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Impairment of mitochondrial tRNA^{Ile} processing by a novel mutation associated with chronic progressive external ophthalmoplegia

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Abstract

We report a sporadic case of chronic progressive external ophthalmoplegia associated with ragged red fibers. The patient presented with enlarged mitochondria with deranged internal architecture and crystalline inclusions. Biochemical studies showed reduced activities of complex I, III and IV in skeletal muscle. Molecular genetic analysis of all mitochondrial tRNAs revealed a G to A transition at nt 4308; the G is a highly conserved nucleotide that participates in a GC base-pair in the T-stem of mammalian mitochondrial tRNA^{Ile}. The mutation was detected at a high level (approx. 50%) in muscle but not in blood. The mutation co-segregated with the phenotype, as the mutation was absent from blood and muscle in the patient's healthy mother. Functional characterisation of the mutation revealed a six-fold reduced rate of tRNA^{Ile} precursor 3' end maturation in vitro by tRNAse Z. Furthermore, the mutated tRNA^{Ile} displays local structural differences from wild-type. These results suggest that structural perturbations reduce efficiency of tRNA^{Ile} precursor 3' end processing and contribute to the molecular pathomechanism of this mutation.

Introduction

Mutations in the mitochondrial genome occur 10- to 17-fold more frequently than in the nuclear genome (Pesole et al., 1999). Mitochondrial tRNA genes (*MTT*) appear to be particularly frequently affected by pathogenesis-related mutations. Although *MTT* only account for approximately 10% of the mitochondrial genome, more than 150 of the so far over 220 disease correlated mutations are located in *MTT* (for a compilation, see MITOMAP: A Human Mitochondrial Genome Database. <http://www.mitomap.org>, 2009). These genes are thus hot-spots for mutation-based mitochondrial pathogenesis. The molecular consequences of these base substitutions with respect to tRNA structure and function are largely unclear, however, and so is the molecular pathology. Furthermore, there is no general rule to distinguish between pathogenic and polymorphic mutations. The mere

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presence of a mutation in a *MTT*, even in a conserved position, is not sufficient to predict its pathogenicity as there are known pathogenic mutations located at non-conserved nucleotides, and polymorphic mutations located at conserved nucleotides (Florentz and Sissler, 2001). Thus, molecular investigations are required to confirm the pathogenicity of mutations in the *MTT* and characterise the underlying molecular mechanism. Presumably, defects in translation of mitochondrial mRNAs would arise from structural changes in the tRNAs. These changes could affect excision of the tRNA by RNase P and tRNase Z, affecting the mRNA function in translation and could also reduce the level of a mature tRNA at the level of removal of the leader and trailer, the addition of CCA that follows endonucleolytic removal of the 3' end trailer by tRNase Z, subsequent aminoacylation and the interaction of the aminoacyl-tRNA with translation factors and the mitoribosome. In this study, we have analyzed the in vitro processing of pre-tRNAs by tRNase Z, a reaction that is central to the maturation and function of the tRNAs in mitochondrial translation (reviewed in Levinger et al. (Levinger et al., 2004)).

In vivo studies are complicated by a varying degree of heteroplasmy and variable nuclear genetic background, and limited by the availability of biopsy material. However, development of in vitro systems which can directly address potential underlying molecular mechanisms enabled us to analyse the effect of mutations in tRNA genes on a critical step in mitochondrial biogenesis (Rossmanith et al., 1995). Chronic progressive external ophthalmoplegia (CPEO) is a common clinical feature of mitochondrial diseases (DiMauro and Schon, 2008). It presents often as ptosis followed by a gradual involvement of the extraocular muscles in combination with variable degrees of oropharyngeal and limb weakness. The underlying genetic basis of CPEO is, however, heterogenous. CPEO is often caused by large-scale mtDNA rearrangements (deletions and/or duplications). Whereas multiple deletions are often observed in autosomal dominant or recessive forms of CPEO (Hirano and DiMauro, 2001), sporadic single deletions are thought to arise during in oogenesis or early embryonic development. Furthermore, in some cases CPEO is maternally inherited due to point mutations in the mtDNA (McFarland et al., 2010). Specifically, an adult patient presenting with CPEO was found to harbour a substitution (m.4308G>A) in the T-stem of mitochondrial tRNA^{Ile} that has not been previously observed. To date 14 substitutions in tRNA^{Ile} have been linked to mitochondrial diseases: m.4267A>G (Taylor et al., 2002), m.4269A>G (Degoul et al., 1998; Hayashi et al., 1994; Kaido et al., 1995; Taniike et al., 1992), m.4274T>C (Borthwick et al., 2006; Chinnery et al., 1997), m.4284G>A (Corona et al., 2002), m.4285T>C (Silvestri et al., 1996), m.4290T>C (Limongelli et al., 2004), m.4291T>C (Wilson et al., 2004), m.4295A>G (Merante et al., 1996), m.4298G>A (Taylor et al., 1998), m.4300A>G (Casali et al., 1995; Taylor et al., 2003), m.4302A>G (Berardo et al., 2010), m.4309G>A (Campos et al., 2002; Franceschina et al., 1998), m.4317A>G (Degoul et al., 1998; Obayashi et al., 1992; Ozawa et al., 1991a; Ozawa et al., 1991b; Tanaka et al., 1990) and m.4320C>T (Santorelli et al., 1995). Seven of them are associated with CPEO (in italics) suggesting that tRNA^{Ile} could be a hotspot for CPEO. We report herein the effects of the m.4308G>A substitution on the endonucleolytic cleavage of the 3' end trailer that is central to pre-tRNA maturation and on the tRNA^{Ile} structure.

Patient, materials and methods

Patient

The 33-year-old woman presented in a peripheral Hospital after her second syncope in a six months interval. History revealed a progressive weight loss in the past two years although the appetite was normal and there were no gastrointestinal symptoms. The BMI at presentation was 16.9 kg/m². She had a general exercise intolerance with problems in stair climbing and amenorrhea. The examination showed a bilateral ptosis, an impairment of

vertical and left gaze whereas right gaze was almost normal. There was no nystagmus and the fundus and visual fields were normal. Further Investigations including audiometry, EEG, brain MRI, echocardiogram, Liquor protein, glucose and lactate were all normal. The electrocardiogram showed ventricular extrasystola without other arrhythmia. The family history was non informative. She had two healthy siblings and was the mother of two healthy children. In the five year follow up the patient developed a clear progression of the muscular symptoms with a complete external ophthalmoplegia and a proximal myopathy with disabling muscular cramps. There further is an increased frequency of neurocardiogenic syncope as documented with a pathological Tilt test. Brain MRI, EEG and Holter ECG remained normal. The patient was treated symptomatically and with a vitamin cocktail, carnitine and coenzyme Q10.

