INTRODUCTION

Referred herein as DDON syndrome, the synonymous condition Mohr–Tranebjaerg syndrome (MTS) was first described nearly sixty years ago as an X-linked condition affecting Scandinavian populations (Jin et al., 1996; Tranebjaerg, 1993; Tranebjaerg et al., 1995). Characterized primarily by progressive deafness in early childhood, this condition also manifests with dystonia, spasticity,
and dysphagia (Bahmad, Merchant, Nadol, & Tranebjaerg, 2007; Ha et al., 2012; Kojovic et al., 2013). Mental disturbances and vision loss with variable onset and progression are also common phenotypes (Tranebjaerg et al., 2001). Located on Xq22, the gene associated with DDON syndrome, TIMM8A (originally called DDP for deafness–dystonia peptide; OMIM#300356) encodes a small protein that localizes to the intermembrane space in mitochondria (Jin et al., 1996; Tranebjaerg et al., 1995). Tim8 forms a complex with other small TIM proteins, to facilitate the import of nuclear-encoded proteins into the mitochondrial inner membrane (Beverly, Sawaya, Schmid, & Koehler, 2008; Hasson et al., 2010; Rothbauer et al., 2001).

The pathogenetic mechanism of DDON is not fully defined. However, several studies have implicated impaired transport through the intermembrane space, and subsequent mitochondrial dysfunction when Tim8 is unable to associate with its binding partner, Tim13, as the primary driver of pathogenesis. Loss of the Tim8/Tim13 complex alters the transport and function of other proteins in the inner mitochondrial membrane, including Tim22 and Tim23 (Hasson et al., 2010; Rothbauer et al., 2001). Notably, downregulation or absence of Tim8 does not affect Tim23 import or levels in patient cell lines (Engl, Florian, Tranebjaerg, & Rapaport, 2012). This and other studies show that loss of TIMM8A results in abnormal mitochondrial morphology but this is not associated with any obvious impact on mitochondrial energetics (Binder et al., 2003; Engl et al., 2012). More recently, loss of Tim8a in neurons was shown to cause defects in Complex IV assembly, priming these cells for apoptotic vulnerability (Kang et al., 2019). Most of the mutations associated with DDON syndrome are frameshifts or premature stops, and there are a few missense mutations reported, including two in the first codon of the gene (Aguirre et al., 2006; Binder et al., 2003; Blesa et al., 2007; Hofmann et al., 2002; Penamora-Destriza et al., 2015; Ujike, Tanabe, Takehisa, Hayabara, & Kuroda, 2001; Wang et al., 2019). Whether these first codon mutations result in the utilization of an alternate start site, or whether they result in the complete loss of protein noted in other DDON syndrome patients, is not known. In this report, we describe a male patient harboring a novel base change in the TIMM8A gene (c.1A>T, p.Met1Leu) with features of DDON syndrome and provide functional studies to confirm the pathogenic status of this variant.

### 1.1 Clinical summary

Our male patient was the product of a nonconsanguineous normal pregnancy. Term delivery was via C-section due to placental hemorrhage and fortunately there were no perinatal problems from delivery. Prior to age 3 years, he met all developmental milestones on time without stagnation or regression. At 3 years, his parents began to notice some regression in his expressive language. He was eventually diagnosed with an auditory neuropathy and at age 6 years received cochlear implants. His most recent ophthalmological examination, at age 6, did not show optic neuropathy or retinal involvement. His neurological examination was otherwise without abnormality; in particular, findings of ataxia or dystonia were absent.

Due to early auditory neuropathy, a massively parallel gene sequencing panel was sent for commercial testing for hearing loss gene abnormalities (Prevention Genetics). A likely pathogenic variant in the TIMM8A gene, c.1A>T, p.Met1Leu was found. On parental testing, mother was found to be a carrier, with low copy numbers of the variant compared to wild type, suggesting that mother may be a mosaic for this genetic change.

### 2 METHODS

#### 2.1 Patient information and ethical compliance

Informed consent was obtained from the parents of the proband prior to participation in the research study. All procedures employed were reviewed and approved by the appropriate institutional review committee in the GGC IRB protocol.

