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To cite this version:

Lambert van den Heuvel, Paulien Smits, Hana Antonicka, P. M. van Hasselt, Woranontee Weraarpachai, et al.. Mutation in subdomain G' of mitochondrial elongation factor G1 is associated with combined OXPHOS deficiency in fibroblasts but not in muscle. European Journal of Human Genetics, 2010, 10.1038/ejhg.2010.208 . hal-00597453

HAL Id: hal-00597453

https://hal.science/hal-00597453v1

Submitted on 1 Jun 2011

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Title: Mutation in subdomain G' of mitochondrial elongation factor G1 is associated with combined OXPHOS deficiency in fibroblasts but not in muscle

Running title: Mitochondrial elongation factor G1 mutation

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Keywords: combined OXPHOS deficiency, mitochondrial translation, EFG1/GFM1

Abstract

The mitochondrial translation system is responsible for the synthesis of 13 proteins required for oxidative phosphorylation (OXPHOS), the major energy-generating process of our cells. Mitochondrial translation is controlled by various nuclear encoded proteins. In 27 patients with combined OXPHOS deficiencies, in whom complex II (the only complex that is entirely encoded by the nuclear DNA) showed normal activities and mutations in the mitochondrial genome as well as polymerase gamma were excluded, we screened all mitochondrial translation factors for mutations. Here we report a mutation in mitochondrial elongation factor G1 (*GFM1*) in a patient affected by severe, rapidly progressive mitochondrial encephalopathy. This mutation is predicted to result in an Arg250Trp substitution in subdomain G' of the elongation factor G1 protein and is presumed to hamper ribosome-dependent GTP hydrolysis. Strikingly, the decrease in enzyme activities of complex I, III and IV detected in patient fibroblasts was not found in muscle tissue. The OXPHOS system defects as well as the impairment in mitochondrial translation in fibroblasts were rescued by over-expressing wild-type *GFM1*, establishing the *GFM1* defect as the cause of the fatal mitochondrial disease. Furthermore, this study evinces the importance of thorough diagnostic biochemical analysis of both muscle tissue and fibroblasts in patients suspected to suffer from a mitochondrial disorder, as enzyme deficiencies can be selectively expressed.

Introduction

Mitochondria harbor a translation system distinct from the one in the cell's cytoplasm. This system is essential for the functioning of the oxidative phosphorylation (OXPHOS) system, and thus for energy production, through the synthesis of 13 proteins of OXPHOS complexes I, III, IV and V. The remaining OXPHOS system subunits as well as all other mitochondrial proteins, including those involved in mitochondrial translation, are encoded by the nuclear DNA. Defects in the mitochondrial translation machinery, due to either mitochondrial (mt) or nuclear (n) DNA mutations, can lead to the dysfunction of multiple mtDNA-reliant OXPHOS complexes. Mitochondrial disorders caused by OXPHOS deficiencies are often devastating diseases that are estimated to occur in 1:5000 live births.[1]

Sequencing of all mitochondrial translation factors (*MTIF2*, *MTIF3*, *TSFM*, *TUFM*, *GFM1*, *GFM2*, *MTRF1*, *MTRF1L*, and *MRRF*) in a cohort of 27 patients with combined OXPHOS enzyme deficits in fibroblasts and/or muscle tissue, in whom complex II showed normal activities and mutations in mtDNA and polymerase gamma were excluded, revealed a novel mutation in elongation factor G1 (gene: *GFM1*, protein: EFG1) in one patient. In this report, we demonstrate that the combined OXPHOS deficiency and mitochondrial protein synthesis defect detected in patient fibroblasts are the result of the *GFM1* mutation, ultimately leading to mitochondrial encephalopathy inducing rapid neurological deterioration, therapy resistant epilepsy and death at two years of age.