All participating individuals gave informed consent. The study protocol was approved by the local ethical commission.

Electron microscopy

Skeletal muscle was fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4), post-fixed with 1% osmium tetroxide, dehydrated through ascending concentrations of alcohol and embedded in Epon812. Ultrathin sections were obtained routinely at 60 nm thickness on a Reichert-Jung Ultracut ultramicrotome equipped with a diamond knife (Diatome, Switzerland), stained with uranylacetate and leadcitrate using EMstain (Leica, Austria) and observed in a Philips CM12 electron microscope at 80 kv equipped with a Morada digital camera using iTEM software.

OXPHOS assays

Skeletal muscle homogenates (600 g supernatants) were prepared as described previously (Birch-Machin and Turnbull, 2001; Rustin et al., 1994). The activities of the individual respiratory chain complexes and the mitochondrial matrix marker enzyme citrate synthase in skeletal muscle homogenates were measured spectrophotometrically with an UV-1601 spectrophotometer (Shimadzu) using 1 ml sample cuvettes thermostatically maintained at 30° C according to Birch-Machin and Turnbull (2001) (Birch-Machin and Turnbull, 2001). Values were estimated by the difference in activity levels measured in the presence and absence of specific inhibitors and are expressed relative to the mitochondrial marker enzyme citrate synthase (mU/mU citrate synthase), determined as described (Shepherd and Garland, 1969).

Molecular genetic analysis

Total DNA extraction from EDTA-blood and skeletal muscle were performed according to standard isolation protocols (Qiagen). Large-scale rearrangements were excluded by long polymerase chain reaction (PCR) as described previously (Kleinle et al., 1997). mtDNA was screened for mutations by PCR and subsequent single strand conformation polymorphism as previously described (Kleinle et al., 1998; Liechti-Gallati et al., 1999). The *tRNA^{Leu}* gene encompassing the mutation was amplified using the forward (nucleotide position nt 4218-4237) and reverse complementary (nt 4516-4497) primers according to the Cambridge sequence of Anderson et al. (Anderson et al., 1981). Direct sequencing of the PCR products was performed on an ABI 3100 DNA Sequencer (Applied Biosystems) using the ABI PRISM BigDye Terminator Cycle Sequencing Kit.

Heteroplasmy of the G4308A mtDNA point mutation was investigated by PCR amplification of a convenient mtDNA fragment using the following primers: forward, 4212-4233; reverse, 4309-4330 (C/A mismatch at position 4311 introducing a *Bgl*III restriction site in mutant mtDNA). Mutant loads were evaluated by fluorescent last cycle

PCR (Valentino et al., 2002) using a 5'-FAM labeled forward primer, *Bgl*II digestion and subsequent separation of fragments on an agarose gel and for quantification on an ABI 3100 DNA Sequencer.

Preparation of tRNA precursor

A 71 nt long oligonucleotide reverse primer (5'-GCGCGGATCCCCGGGTTTCGATTCTCATAGTCCTAGAAATAAGGGGGTTTATGCTCTTATTATTTACTCTATC-3'; the mismatched nucleotide for mutagenesis to produce m.4308G>A is double-underlined) was used to reconstruct the *Bam*HI subcloning site, *Sma*I runoff site, 20 nt natural sequence of 3' end trailer, the entire T arm and variable including the m.4308G>A (50G>A) substitution. The 33 nt long universal forward primer (5'-GCGCGAATTCTAATACGACTCACTATAGGGAGA-3') reconstructed the *Eco*RI subcloning site, T7 promoter and strong start (GGGAGA). The template for amplification was wild type tRNA^{Ile} including the hammerhead for cleavage at +1 of the tRNA (original hammerhead construct was a gift of S. Kelley (Kelley et al., 2001)). The amplified DNA segments were digested with *Eco*RI/*Bam*HI, ligated into the subcloning vector, and confirmed by sequencing (Genewiz). Runoff templates were prepared by *Sma*I digestion.

Unlabeled transcripts were prepared with T7 RNA polymerase as previously described (Fechter et al., 1998), followed by phenol/chloroform deproteinization and ethanol precipitation before hammerhead self-cleavage of tRNA^{Ile} at the 5' side of +1. tRNA precursors were purified from the hammerhead on denaturing 6% polyacrylamide gels, visualized by UV shadowing, extracted from gel slices by diffusion for 30 min at 37°C and recovered by ethanol precipitation. Concentration of recovered tRNA was determined by reading A₂₆₀, using a conversion factor of 875 000 A₂₆₀ M⁻¹.

RNA labeling

tRNA precursors were labeled at their 5' ends with T4 polynucleotide kinase and [γ -³²P]ATP for 30 min at 37°C using RNasin as a ribonuclease inhibitor, gel purified, visualized by autoradiography, and recovered from the gel as described above.

tRNA refolding

Both labeled and unlabeled tRNAs were re-folded by heating in water for 2 min at 90°C, mixing briefly with an equal volume of heated buffer to a final concentration of 25 mM Tris-HCL pH 7.5, 250 mM KCL, 5 mM MgCl₂ and 5% glycerol, and cooling to room temperature for 5 min.

3' End processing

Human tRNase Z^L was baculovirus expressed from a glycine at position 50 and affinity purified as previously described (Yan et al., 2006). Within the first 50 residues, tRNase Z^L has a presumed mitochondrial targeting sequence with a predicted cleavage site at residue 30. There is also a methionine at r16 that could be the translation start for a nuclear-targeted tRNase Z. tRNase Z has been expressed from +1, 16, 31, 45, 50, 55 and 60, and the processing activity on a nuclear-encoded substrate was found to be the same, except that the +1 form expressed appeared to be lower in yield and more subject to degradation (Hopkinson and Levinger, unpublished).

tRNase Z^L processing reactions (25 μ l) were performed with labeled WT and m.4308G>A pre-tRNA^{Ile} substrates using 25 mM K-MOPS pH 6.75, 2 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol, 4 units/ml RNasin⁺ and 100 μ g/ml bovine serum albumin at 37°C. Reactions were sampled and analyzed after 5, 10 and 15 minutes by transferring 5 μ l to 2.5 μ l of formamide-marker dye mix and electrophoresing on 6% denaturing polyacrylamide gels.