#### 2.2 SDS-PAGE and western blotting

Fractionated samples from both patient and control cell lines were resolved on a 15% SDS-PAGE gel followed by transfer to nitrocellulose membranes and blocked using 5% nonfat powdered milk in TBS-T solution for 1 hr at room temperature. The following primary antibodies were utilized at specific dilutions as indicated: Proteintech rabbit polyclonal TIMM8A antibody at (1:1,000, 24 hr, 4°C); Santa Cruz mouse monoclonal GAPDH (6C5) antibody (1:10,000, overnight, 4°C); Santa Cruz mouse monoclonal Lamin A/C (636) antibody (1:500, overnight, 4°C); Santa Cruz mouse monoclonal Cytochrome C (7H8) antibody (1:200, overnight, 4°C); and Proteintech rabbit polyclonal TIM13 antibody (1:500 dilution, 12 hr, 4°C). Secondary antibodies used were anti-mouse-HRP (1:3,000, 1 hr, room temperature) and anti-rabbit-HRP (1:1,000, 1 hr, room temperature). Ponceau S staining was performed to visualize total protein load.

#### 2.3 Isolation of nuclear/mitochondria by subcellular fractionation

A subcellular fractionation protocol was utilized in order to enhance the signal of the TIMM proteins trapped within the
mitochondrial intermembrane. Patient cell lines were cultured, fractionated into two lysates, with the first containing both nuclei and mitochondria and a second lysate including cytoplasmic and cellular membrane contents. All steps were performed on ice and centrifugation at 4°C. Subcellular fractionation buffer (SF buffer) containing 250 mM sucrose, 10 mM KCl, 20 mM HEPES (pH 7.4), 1.5 mM MgCl2, and 1 mM EDTA, 1 mM DTT, and protease inhibitor cocktail, was added to fibroblasts and lymphoblasts to generate the cell homogenates. Homogenates were passed through a 25-gauge needle ten times before centrifugation at 8,000 rpm (10,000 g) for 12 min resulting in a pellet containing the nuclear and mitochondrial material and a supernatant fraction. RIPA buffer was added to the fractionated cellular components to solubilize the proteins, and a BCA assay performed to quantify total protein concentration in each fraction.

### 2.4 Mitochondrial staining

Fibroblasts were seeded onto coverslips in a twelve-well cell culture plate. After washing with DPBS containing calcium and magnesium, the cells were fixed to the slides with the addition of 3.7% formaldehyde for 10 min followed by washing 4 times in DPBS containing calcium and magnesium. Permeabilization was achieved with the addition of 1 ml of 0.1% Triton-X in DPBS for 10 min at room temperature, and then, wells were washed three times with DPBS containing calcium and magnesium. Fixed coverslips were blocked using 3% bovine serum albumin and incubation for 1 hr at room temperature on a rotator followed by washing with DPBS. A rabbit polyclonal anti-P5CS (ALDH18A1) antibody (1:250 dilution) was used to label mitochondria in staining buffer for one hour at room temperature followed by incubation with an Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Abcam, 1:500 dilution) in the dark for 1 hr. After washing, the coverslips were mounted with Prolong Gold™ and visualized using an Olympus FV3000 confocal microscope. To quantify the percentage of elongated mitochondria, images from 8 different fields of cells were obtained and the percentage of elongated mitochondria relative to the total mitochondria scored was determined in each field. These percentages were then averaged, and statistical significance calculated using a Student's t test.