Materials and methods

Case report

The female patient was born at term as the second of a dizygotic twin after an uneventful gestation and birth. She was small for gestational age, with a birth weight of 2330 grams at 40 weeks of gestation. Parents were consanguineous (second cousins). Two days after birth she was briefly admitted to the hospital because of feeding problems and reduced consciousness that were attributed to an infection. Cultures of urine and blood, however, remained negative. In the following months she developed very slow compared with her healthy twin-sister. In addition, she was severely hypotonic, made poor eye contact and continued to have significant feeding problems. Seizures were noted from the age of 8 weeks onwards. Examination at the age of 3 months showed a borderline microcephalic girl (head circumference was -2.5 SD) with wandering eye movements (suggesting delayed visual maturation) and without dysmorphic features. Axial hypotonia was accompanied by hypertonia of the extremities with brisk tendon reflexes. Liver function tests, including bilirubin levels, were normal, except for a repeatedly low alanine aminotransferase (ALAT range 3-15 U/l, *N*: 10-50 U/l; ASAT range 24-68 U/l, *N*: 20-65 U/l). Blood ammonia was normal. Biochemical examination revealed elevated lactate levels in blood (4.9 mmol/l, *N*: <2.1mmol/l) and cerebrospinal fluid (CSF) (2.5 mmol/l, *N*: <2.1 mmol/l) as well as an increased plasma lactate:pyruvate ratio (22, *N*: 12-18). Amino acids in plasma and CSF were normal. Urine organic acids showed increased excretion of lactate (454 mmol/mol creatinine, *N*: <156 mmol/mol creatinine), but no other abnormalities. Ophthalmologic and cardiological investigations were normal. Neuroimaging revealed a small frontal

cortex, a thin corpus callosum and delayed myelination. Multifocal epileptic discharges
9 were found on electroencephalogram (EEG): a disturbed background pattern with multifocal spikes and waves, no clear hypsarrythmia; seizures consisting of 1-3 seconds of background suppressions with high frequency discharge, predominantly over occipital areas, resulting in nystagmus; occasionally a tonic component was seen, resembling infantile spasm. A muscle biopsy was performed on suspicion of a mitochondrial disease. Histological examination of the skeletal muscle showed normal mitochondrial morphology without ragged-red fibers. On follow-up she deteriorated. She lost the ability to suck within the first year of life. Microaspirations persisted after introducing tube feeding and induced recurrent pneumonias. In addition, she developed hepatomegaly (4 cm below the right costal margin at the age of 8 months). The epilepsy
9 progressed, the EEG evolving into a Lennox-Gastaut syndrome, and was resistant to
12 various anti-epileptic drugs (vigabatrin, nitrazepam, ACTH), while a ketogenic diet had only a modest favorable effect. She died at the age of 2 years of respiratory insufficiency secondary to pneumonia. The parents did not consent to perform a postmortem.

Cell culture

Human skin fibroblasts were cultured in M199 medium (Gibco, Breda, The Netherlands) supplemented with 10% fetal calf serum and antibiotics. For complementation experiments, primary skin fibroblasts were immortalized with a retroviral vector expressing the E7 gene of type 16 human papilloma virus and another retroviral vector expressing the protein component (htert) of human telomerase.[2] Patient

and control skin fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum.

Enzyme measurements

The activities of the OXPHOS enzyme complexes were measured in skin fibroblasts and muscle tissue as described previously.[3,4] The pyruvate oxidation and ATP production rates were determined in muscle tissue.[5]

Sequence analysis

Total RNA was isolated from cultured skin fibroblasts using RNA-Bee (Tel-Test Texas, USA); complementary DNA (cDNA) was prepared using superscript II reverse transcriptase (Invitrogen, Breda, The Netherlands). Sequence analysis of the open reading frame of *GFM1* (GenBank accession number NM_024996) was performed in 4 overlapping fragments (primer sequences are available on request). The presence of the mutation was confirmed on genomic DNA, isolated from patient fibroblasts and from blood from control subjects and the patient's parents using salt extraction,[6] with the primers 5'-CATTACTCTTATTTGCCATTTG-3' (forward) and 5'-GGGTCCTCAATAACTTTTC-3' (reverse). Both strands were used for direct sequencing on a 3130*xl* Genetic Analyzer, using the BigDye terminator v1.1 cycle sequencing kit according to the manufacturer's protocol (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Sequence data were analyzed with Sequencher 4.8 software (Gene Codes, Michigan, USA) by comparison with the reference sequence of *GFM1*.