Gels were dried and exposed overnight using a storage phosphor screen which was scanned with the Typhoon and analyzed using ImageQuant. Efficiency experiments without added unlabeled substrates (data not shown) approximate zero order kinetics in which % product/minute of reaction ($V/[S]$) is proportional to V . Reaction velocities for variant and wild type pre-tRNA substrates were matched by adjusting enzyme concentrations.

Michaelis-Menten experiments were performed using unlabeled pre-tRNA substrate at 2, 5, 10, 20 and 50 nM with a constant (trace) concentration of labeled substrate. To obtain linear processing reactions over a range of substrate concentrations, tRNase Z was used at 2.5 pM for wild type pre-tRNA^{Ile} and at 12.5 pM for m.4308G>A pre-tRNA^{Ile}. As % product/minute ($V/[S]$) decreases with increasing $[S]$, V (obtained by multiplying $V/[S]$ by $[S]$) increases (Figure 5B, C). Each variant tRNA was analyzed in two or more kinetic experiments. Comparisons to determine relative k_{cat}/K_M (Figure 3, 4; Table 2) were made between results of variant and wild type experiments performed on the same day.

Structure probing

Structure probing of mitochondrial tRNA^{Ile} was performed substantially as previously reported (Levinger et al., 2003). For alkaline ladders, the end-labeled tRNA was incubated at 90°C for 10 min in 50 mM NaHCO₃, pH 9. For semi-denaturing (SD) ribonuclease (RNase) T1 reactions, the labeled tRNA was incubated at 50°C for 5 min in 12.5 mM Na citrate, pH 4.5, 3.5 M urea, 0.5 mM EDTA. Unlabeled *Escherichia coli* tRNA was used as a carrier at a final concentration of 1 µg/10 µl reaction. To achieve native conditions, tRNAs were refolded as described above for processing, and incubated in the processing buffer (but without RNasin) for 5 min at 37°C with the structure probing nucleases. Final concentrations were 1.0 and 2.5 × 10⁻² U/µl (RNase T1; US Biochemical), 0.4 and 1 × 10⁻² U/µl (RNase V1; Pierce), 0.4 and 1 × 10⁻² U/µl (RNase If; New England Biolabs) or 0.8 and 2 × 10⁻⁷ U/ml (RNase A; Sigma). Native reactions were performed using processing buffer (with Tris-HCl pH 7 substituted for K-MOPS pH 6.75) at 37°C for 5 min. Reactions were terminated by placing samples at -20°C. For electrophoresis on 30 X 40 cm, 6, 8 or 10% denaturing polyacrylamide gels, 5 µl of formamide-marker dye mix was added and 7.5 µl samples were loaded directly, without heating, and electrophoresed until the bromophenol blue migrated to 10 cm above the bottom. Imaging and analysis were performed as for processing gels.

Results

Ultrastructural examinations and biochemical assays

Electron microscopy examination showed myofibers containing increased amounts of lipid droplets and glycogen. In these fibers mitochondria were strikingly abnormal in appearance, abundance, and distribution (Fig. 1). While enlarged mitochondria were not present in all fibers; when present, however, the vast majority of mitochondria showed an aberrant morphology. They had disturbed internal organisation. Many exhibit concentrically arranged cristae and contained the classic “parking lot” inclusions. Further, subsarcolemmal aggregates of enlarged mitochondria are seen, producing the appearance of ragged red fibers. Subsequent measurement of the respiratory chain activities on muscle homogenate demonstrated a partial deficiency of the respiratory complexes I, III and IV containing mtDNA-encoded subunits, with normal SDH activity, whereas complex V remained in the lower reference range (Table 1).

Genetic analysis

The clinical features and the histological and biochemical results from the patient’s muscle biopsy supported the diagnosis of a mitochondrial disorder, prompting us to analyse the

mitochondrial genome. Large-scale rearrangements of mtDNA, which are the most frequent cause of CPEO, were excluded using long PCR (Kleinle et al., 1997). All 22 tRNAs were subjected to modified SSCP analysis followed by direct sequencing of polymorphic SSCP-fragments. We detected a novel G to A point mutation at nt 4308 in the mitochondrial tRNA^{Ile}-gene (Fig. 2A) which changes a nucleotide in the T-stem, disrupting a GC base pair close to the V-loop – T-stem boundary, which is strictly conserved in mammals. The mutation was present in heteroplasmic state in the patient's skeletal muscle but not in blood (Fig. 2B). Quantification of the mutation using a last fluorescence cycle revealed 47% heteroplasmy. Mutated mtDNA was found neither in the mother's blood nor in her skeletal muscle (Fig. 2B), suggesting a *de novo* mutation.

Mitochondrial tRNA^{Ile} precursor 3' end processing

Endonucleolytic removal of the 3' end trailer by tRNase Z is central to the maturation of human mitochondrial tRNA precursors. Precursor 3' end processing kinetics were investigated using wild-type and G4308A mutant tRNA^{Ile} precursor containing a mature 5' end and a 20 nt 3' end trailer (Fig. 3A; arrow indicates the expected tRNase Z cleavage site). Figure 3B and C illustrate the effect of the G4308A substitution on tRNase Z processing. Based on efficiency experiments (not shown), the tRNase Z reaction with m.4308G>A was performed at a 5X higher tRNase Z concentration than for WT to approximately match $V_{\max}$ for WT tRNA^{Ile} and G4308A substrates. This leads to a ~6X lower K_{cat} for m.4308G>A (Table 2). The G4308A mutant tRNA^{Ile} causes relatively little effect on K_M (Table 2). The combined result is a ~ 5-fold overall reduction in processing efficiency (K_{cat}/K_M) relative to wild-type (Table 2). Comparison with the previously identified mutation m.4309G>A (Franceschina et al., 1998) (also associated with CPEO) revealed a 2-fold more pronounced 3' end processing deficiency than for the m.4308G>A mutant (Table 2 and Fig 4). K_{cat} relative to wild-type was 2 fold lower for m.4308G>A (0.15) than for m.4309G>A (0.32) (Table 2), whereas K_M of m.4308G>A was slightly lower than wild-type compared with a slight increase in K_M for m.4309G>A (Table 2). Along with the 5-fold lower 3' end processing efficiency for the m.4308G>A mutant, we confirmed a 3-fold decrease in processing efficiency of the m.4309G>A mutant (Table 2).