### 3 RESULTS

#### 3.1 Genetic and biochemical characterization

Patients with DDON syndrome typically bear loss of function mutations in the TIMM8A gene, although some missense mutations have also been reported that impact the start codon. Massive parallel sequencing using an auditory neuropathy panel and buccal cells of the patient uncovered an A>T change in the initiation codon, which can in theory alter both translation initiation and possibly TIMM8A transcription. To study whether the patient's TIMM8A mutation impairs translation of the protein, we first performed Western blot analysis on patient fibroblasts. The results of this experiment show that the patient's fibroblasts...
make no detectable Tim8a protein, consistent with the fact that
the mutation disrupts the start codon of the TIMM8A transcript
(Figure 1a). There was no evidence of a truncated peptide in
these cells that might indicate the usage of an alternate start site,
although we cannot rule out that another start site is used but
produces an unstable peptide. We also examined Tim8a levels
in patient lymphoblasts to determine whether loss of the protein
was observed in another cell type. Both fibroblasts and lympho-
blasts were lysed using a detergent-free, subcellular fractionation
buffer, and the mitochondria and nuclei fraction separated from
the cytosolic fraction. Analysis of marker proteins for the nuclear/
mitochondrial fraction (Lamin A/C) and cytosol (GAPDH) dem-
onstrate the fidelity of the fractionation, and Ponceau S staining
shows an equivalent amount of total protein was resolved. The
results in Figure 1b show that regardless of the cell type, the pa-
tient does not make any detectable levels of the Tim8 protein. We
next assessed whether loss of the Tim8a protein would alter the
steady-state level of its binding partner Tim13. Prior reports dem-
onstrated variable loss of Tim13 in DDONS/MTS patient cells. Western
blot of fibroblast lysates showed a striking reduction, but not absence, of Tim13 in the patient cells (Figure 1c). The
reduction of Tim13 is at the level of the protein as qPCR analysis
revealed equivalent transcript abundance in control and patient
fibroblasts (Figure 2). However, TIMM8A transcript was reduced
roughly 50% in the patient fibroblasts, suggesting that the A>T
base change may impact the transcription of the TIMM8A gene.

Tim8a and Tim13 are part of a small protein complex
involved in the import of proteins through the inner mito-
chondrial membrane. Loss of this import has been shown to
cause altered mitochondrial morphology (Binder et al., 2003;
Engl et al., 2012). Using an antibody to the mitochondrial
enzyme, pyrroline 5-carboxylate synthase (P5CS), we stained
control and patient fibroblasts to ask whether morphology of
this organelle was abnormal (Figure 3). Our results show a
clear increase in elongated mitochondria in the patient. These
elongated mitochondria, which have a cigar-like (as opposed
to a globular) appearance in the patient cells, may arise due to
increased fusion—a phenotype that could reflect the failure
to import specific inner membrane proteins or another Tim8-
dependent function.

4 DISCUSSION

With the addition of a novel frameshift variant described in
2019 (Wang et al., 2019), approximately fifteen pathogenic
variants in the TIMM8A gene resulting in DDON have been
documented in the literature. The variant described in this
report of a c.1A>T start loss bears similarity to earlier
start loss cases (Binder et al., 2003; Merchant et al., 2001;
Penamora-Destriza et al., 2015; Tranebjærg, 2012). In
all three cases, the methionine required to initiate protein

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**FIGURE 2** Quantitative PCR analysis of TIMM8A and TIM13 transcript abundance in WT and P1 fibroblasts, normalized to RPL4 transcript abundance. Data represent the average of three independent analyses

**FIGURE 3** (a) Immunostaining of the mitochondrial enzyme pyrroline 5-carboxylate synthase (P5CS) in WT and P1 fibroblasts. (b) Quantification of the percentage of elongated mitochondria relative to the total mitochondria in eight cell fields across two independent experiments. 60–80 mitochondria per cell field were counted. ***p < .001
translation is replaced leading to a complete absence of the Tim8a/DDP protein. The absence or variant forms of the TIMM8A gene product appear to only significantly influence the function of neurons within specific neuronal populations. Roesch et al. revealed elevated levels of both Tim8 and Tim13 within large neurons of the brain in mouse models of specific structures including components of the basal ganglia, brainstem, and the Purkinje cells of the cerebellum (Roesch, Hynds, Varga, Tranebjærg, & Koehler, 2004). Implication of these structures may correlate with the symptomatology in DDON patients, including the characteristic dystonia and variable neuropsychological features.

Rates of mitochondrial fusion and fission directly dictate the organelle’s morphology and function. The balanced intramitochondrial contents achieved by proper fission and fusion are required for normal functioning (Chen et al., 2010). Beyond its function of shuttling proteins across the mitochondrial intermembrane space, Arnoult et al. characterized the role of the C-terminal portion of Tim8a interacting with the well-characterized dynamin-related protein 1 (DRP1) during programmed cell death (PCD) of the mitochondria (Arnoult et al., 2005). They demonstrated extensive fragmentation of mitochondria occurs during PCD, which is accomplished through increased rates of fission when DRP1 localizes to the mitochondria via its interaction with Tim8a. These authors concluded that this DRP1-mediated mitochondrial fission occurs during caspase-independent apoptosis. HeLa cells harboring a dominant-negative mutant of DRP1 demonstrated an elongated morphological appearance with fused interconnected tubules because of the inhibition of mitochondrial fission (Arnoult et al., 2005). Engl et al. published work documenting that both DDON patient fibroblasts featuring the c.116delT variant in the TIMM8A gene and HeLa cells transfected with siRNA targeting TIMM8A yielded mitochondrial with similar elongated morphology (Engl et al., 2012).