Computational modeling

The structures of the wild-type and mutant EFG1 proteins were based on the crystal structure of *Thermus thermophilus* EFG.[7] Modeling, energy minimization and analysis were performed using the YASARA & WHAT IF Twinset software.[8] The human EFG1 and *T. thermophilus* EFG proteins share 45% sequence identity; the first 43 EFG1 residues could not be modeled. Molecular figures were drawn with the POV-Ray module in YASARA.

cDNA constructs and virus production and infection

A retroviral vector containing the cDNA sequence of *GFM1* was created with the Gateway cloning system (Invitrogen, Carlsbad, CA, USA). The *GFM1* cDNA was amplified from I.M.A.G.E. clone No. 5574223 (ATCC) using specific primers and this PCR construct was subsequently cloned into a Gateway-modified retroviral expression vector, pBABE. The fidelity of the cDNA clone was confirmed by automated DNA sequencing. The retroviral construct was transiently transfected into Phoenix packaging cell line using the HBS/$Ca_3(PO_4)_2$ method (http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). Patient and control fibroblasts were infected 48 h later by exposure to virus-containing medium in the presence of 4 μg/ml of polybrene, as described.[2]

Blue-native and SDS-PAGE analysis

Blue-native PAGE was used for separation of the OXPHOS complexes on 6–15% polyacrylamide gradient gels.[9] Mitoplasts, which were prepared from fibroblasts by treatment with 0.8 mg of digitonin/mg of protein, were solubilized with 1% lauryl

maltoside, and 20 μg of the solubilized protein was used for electrophoresis.[10] Assembly of the OXPHOS complexes was investigated by immunoblot analysis using monoclonal antibodies against subunits of complexes II–V (Molecular Probes/Invitrogen, Carlsbad, CA, USA) and a polyclonal anti-ND1 antibody for complex I detection (a kind gift of A. Lombes, Paris).

One-dimensional 10% SDS-PAGE analysis was performed as described previously.[11] A polyclonal antibody directed against EFG1[12] and a monoclonal antibody against the 70 kDa subunit of complex II (Molecular Probes, Leiden, The Netherlands) were used for detection.

Pulse labeling of mitochondrial translation products

^{35}S labeling of mitochondrial translation was performed as described elsewhere.[13] In short, cells were labeled for 60 min at 37 ºC in methionine-free DMEM with 200 μCi/ml of a [^{35}S]-methionine and -cysteine mixture (Perkin Elmer, Woodbridge, ON, Canada) and 100 μg/ml emetine, and subsequently chased for 10 min in regular DMEM. Total cellular protein (50 μg) was resuspended in loading buffer containing 93 mM Tris–HCl (pH 6.7), 7.5% glycerol, 1% SDS, 0.25 mg bromophenol blue per ml and 3% mercaptoethanol, sonicated for 3–8 s, and run on 15–20% polyacrylamide gradient gels.

Results

Patient fibroblasts showed decreased enzyme activities of OXPHOS complexes I, III and IV, whereas complex II activity was normal (Table 1). Strikingly, skeletal muscle biochemistry revealed normal enzyme activities for OXPHOS complexes I and IV while complex III was only moderately decreased compared to the lowest reference range value. The capacity of the mitochondrial energy generating system, however, was clearly reduced in muscle tissue, indicated by impairments in pyruvate oxidation and ATP production rates (Table 2).

After excluding mutations in mtDNA and polymerase gamma (*POLG* and *POLG2*), the nine known mitochondrial translation factors (*MTIF2*, *MTIF3*, *TSFM*, *TUFM*, *GFM1*, *GFM2*, *MTRF1*, *MTRF1L*, and *MRRF*) were sequenced. This revealed a homozygous C to T transition at position 748 of the coding sequence of *GFM1* (Fig. 1A). The mutation was not present in any reported human EST or in 100 control subjects and was found heterozygous in both parents (Fig. 1A). This mutation is predicted to result in an Arg250Trp substitution in helix A of the G' subdomain, which is part of domain I of the EFG1 protein (Fig. 1B). Sequence alignment of EFG1 orthologs in several species shows that this amino acid is highly conserved from human to bacteria (Fig. 1C). Immunoblot analysis revealed a severe reduction in the steady-state level of EFG1 in patient fibroblasts (Fig. 1D). Figure 2 depicts how the Arg residue is positioned on the periphery of the protein and forms a hydrogen bond with Glu 153; this bond is lost when the Trp residue is introduced. Furthermore, a positive charge, which could be important for interactions between helix A and other proteins, is