Effect of the m.4308G>A substitution on tRNA^{Ile} precursor structure

Secondary structure probing nucleases were used to investigate precursor tRNA^{Ile} folding and to search for structural differences between the m.4308G>A mutant and wild-type tRNA^{Ile}. Ribonuclease T1 is specific for G residues, ribonuclease A for single stranded pyrimidines, Ribonuclease I_f for single stranded RNA and ribonuclease V1 for paired stems and otherwise structured s (note, however, that V1 cleavage as a literal indicator of helicity can be misleading, missing some helices and cutting to the sides of others (Maizels et al., 1999); reliable comparisons between mutant and wild-type V1 data can nonetheless be made). The alternating I_f and V1 patterns are consistent with the stem-loop structure characteristic of the canonical tRNA cloverleaf and the 3' end trailer is also apparently structured, as previously reported (Levinger et al., 2003).

Ribonuclease T1 used under semi-denaturing (Fig. 4A lane 2 and 4B lane 2) as well as native conditions (Fig. 4A lanes 3–4 and 4B lanes 3–4) can reveal differences in exposure of Gs which arise from changes in tRNA folding. No differences in T1 susceptibility were observed between wild-type and m.4308G>A mutant precursor, but absence of G50 from the m.4308G>A pattern confirms the m.4308G>A substitution. Ribonuclease A and I_f patterns are consistent with the cloverleaf structure of tRNA^{Ile} (Fig. 4A). The bottom of the T stem (nucleotides 49–53, Fig. 4B) displays low V1 sensitivity; only nucleotide 50 is a strong V1 site in the wild-type tRNA^{Ile} precursor (Fig. 4C). Most structural changes in the m.4308G>A precursor relative to wild-type are observed at a hinge position between the T

arm and acceptor stem (Fig. 4B and C, indicated by a dot). Decreased V1 susceptibility of the m.4308G>A mutant is observed at the site of the substitution (A50) and increased V1 susceptibility is observed at U56 in the T loop and U65 at a hinge position between the T arm and acceptor stem. These changes in nuclease V1 cleavage are superimposed on the tRNA^{Ile} secondary structure in Figure 5A.

The tRNA^{Ile} T stem contains the 52-62 A-C apposition which weakens the T stem (as suggested by Kelley et al., 2001 (Kelley et al., 2001)) and would be expected to increase the disturbance of T-stem structure arising from the m.4308G>A mutation. Manual folding allows us to suggest a somewhat extreme refolding involving the V-loop, T arm and acceptor stem produced by sliding one nucleotide from the top of the T stem into the T loop (producing an 8 nt T loop; Figure 5B) which generates a 10 bp T-stem including one A-C apposition. With no acceptor stem, this structure would not be a substrate for tRNA end-processing enzymes including tRNase Z. Structural modeling using Mfold (Zuker, 2003) predicts that destabilization of the T stem by the G4308A mutant would lead to a large open loop consisting of the V-loop and the entire T-arm, while the acceptor stem remains intact (Figure 5C). This structure, lacking a T-stem to coaxially stack on the acceptor stem, would also fail as a tRNase Z substrate.

Discussion

Various causes have been found for sporadic CPEO, including both large rearrangements and point mutations in mtDNA. In a patient presenting with CPEO, we identified a novel point mutation in mitochondrial tRNA^{Ile} which could interfere with formation of the T stem and stabilize an alternate secondary structure. The following features substantiate the pathogenic nature of the m.4308G>A mutation. The lack of large rearrangements in the mtDNA suggests that primary mutations in a nuclear gene (e.g. POLG, POLG2, Twinkle and ANT1) is unlikely (Spinazzola and Zeviani, 2009; Tuppen et al., 2010). The mutation was associated with clinical symptoms and was present in a heteroplasmic form in the muscle tissue, consistent with a general criterion for mtDNA. Electron microscopy showed the typical features of a mitochondrial cytopathy (Mierau et al., 2004). Morphologically, there were ultrastructural mitochondrial abnormalities, including deranged internal architecture and crystalline inclusions which have not only been observed in PEO patients (Mitsumoto et al., 1983) but also in other diseases linked to defective mitochondrial function such as MERRF (Suomalainen, 1997), Kearns-Sayre Syndrome (McKechne et al., 1985), myopathy (Frey and Mannella, 2000; Zanssen et al., 1997), and encephalopathy (Kaido et al., 1995). Biochemically, we found a multiple partial respiratory chain enzyme deficiency, affecting the activities of complexes I, III and IV. The m.4308G>A variant was not found in analysis of more than 2700 individuals (Ingman and Gyllensten, 2006), strongly suggesting that it is not a neutral polymorphism. A general approach to demonstrate the pathogenicity of a newly identified point mutation in the mtDNA includes single fibre PCR to show a co-segregation of the mutation with COX-negative fibres. However, in this particular case, the amount of native muscle biopsy remaining after electron microscopy, biochemical analysis and genetic testing, was insufficient to obtain good quality sections for accurate qualitative assessment of COX-negative fibers, prompting us to investigate the pathogenicity of the newly identified variant at a functional level. Mitochondrial tRNAs are embedded in long primary transcripts, punctuating the two ribosomal RNAs and 11 mRNAs (Anderson et al., 1981). Production of mature, functional mitochondrial RNAs requires endonucleolytic cleavage of the precursors at the 5' and/or 3' ends of the tRNAs (Montoya et al., 1981; Ojala et al., 1981). tRNA^{Ile} 5' end and ND1 3' end have an overlap of 1 nt, which therefore requires the addition of an A after processing to complete the ND1 translation termination codon. At its 3' end, tRNA^{Ile} has a 70 nt spacer followed by tRNA^{Met}, making the reductions in tRNase Z processing efficiency due to pathogenesis-related mutations still

more remarkable. This is also observed for tRNA^{Ser(UCN)} which is followed by an even longer 2.5 kb spacer at its 3' end (Levinger et al., 2001). In these instances, the molecular defect is probably the reduced quantity and impaired function of the affected aminoacyl tRNA that causes a deficiency in translation of mitochondrial messages, leading to the pathology.

