whereas recessive variants, including the Pro724Leu variant, are less potent in inducing ER stress.
Discussion

We report five patients with a novel congenital syndrome characterised by neonatal or infancy-onset diabetes, congenital cataracts and congenital sensorineural deafness caused by heterozygous de novo WFS1 missense mutations. Functional analysis suggests that these dominant mutations tend to aggregate in the ER and induce robust ER stress. It is likely that strong ER stress induced by these dominant WFS1 mutations causes early cell death (probably starting in utero), resulting in the severe syndrome identified in our patients.

The clinical features identified in our patients are distinct from those described in patients with “classical” Wolfram syndrome, with the presentation of symptoms at such an early age being the major difference. All our patients were diagnosed with diabetes before 12 months of age, with 2 patients being diagnosed in the neonatal period. In contrast patients with genetically confirmed recessive Wolfram syndrome develop insulin-dependent diabetes at a median age at diagnosis of 6 years (range 1-32 years) (22). Diabetes is often the presenting feature of Wolfram syndrome, with optic atrophy and deafness appearing months to years later (median age 11 and 14 years respectively). In contrast, our patients were all diagnosed with sensorineural deafness soon after birth. Four out of five patients in our cohort also had eye manifestations, but only two of them had optic atrophy. In fact congenital cataracts were the most common eye feature in our patients (diagnosed in 4/5). Although cataracts have been reported in some patients with Wolfram syndrome, they are not a consistent feature of the disease and they are not congenital. Interestingly, isolated congenital nuclear cataracts have been previously reported in one family with a heterozygous WFS1 mutation (13) (Figure 1), highlighting the importance of WFS1 in function and/or development of the eye lens. The fourth cardinal feature of Wolfram syndrome, diabetes insipidus, is also the least consistent, being diagnosed in ~40% of Wolfram syndrome patients, at a median age of 13 years (22). Currently none of our patients has diabetes insipidus, but they are all younger than the median age that this is diagnosed. We therefore cannot exclude the possibility that they might develop this feature in the future. Clinical follow up in these patients will be needed also to establish the presence of any developmental and/or growth delay. Another striking difference between our patients and typical Wolfram syndrome cases
is the birth weight. Patients with “classical” Wolfram syndrome generally have a normal weight at birth (unpublished data from the Exeter monogenic diabetes database), whilst all our patients have birth weight below the median for their gestational age and sex, which is consistent with impaired insulin secretion in utero.

The novel syndrome we are reporting is genetically different from “classical” Wolfram syndrome as it results from spontaneous heterozygous mutations rather than being recessively inherited. Additional dominantly acting WFS1 mutations have been previously reported to cause autosomal dominant forms of both optic atrophy and hearing loss (10-12), low frequency hearing loss (8; 9), congenital cataracts (13), and isolated adult-onset diabetes (7). In these cases the dominant phenotype is less severe than typically seen in classical recessive Wolfram Syndrome (Figure 1).

The phenotype observed in our patients is more severe than that resulting from homozygous complete loss of function mutations, suggesting that it is not just loss of WFS1 activity that results in this phenotype. In vitro functional studies showed that the dominant mutations we report may act through a different mechanism than recessive Wolfram syndrome mutations, as they strongly induce ER stress both basally and in the presence of thapsigargin (Figure 5). Protein aggregation is unlikely to play a major role in the pathogenesis of recessive Wolfram syndrome, since a) this condition is known to often result from complete absence of WFS1 protein caused by recessive null mutations and b) because heterozygous carriers of Wolfram syndrome-causing missense mutations are clinically unaffected. On the contrary, functional data on the dominant mutations identified in our patients, support a role for protein aggregation. Taken together, our results suggest that the dominant mutations actively induce ER stress. Additional functional studies on beta cell lines and primary islets are needed to determine whether the combined toxic effects of protein aggregation and ER stress have a direct effect on insulin secretion, possibly as a consequence of beta cell death. Recessive missense and null WFS1 mutations result in Wolfram syndrome due to inability to regulate the unfolded protein response rather than directly inducing ER stress. Functional studies on the mutation causing autosomal dominant adult-onset diabetes without other features (p.Trp314Arg) suggest that this mutation is also functionally different from the mutations we studied, as it impairs the protein’s ability
to suppress the ER stress response after its activation (7). The mechanisms underlying the broad phenotypic spectrum caused by specific dominantly acting mutations in WFS1 are currently unknown. Further studies investigating the effect of specific dominant and recessive WFS1 mutations in other cell lines and animal models are needed to further our understanding of the pathophysiology underlying this broad spectrum of disease.