lost due to the mutation. Additionally, the large and aromatic Trp side chain could interfere with these protein interactions.

To investigate whether the lack of functional EFG1 protein was responsible for the combined OXPHOS system defect, we transduced patient fibroblasts with a retroviral vector expressing the wild-type *GFM1* cDNA. Patient fibroblasts displayed markedly reduced levels of fully assembled OXPHOS complexes (Fig. 3B), corresponding to the deficiencies detected using enzyme activity assays (Table 1), as well as a clear impairment in mitochondrial protein synthesis (Fig. 3A). These phenotypes were rescued by over-expressing wild-type *GFM1* (Fig. 3A and B). The mitochondrial translation rate increased from 51% to 92% of control levels and the activity of complex IV was restored from 20% to 88% of the activity in control fibroblasts.

Discussion

A homozygous mutation in subdomain G' of translation elongation factor G1 (Arg250Trp) led to a combined OXPHOS deficiency and a clinical picture consistent with severe, rapidly progressive mitochondrial encephalopathy in our patient.

EFG1 is a GTPase that catalyzes the translocation step of mitochondrial protein synthesis, during which the deacylated tRNA moves from the ribosomal peptidyl (P) to the exit (E) site, the peptidyl-tRNA moves from the acceptor (A) to the P site and the mRNA is advanced by one codon, preparing for a new elongation cycle.[14] EFG1 consists of five domains; GTP-binding domain I (or G) contains a large insert, subdomain G', which is indirectly required for translocation. The Arg250Trp mutation replaces a highly conserved arginine residue by tryptophan in helix A of subdomain G', which possibly disturbs protein structure due to the loss of a stabilizing hydrogen bond and might hamper interaction of helix A with other proteins. The G' domain of bacterial EF-G has been demonstrated to interact with ribosomal protein L7/L12 (L7 and L12 refer to the *N*-acetylated and unacetylated forms of the same protein).[15,16] Multiple copies of L7/L12 together with protein L10 make up a peripheral stalk of the large ribosomal subunit; the end of the stalk contains the C-terminal domain (CTD) of L7/L12.[17] The CTD of L7/L12 promotes recruitment of EF-G to the ribosome, greatly stimulates its GTP hydrolysis activity and controls the release of inorganic phosphate.[17,18] Research by Nechifor and Wilson indicated that helix A of EF-G domain G' is directly involved in ribosome-activated GTP hydrolysis and thereby exerts indirect effects on translocation.[19] Mutating the conserved residue 224 or 228 in helix A of *Escherichia coli* EF-G (corresponding to amino acids 255 and 259 in human EFG1),

thus disrupting electrostatic interactions with L7/L12, resulted in severe defects in GTP hydrolysis in the presence of the ribosome (without effects on basal GTPase activity) and smaller defects in ribosomal translocation. Hence, the Arg250Trp mutation in EFG1 reported in the current study is presumed to hamper ribosome-dependent GTP hydrolysis, causing impairments in mitochondrial translation and oxidative phosphorylation and consequently fatal mitochondrial encephalopathy.