For functional studies of the m.4308G>A substitution, we initially investigated the effect of the m.4308G>A substitution on tRNase Z processing and observed a five fold reduction in processing efficiency. On the basis of this reduced processing efficiency, we performed Michaelis-Menten kinetics on wild-type and two mutants (m.4308G>A and m.4309G>A). The latter has previously been described to be associated with CPEO (Franceschina et al., 1998) and resulted in a pathology-related reduction in 3' end processing (Levinger et al., 2003). Both mutants display substantially reduced 3' end processing efficiency, due principally to a lower k_{cat} . Whereas the m.4309G>A mutant decreases 3' end processing about 3 fold, the G4308A mutant has 5 fold reduced 3' end processing efficiency.

Wild-type and m.4308G>A mutant structures were compared to search for structural reasons for reduced processing efficiency. The mutation disrupts a strictly conserved GC base pair in the T stem of tRNA^{Ile} close to the V-Loop – T-stem boundary. The effect of the G4308A substitution on tRNA^{Ile} structure (Figures 5, 6) and tRNase Z processing combine to suggest an overall reduction in availability of Ile-tRNA^{Ile} for translation. Computer analysis and visual inspection of mutant tRNA^{Ile} suggest that the m.4308G>A mutation destabilises the already weak T stem. While no single secondary structure completely explains the changes in V1 nuclease susceptibility, the three-headed arrow between Figure 6A, B and C indicates a shift in the equilibrium away from the coaxially stacked acceptor stem and T stem that is required of a tRNase Z substrate in favour of altered structures. The m.4308G>A mutation thus severely affects secondary and tertiary structure of tRNA^{Ile}, thereby reducing its function.

End processing reactions by RNase P, tRNase Z and CCA-adding enzyme require an intact, coaxially stacked acceptor stem and T domain (Levinger et al., 2003). Thus, the location of the m.4308G>A mutation and its implication in structural, functional and physiological effects are consistent with interference of the mutated tRNA^{Ile} with cleavage by tRNase Z, contributing to pathology.

Interestingly, while our manuscript was in the process of revision, we became aware of a publication (Sihem et al., 2010) in which the authors found the m.4308G>A in a patient with sporadic CPEO, suggesting an association of the m.4308G>A mutation with this clinical phenotype.

Genetic counseling for a mtDNA mutation is generally a difficult task as factors determining the transmission of mtDNA mutations are largely unknown. Thus, the likelihood that the m. 4308G>A mutation would be transmitted to the patient's two children is difficult to predict (Elson et al., 2009). The mutation found in the patient is confined to muscle and the absence of the mutation in blood suggests that it is not a germline mutation. However, there is no guarantee that the patient's oocytes are mutation-free. To fully exclude transmission of the mutation, a muscle biopsy should be performed on the presently unaffected children. However, a muscle biopsy in unaffected individuals is not standard practice and therefore, the status of her children remains unknown.

In summary, this study adds m.4308G>A to the growing list of point mutations associated with CPEO and further emphasizes the importance of analyzing all the 22 tRNAs in sporadic CPEO patients after exclusion of large deletions. It also demonstrates the need of a muscle biopsy when no mutations can be detected in DNA extracted from blood. Our data

provide a further example in support of the thesis that inefficient 3' end processing of mutant tRNAs can contribute to pathology (Levinger et al., 2004). CPEO may be associated with mtDNA deletions or related to point mutations within one or another of the tRNA genes. A link between these two mitochondrial genome-based causes could be presumed to arise from a decrease in mitochondrial protein synthesis which would cause the multiple partial defects in respiratory chain activity.

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References

- Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. *Nature*. 1981; 290:457–465. [PubMed: 7219534]
- Berardo A, Coku J, Kurt B, DiMauro S, Hirano M. A novel mutation in the tRNA^{Ile} gene (MTTI) affecting the variable loop in a patient with chronic progressive external ophthalmoplegia (CPEO). *Neuromuscul Disord*. 2010; 20:204–206. [PubMed: 20149659]
- Birch-Machin MA, Turnbull DM. Assaying mitochondrial respiratory complex activity in mitochondria isolated from human cells and tissues. *Methods Cell Biol*. 2001; 65:97–117. [PubMed: 11381612]
- Borthwick GM, Taylor RW, Walls TJ, Tonska K, Taylor GA, Shaw PJ, Ince PG, Turnbull DM. Motor neuron disease in a patient with a mitochondrial tRNA^{Ile} mutation. *Ann Neurol*. 2006; 59:570–574. [PubMed: 16358336]
- Campos Y, Garcia A, Lopez A, Jimenez S, Rubio JC, Del Hoyo P, Bustos F, Martin MA, Cabello A, Ricoy JR, Arenas J. Cosegregation of the mitochondrial DNA A1555G and G4309A mutations results in deafness and mitochondrial myopathy. *Muscle Nerve*. 2002; 25:185–188. [PubMed: 11870684]
- Casali C, Santorelli FM, D'Amati G, Bernucci P, DeBiase L, DiMauro S. A novel mtDNA point mutation in maternally inherited cardiomyopathy. *Biochem Biophys Res Commun*. 1995; 213:588–593. [PubMed: 7646516]
- Chinnery PF, Johnson MA, Taylor RW, Durward WF, Turnbull DM. A novel mitochondrial tRNA isoleucine gene mutation causing chronic progressive external ophthalmoplegia. *Neurology*. 1997; 49:1166–1168. [PubMed: 9339712]
- Corona P, Lamantea E, Greco M, Carrara F, Agostino A, Guidetti D, Dotti MT, Mariotti C, Zeviani M. Novel heteroplasmic mtDNA mutation in a family with heterogeneous clinical presentations. *Ann Neurol*. 2002; 51:118–122. [PubMed: 11782991]
- Degoul F, Brule H, Cepanec C, Helm M, Marsac C, Leroux J, Giege R, Florentz C. Isoleucylation properties of native human mitochondrial tRNA^{Ile} and tRNA^{Ile} transcripts. Implications for cardiomyopathy-related point mutations (4269, 4317) in the tRNA^{Ile} gene. *Hum Mol Genet*. 1998; 7:347–354. [PubMed: 9466989]
- DiMauro S, Schon EA. Mitochondrial disorders in the nervous system. *Annu Rev Neurosci*. 2008; 31:91–123. [PubMed: 18333761]
- Elson JL, Swalwell H, Blakely EL, McFarland R, Taylor RW, Turnbull DM. Pathogenic mitochondrial tRNA mutations--which mutations are inherited and why? *Hum Mutat*. 2009; 30:E984–E992. [PubMed: 19718780]
- Fechter P, Rudinger J, Giege R, Theobald-Dietrich A. Ribozyme processed tRNA transcripts with unfriendly internal promoter for T7 RNA polymerase: production and activity. *FEBS Lett*. 1998; 436:99–103. [PubMed: 9771901]
- Florentz C, Sissler M. Disease-related versus polymorphic mutations in human mitochondrial tRNAs. Where is the difference? *EMBO Rep*. 2001; 2:481–486. [PubMed: 11415979]