Neurons function as highly polarized cells that require a sufficient amount of mitochondria within specific locations to carry out its actions such as axonal transport and synaptic transport. This polarity requires DRP1-directed mitochondrial fission during development to appropriately distribute mitochondria and represents an essential requirement for neuronal viability. While expendable in most other cells of the body such as fibroblasts, DRP1 depletion prevents mitochondrial survival and proliferation in the developing neuron so that their processes fail to generate the appropriate synaptic formation with other neurons (Kageyama et al., 2012). Additionally, it was shown that mitochondrial fission enables postmitotic neuron survival for a short time by reducing damage by oxidative species. Purkinje cells of the cerebellum in DRP1 deficient mice increased fusion and caused mitochondria tubules to elongate. Likewise, hippocampal primary neurons in rats with suppressed DRP1 expression slowed cell death and fragmentation of mitochondria when compared to overexpression of DRP1, but ultimately did not protect against necrotic death of the neuron (Estaquier & Arnoult, 2007).

Based upon these findings, one theory to account for DDON syndrome pathogenesis would be that the primary neurons of these sensory pathways along with other cortical tracts require an advanced level of regulation of their mitochondrial morphology via the Tim8 and DRP1 interaction to ensure the appropriate distribution of mitochondria as these primary neurons further differentiate. Failure of proper localization of DRP1 to the mitochondria due to the absence of the Tim8 protein leading to subsequent loss of mitochondrial fission would cause an accumulation of elongated mitochondria. Further differentiation of these primary neurons as the brain matures would not allow for proper recycling of mitochondria so that accumulation of damaged mitochondria would lead to death of these primary neurons, mirroring the postmortem findings described above. This Tim8/DRP1 interaction must play a central role in the primary neurons of the auditory and visual sensory pathways so that in its absence, degeneration occurs to produce the hallmark DDON symptomology and histopathologic findings.

Optic atrophy protein 1 (Opa1) is a well-characterized mediator of mitochondrial fusion that is localized to the mitochondrial inner membrane. Its function is in direct opposition to that of DRP1 and contains splice variants that are specific to tissues located in the retina and cochlea. Mutations in Opa1 resulting in autosomal-dominant optic atrophy plus syndrome (ADOA+) bear a striking resemblance to that of DDON (Davies et al., 2007; Yu-Wai-Man, Griffiths, & Chinnery, 2011; Yu-Wai-Man et al., 2010). Here, however, optic atrophy with visual impairment is the initial manifestation during childhood, followed by sensorineural hearing loss in late childhood to young adulthood. Mitochondria in ADOA+ display morphological abnormalities characterized by increased fragmentation resulting in smaller, more punctuated mitochondria. This morphological difference is attributed to increased rates of mitochondrial fission due to the lack of Opa1 mediated fusion. This has been demonstrated in both retinal and nonretinal cells such as fibroblasts (Davies et al., 2007).

Advancements in the therapies that hold the potential to treat single gene-causing disorders represent an exciting frontier in clinical research. DDON syndrome results from mutations of a single gene, TIMM8A, and the novel variant described in this case report as a start loss located at the first codon may be a candidate for read through pharmaceuticals (Bello & Pegoraro, 2016; Keeling, Xue, Gunn, & Bedwell, 2014; Rowe & Clancy, 2009). These drugs are characterized
by binding to ribosomes to induce a translational “read-through” of premature stop codons to generate a full-length protein. The drug ELX-02 is an investigational synthetic eukaryotic ribosome-selective glycoside currently undergoing clinical trials to assess its use in CF patients currently within the United States (Leubitz et al., 2019). Whether this compound, or other drugs being considered for the treatment of optic neuropathy, will prove effective requires further investigation.

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CONFLICT OF INTEREST
The authors declare no conflict of interest as part of this work. This manuscript does not contain any shared data.

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