The results of our functional studies suggest a direct genotype-phenotype correlation within the dominant mutations we report, which could be explained by the differences in functional impact seen in the in vitro experiments. The 3 patients with the p.Glu809Lys mutation included the 2 cases with the most severe clinical phenotype (diabetes diagnosed before 6 months and additional severe neurological features). Cells transfected with the WFS1 construct harbouring the p.Glu809Lys mutation, showed the highest level of ESRE reporter activity both at baseline and, after induction of ER stress in the luciferase assay experiments (Figure 5). The heterozygous p.Glu809Lys mutation has been identified previously in 2 patients with features that included early-onset diabetes (diagnosed at 6 months and 2 years respectively), deafness and eye abnormalities (cataracts and optic atrophy) (4; 6). In both cases parental samples were not available for testing and the authors hypothesised the presence of a second, undetected WFS1 mutation. We suggest that these two patients have the same autosomal dominant syndrome we report. Patient 5, who is heterozygous for the previously reported p.His313Tyr mutation, does not have the full triad of symptoms identified in the other patients: he has early-onset diabetes, congenital deafness and severe hypotonia but no cataracts or other eye features. In keeping with this being a less severe dominant phenotype three previously reported patients had diabetes occurring outside the first year of life and no reports of eye abnormalities (5; 18). The delayed manifestation of disease in these patients is consistent with the results of our in vitro experiments where the p.His313Tyr mutation resulted in a lower level of ER stress compared to the p.Glu809Lys mutation (Figure 5). Further studies looking at the evolution of disease in patients with this rare syndrome will be needed to precisely define a genotype/phenotype relationship.

In conclusion, we describe a novel congenital syndrome characterized by neonatal/early-onset diabetes, sensorineural deafness and congenital cataracts caused by dominant WFS1 mutations. Our
findings highlight a new disease mechanism linked to $WFS1$ mutations and establish this gene as a cause of syndromic neonatal/early-onset diabetes. Elucidation of the complex roles played by $WFS1$ in regulation of insulin secretion and endoplasmic reticulum stress will be fundamentally important for current studies looking into therapeutic options for patients with Wolfram syndrome (23).
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ATH is the guarantor of the clinical and genetic work and FU is the guarantor of the functional work. Both had had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Author Contributions

ATH, SE, FU and JM participated in study conception and design. EDF, SEF, MBJ and SE performed the genetic analysis. ATH analyzed the clinical data. GJ and RC performed bioinformatic analysis. JM, TY, and DA participated in data acquisition. JM, DA, and FU participated in data analysis and interpretation. EDF, SEF and ATH wrote the first draft of the manuscript. RC, SEF, SE, JM, TY, DA, and FU participated in manuscript improvement. FA, MM, NL, VO, and OP collected patients’ samples and clinical data. All authors reviewed the manuscript.
References


Supplementary Figure 1. GFP fluorescence of INS-1 cells transiently transfected with dominant and recessive WFS1 mutants. INS1 823/13 cells were transfected with GFP-tagged empty vector (pEGFP-N1), wild-type WFS1 (WT), mutant c.937C>T p.His313Tyr WFS1 (p.H313Y), mutant c.2425G>A p.Glu809Lys WFS1 (p.E809K), mutant c.2489A>C p.Glu830Ala WFS1 (p.E830A), or mutant c.2341C>T p.Pro724Leu WFS1 (p.P724L) expression plasmids. Representative fluorescence (left), DAPI (middle) and merged (right) images of INS1 832/13 cells expressing WFS1 mutants are shown n=4; magnification is 40X.