- Franceschina L, Salani S, Bordoni A, Sciacco M, Napoli L, Comi GP, Prella A, Fortunato F, Hadjigeorgiou GM, Farina E, Bresolin N, D'Angelo MG, Scarlato G. A novel mitochondrial tRNA(Ile) point mutation in chronic progressive external ophthalmoplegia. *J Neurol*. 1998; 245:755–758. [PubMed: 9808249]
- Frey TG, Mannella CA. The internal structure of mitochondria. *Trends Biochem Sci*. 2000; 25:319–324. [PubMed: 10871882]
- Hayashi J, Ohta S, Kagawa Y, Takai D, Miyabayashi S, Tada K, Fukushima H, Inui K, Okada S, Goto Y, et al. Functional and morphological abnormalities of mitochondria in human cells containing mitochondrial DNA with pathogenic point mutations in tRNA genes. *J Biol Chem*. 1994; 269:19060–19066. [PubMed: 7518448]
- Hirano M, DiMauro S. ANT1, Twinkle, POLG, and TP: new genes open our eyes to ophthalmoplegia. *Neurology*. 2001; 57:2163–2165. [PubMed: 11756592]
- Ingman M, Gyllenstein U. mtDB: Human Mitochondrial Genome Database, a resource for population genetics and medical sciences. *Nucleic Acids Res*. 2006; 34:D749–D751. [PubMed: 16381973]
- Kaido M, Fujimura H, Taniike M, Yoshikawa H, Toyooka K, Yorifuji S, Inui K, Okada S, Sparaco M, Yanagihara T. Focal cytochrome c oxidase deficiency in the brain and dorsal root ganglia in a case with mitochondrial encephalomyopathy (tRNA(Ile) 4269 mutation): histochemical, immunohistochemical, and ultrastructural study. *J Neurol Sci*. 1995; 131:170–176. [PubMed: 7595643]
- Kelley SO, Steinberg SV, Schimmel P. Fragile T-stem in disease-associated human mitochondrial tRNA sensitizes structure to local and distant mutations. *J Biol Chem*. 2001; 276:10607–10611. [PubMed: 11110797]
- Kleinle S, Schneider V, Moosmann P, Brandner S, Krahenbuhl S, Liechti-Gallati S. A novel mitochondrial tRNA(Phe) mutation inhibiting anticodon stem formation associated with a muscle disease. *Biochem Biophys Res Commun*. 1998; 247:112–115. [PubMed: 9636664]
- Kleinle S, Wiesmann U, Superti-Furga A, Krahenbuhl S, Boltshauser E, Reichen J, Liechti-Gallati S. Detection and characterization of mitochondrial DNA rearrangements in Pearson and Kearns-Sayre syndromes by long PCR. *Hum Genet*. 1997; 100:643–650. [PubMed: 9341886]
- Levinger L, Giege R, Florentz C. Pathology-related substitutions in human mitochondrial tRNA(Ile) reduce precursor 3' end processing efficiency in vitro. *Nucleic Acids Res*. 2003; 31:1904–1912. [PubMed: 12655007]
- Levinger L, Jacobs O, James M. In vitro 3'-end endonucleolytic processing defect in a human mitochondrial tRNA(Ser(UCN)) precursor with the U7445C substitution, which causes non-syndromic deafness. *Nucleic Acids Res*. 2001; 29:4334–4340. [PubMed: 11691920]
- Levinger L, Morl M, Florentz C. Mitochondrial tRNA 3' end metabolism and human disease. *Nucleic Acids Res*. 2004; 32:5430–5441. [PubMed: 15477393]
- Liechti-Gallati S, Schneider V, Neeser D, Kraemer R. Two buffer PAGE system-based SSCP/HD analysis: a general protocol for rapid and sensitive mutation screening in cystic fibrosis and any other human genetic disease. *Eur J Hum Genet*. 1999; 7:590–598. [PubMed: 10439967]
- Limongelli A, Schaefer J, Jackson S, Invernizzi F, Kirino Y, Suzuki T, Reichmann H, Zeviani M. Variable penetrance of a familial progressive necrotising encephalopathy due to a novel tRNA(Ile) homoplasmic mutation in the mitochondrial genome. *J Med Genet*. 2004; 41:342–349. [PubMed: 15121771]
- Maizels N, Weiner AM, Yue D, Shi PY. New evidence for the genomic tag hypothesis: archaeal CCA-adding enzymes and tDNA substrates. *Biol Bull*. 1999; 196:331–333. discussion 333–334. [PubMed: 10390831]
- McFarland R, Taylor RW, Turnbull DM. A neurological perspective on mitochondrial disease. *Lancet Neurol*. 2010; 9:829–840. [PubMed: 20650404]
- McKechne NM, King M, Lee WR. Retinal pathology in the Kearns-Sayre syndrome. *Br J Ophthalmol*. 1985; 69:63–75. [PubMed: 3965031]
- Merante F, Myint T, Tein I, Benson L, Robinson BH. An additional mitochondrial tRNA(Ile) point mutation (A-to-G at nucleotide 4295) causing hypertrophic cardiomyopathy. *Hum Mutat*. 1996; 8:216–222. [PubMed: 8889580]

- Mierau GW, Tyson RW, Freehauf CL. Role of electron microscopy in the diagnosis of mitochondrial cytopathies. *Pediatr Dev Pathol*. 2004; 7:637–640. [PubMed: 15630535]
- Mitumoto H, Aprille JR, Wray SH, Nemni R, Bradley WG. Chronic progressive external ophthalmoplegia (CPEO): clinical, morphologic, and biochemical studies. *Neurology*. 1983; 33:452–461. [PubMed: 6300733]
- Montoya J, Ojala D, Attardi G. Distinctive features of the 5'-terminal sequences of the human mitochondrial mRNAs. *Nature*. 1981; 290:465–470. [PubMed: 7219535]
- Obayashi T, Hattori K, Sugiyama S, Tanaka M, Tanaka T, Itoyama S, Deguchi H, Kawamura K, Koga Y, Toshima H, et al. Point mutations in mitochondrial DNA in patients with hypertrophic cardiomyopathy. *Am Heart J*. 1992; 124:1263–1269. [PubMed: 1442494]
- Ojala D, Merkel C, Gelfand R, Attardi G. The tRNA genes punctuate the reading of genetic information in human mitochondrial DNA. *Cell*. 1980; 22:393–403. [PubMed: 7448867]
- Ojala D, Montoya J, Attardi G. tRNA punctuation model of RNA processing in human mitochondria. *Nature*. 1981; 290:470–474. [PubMed: 7219536]
- Ozawa T, Tanaka M, Ino H, Ohno K, Sano T, Wada Y, Yoneda M, Tanno Y, Miyatake T, Tanaka T, et al. Distinct clustering of point mutations in mitochondrial DNA among patients with mitochondrial encephalomyopathies and with Parkinson's disease. *Biochem Biophys Res Commun*. 1991a; 176:938–946. [PubMed: 2025303]
- Ozawa T, Tanaka M, Sugiyama S, Ino H, Ohno K, Hattori K, Ohbayashi T, Ito T, Deguchi H, Kawamura K, et al. Patients with idiopathic cardiomyopathy belong to the same mitochondrial DNA gene family of Parkinson's disease and mitochondrial encephalomyopathy. *Biochem Biophys Res Commun*. 1991b; 177:518–525. [PubMed: 2043137]
- Pesole G, Gissi C, De Chirico A, Saccone C. Nucleotide substitution rate of mammalian mitochondrial genomes. *J Mol Evol*. 1999; 48:427–434. [PubMed: 10079281]
- Rossmannith W, Tullo A, Potuschak T, Karwan R, Sbisá E. Human mitochondrial tRNA processing. *J Biol Chem*. 1995; 270:12885–12891. [PubMed: 7759547]
- Rustin P, Chretien D, Bourgeron T, Gerard B, Rotig A, Saudubray JM, Munnich A. Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta*. 1994; 228:35–51. [PubMed: 7955428]
- Santorelli FM, Mak SC, Vazquez-Acevedo M, Gonzalez-Astiazaran A, Ridaura-Sanz C, Gonzalez-Halphen D, DiMauro S. A novel mitochondrial DNA point mutation associated with mitochondrial encephalocardiomyopathy. *Biochem Biophys Res Commun*. 1995; 216:835–840. [PubMed: 7488201]
- Shepherd D, Garland PB. The kinetic properties of citrate synthase from rat liver mitochondria. *Biochem J*. 1969; 114:597–610. [PubMed: 5820645]
- Sihem S, Chebel S, Mancuso M, Petrozzi L, Siciliano G, Frihayed M, Hentati F, Amouri R. A novel mitochondrial tRNA(Ile) point mutation associated with chronic progressive external ophthalmoplegia and hyperCKemia. *J Neurol Sci*. 2010
- Silvestri G, Servidei S, Rana M, Ricci E, Spinazzola A, Paris E, Tonali P. A novel mitochondrial DNA point mutation in the tRNA(Ile) gene is associated with progressive external ophthalmoplegia. *Biochem Biophys Res Commun*. 1996; 220:623–627. [PubMed: 8607814]
- Spinazzola A, Zeviani M. Disorders from perturbations of nuclear-mitochondrial intergenomic cross-talk. *J Intern Med*. 2009; 265:174–192. [PubMed: 19192035]
- Suomalainen A. Mitochondrial DNA and disease. *Ann Med*. 1997; 29:235–246. [PubMed: 9240629]
- Tanaka M, Ino H, Ohno K, Hattori K, Sato W, Ozawa T, Tanaka T, Itoyama S. Mitochondrial mutation in fatal infantile cardiomyopathy. *Lancet*. 1990; 336:1452. [PubMed: 1978914]
- Taniike M, Fukushima H, Yanagihara I, Tsukamoto H, Tanaka J, Fujimura H, Nagai T, Sano T, Yamaoka K, Inui K, et al. Mitochondrial tRNA(Ile) mutation in fatal cardiomyopathy. *Biochem Biophys Res Commun*. 1992; 186:47–53. [PubMed: 1632786]
- Taylor RW, Chinnery PF, Bates MJ, Jackson MJ, Johnson MA, Andrews RM, Turnbull DM. A novel mitochondrial DNA point mutation in the tRNA(Ile) gene: studies in a patient presenting with chronic progressive external ophthalmoplegia and multiple sclerosis. *Biochem Biophys Res Commun*. 1998; 243:47–51. [PubMed: 9473477]

- Taylor RW, Giordano C, Davidson MM, d'Amati G, Bain H, Hayes CM, Leonard H, Barron MJ, Casali C, Santorelli FM, Hirano M, Lightowlers RN, DiMauro S, Turnbull DM. A homoplasmic mitochondrial transfer ribonucleic acid mutation as a cause of maternally inherited hypertrophic cardiomyopathy. *J Am Coll Cardiol*. 2003; 41:1786–1796. [PubMed: 12767666]
- Taylor RW, Schaefer AM, McFarland R, Maddison P, Turnbull DM. A novel mitochondrial DNA tRNA(Ile) (A4267G) mutation in a sporadic patient with mitochondrial myopathy. *Neuromuscul Disord*. 2002; 12:659–664. [PubMed: 12207935]
- Tuppen HA, Blakely EL, Turnbull DM, Taylor RW. Mitochondrial DNA mutations and human disease. *Biochim Biophys Acta*. 2010; 1797:113–128. [PubMed: 19761752]
- Valentino ML, Avoni P, Barboni P, Pallotti F, Rengo C, Torroni A, Bellan M, Baruzzi A, Carelli V. Mitochondrial DNA nucleotide changes C14482G and C14482A in the ND6 gene are pathogenic for Leber's hereditary optic neuropathy. *Ann Neurol*. 2002; 51:774–778. [PubMed: 12112086]
- Wilson FH, Hariri A, Farhi A, Zhao H, Petersen KF, Toka HR, Nelson-Williams C, Raja KM, Kashgarian M, Shulman GI, Scheinman SJ, Lifton RP. A cluster of metabolic defects caused by mutation in a mitochondrial tRNA. *Science*. 2004; 306:1190–1194. [PubMed: 15498972]
- Yan H, Zareen N, Levinger L. Naturally occurring mutations in human mitochondrial pre-tRNA^{Ser}(UCN) can affect the transfer ribonuclease Z cleavage site, processing kinetics, and substrate secondary structure. *J Biol Chem*. 2006; 281:3926–3935. [PubMed: 16361254]
- Zanssen S, Molnar M, Schroder JM, Buse G. Multiple mitochondrial tRNA(Leu[UUR]) mutations associated with infantile myopathy. *Mol Cell Biochem*. 1997; 174:231–236. [PubMed: 9309693]
- Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. 2003; 31:3406–3415. [PubMed: 12824337]

Figure 1. Electron microscopy of cross section from limp muscle biopsy

The cross section shows a region of an unaffected and an affected myofiber. The affected fiber shows subsarcolemmal accumulation of mitochondria producing the appearance of ragged red fibers. A significant proportion of mitochondria are enlarged, display with deranged cristae and contain paracrystalline material in the form of the classic “parkin lot” inclusions. The magnified area shows morphologically abnormal mitochondria located between the contractile elements which are absent in the health myofiber.

Figure 2. Analysis of the m.4308G>A mutation in the *tRNA^{Ile}* gene

A) Sequence chromatogram of the encompassing the m.4308G>A mutation (arrow) from patient muscle. B) RFLP of the Mutation m.4308G>A in blood and skeletal muscle of the patient and her mother. Corresponding bands to the WT and m.4308G>A substitutions are indicated at right.

Figure 3. tRNase Z^L processing kinetics with the Wild Type and G4308A mutant of human mitochondrially encoded pre-tRNA^{Ile}

A) Secondary structure of pre-tRNA^{Ile}. The tRNase Z substrate has a 20 nt 3' end trailer. The m.4308G>A substitution and the tRNase Z processing site are identified with arrows. B) Processing kinetics of wild type pre-tRNA^{Ile} and the m.4308G>A mutant with tRNase Z^L. Reactions were performed with unlabeled pre-tRNA^{Ile} at 2, 5, 10, 20 and 50 nM and with a constant trace concentration of labeled pre-tRNA^{Ile}. The appropriate concentration of enzyme to use was first determined by analyzing the % product/minute (equivalent to V/[S]) obtained with the wild type and mutant substrates. Reactions were sampled at 5, 10 and 15 minute intervals as indicated by brackets below the panel. -Subst and -Prod designations on the right identify substrate and product bands. C) Eadie-Hofstee plots of wild type tRNA^{Ile} and the G4308A mutant. Equations are displayed on the Eadie-Hofstee plots (slope is $-K_M$, y-intercept is V_{max} , with units of $\times 10^{-8}$ M and $\times 10^{-10}$ M/min., respectively).

Figure 4. Structure probing of tRNA^{Ile} wild type and m.4308G>A

Panels A, B: denaturing 6% polyacrylamide gels of pre-tRNA^{Ile} wild type (left) and the G4308A variant (right). Lanes 0–10 are identified by designations below the gel. Designations at left are nucleotide numbers based on the canonical tRNA positions. Open and filled dots indicate nucleotide positions that display a structure different from the wild type. Panel C: ImageQuant traces of wild type and G4308A cleaved with RNase V1.

Figure 5. The m.4308G>A substitution in tRNA^{Ile} causes structural rearrangements

A) Changes in V1 susceptibility are identified with dots in the secondary structure. Decreased V1 susceptibility of the G4308A mutant is observed at the site of the substitution and increased V1 susceptibility is observed at U56 in the T loop and U65 at a hinge position between the T arm and acceptor stem. The arrow indicates the 3' end processing site. Possible structural rearrangements of G4308A tRNA^{Ile} include B) a long T stem and loop, a reduced variable and practically no acceptor stem and C) a completely open T-arm/Variable arm with an intact acceptor stem. The dashed arrow at the normal tRNase Z cleavage site indicates that tRNase Z would not be expected to cleave a tRNA with either of these structural rearrangements. The three-headed arrow between (A, B, C) indicates a continuum

of secondary structures favoring those that would produce an unfavorable substrate for tRNase Z (see text for further interpretation).

Table 1

Respiratory chain enzyme activities from patient muscle homogenate.

Enzymes	Patient	Controls (n=31)
Complex I	0.09	0.12 – 0.28 (0.19+/- 0.04)
Complex II	0.14	0.14 – 0.36 (0.21+/- 0.05)
Complex III	0.34	0.55 – 1.11 (0.78+/- 0.15)
Complex IV	0.49	0.57 – 1.77 (1.16+/- 0.28)
Complex V	0.20	0.19 – 0.65 (0.39+/- 0.13)
Citrate synthase	112	70 – 169 (105+/- 25)

Mean value (+/- 1SD) of the activities of the respiratory chain complexes are normalized to citrate synthase (CS). Activities are expressed as mU/mg mitochondrial protein and as a percentage of the control mean. Profound deficiencies below the range of controls are shown in bold. n=number of controls.

tRNase Z^L Processing Kinetics with Wild Type tRNA^{Ile} and Pathogenesis-Associated T Stem Mutant Substrates

Table 2

tRNA ^{Ile}	k_{cat}^a	K_M^b	k_{cat}/K_M^c	k_{cat} Re WT	K_M Re WT	k_{cat}/K_M Re WT ^d
WT	1.6± 0.17	1.3± 0.31	1.7± 0.5	N/A	N/A	N/A
m.4308G>A	0.24± 0.01	0.79± 0.05	0.31± 0.003	0.15± 0.03	0.77± 0.06	0.19± 0.02
m.4309G>A	0.48± 0.06	1.3± 0.25	0.43± 0.14	0.32± 0.04	1.21± 0.43	0.31± 0.06

^a min⁻¹

^b X 10⁻⁸ M

^c X 10⁸ M/min

^d Re WT (n-fold reduction *re* wild type) refers to the ratio of processing efficiencies; the mean k_{cat}/K_M (Mutant) / k_{cat}/K_M (WT) obtained in parallel kinetic experiments.

Averages of two or three Michaelis-Menten experiments with each variant are presented; wild type and variant experiments were performed the same day. +/- indicates standard error. Columns 1–7 are identified in the second row. Relative k_{cat}/K_M for the variants in column 7 is the n-fold reduction relative to wild type.