Previously, three other EFG1 defects have been described. The first patient harbored a homozygous mutation in the GTP-binding domain I (Asn174Ser),[20] and subsequent reports unveiled compound heterozygous missense and nonsense mutations affecting other domains of EFG1: Ser321Pro (on the boundary between domains I and II) + Leu607X (predicted to remove domain V)[12] and Met496Arg (in domain III) + Arg47X (predicted to eliminate basically all domains).[21] Mitochondrial translation was impaired in a similar manner in all patients: a severe overall decrease in the rate of mitochondrial translation with the expression of subunits ND5, ND6, COX I, II and III generally being the lowest. ND3 expression, on the contrary, was often increased compared with control levels. The previously reported patients all presented with OXPHOS enzyme deficiencies in muscle as well as fibroblasts, in contrast to our patient, in whom decreased enzyme activities were detected merely in fibroblasts. Remarkably, the muscular symptoms were relatively mild in all cases. Lowered respiratory chain activities in fibroblasts with normal values in muscle tissue is an uncommon finding in mitochondrial disorders, whereas the opposite is often observed. It should be noted that the capacity of the mitochondrial energy generating system was affected substantially in muscle tissue of our patient. The sensitivity of the assays used to determine this capacity, such as pyruvate oxidation, is much higher than of the

standard enzyme activity measurements.[22] However, the mechanism underlying the (near) normal enzyme activities in muscle is currently unclear. Adaptive processes (e.g. compensatory changes in the levels of other translation factors), which can differ quantitatively and qualitatively between tissues and patients, are likely involved.

Two of the previous reports on EFG1 defects described a very severe phenotype characterized by intrauterine growth retardation, microcephaly, hypertonicity and profound liver dysfunction, resulting in liver failure and death within the first weeks or months.[12,20] The clinical symptomatology in our patient is reminiscent of the somewhat milder phenotype described by Valente et al., which was characterized by feeding problems, axial hypotonia, increased limb muscle tone and infantile onset epilepsy.[21] Notably, liver dysfunction was absent, as in our patient. Whereas β-hydroxybutyrate in blood and urine were normal in our patient, Valente et al. reported increased levels. The biochemical findings appear to correlate with disease severity: our patient showed lower blood lactate levels (4.9 versus 17 mmol/l) and lactate:pyruvate ratios (22 versus 38) compared with the first reported EFG1 patient,[20] suggesting a more modest disturbance of mitochondrial functioning.

The patient investigated here was part of a cohort of 27 patients with combined OXPHOS deficiencies. Sequence analysis of all mitochondrial translation factors additionally revealed a homozygous mutation in elongation factor Ts (*TSFM*) in one patient: Arg333Trp, which has been reported before in two unrelated patients.[23] All three patients presented with extensive lactic acidosis and muscular hypotonia within 3 days after birth, displayed combined complex I, III and IV deficiencies in fibroblasts as well as muscle tissue, and died in the first few months of life. Whereas the mutation in *TSFM* was associated with either mitochondrial encephalomyopathy or hypertrophic

cardiomyopathy in the previously published cases, our patient was affected by both. Additionally, hepatomegaly was observed.

The current study evinces the importance of thorough diagnostic biochemical analysis of muscle tissue as well as fibroblasts in patients suspected to suffer from a mitochondrial disorder, since OXPHOS defects do not always manifest evidently in both tissues. Future research should elucidate the compensatory and regulatory mechanisms, at the mitochondrial, cellular and tissue levels, responsible for the differences in phenotypic presentation of mitochondrial disorders due to *GFM1* and other mitochondrial translation factor mutations.

Acknowledgements

This work was supported by the European Union's Sixth Framework Program, contract number LSHMCT-2004-005260 (MITOCIRCLE). We thank Benigna Geurts (Nijmegen Center for Mitochondrial Disorders) for performing sequence analysis of *GFM1* on control samples.

Conflict of interest

The authors declare no conflict of interest.

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Tables

Table 1: OXPHOS enzyme activities in skeletal muscle and skin fibroblasts

	Activities (mU/U citrate synthase) in			
	Skeletal muscle		Skin fibroblasts	
Complex	Patient	Reference range	Patient	Reference range
I	79	70 – 250	**34**	100 – 310
II	97	67 – 177	595	520 – 845
III	**2151**	2200 – 6610	**1038**	1320 – 2610
II+III	319	300 – 970	122	110 – 470
IV	1103	810 – 3120	**168**	680 – 1190

Samples were 600 g of supernatant of skeletal muscle or mitochondrial enriched fraction from fibroblasts.

Decreased activities as compared to controls are in bold.

Table 2: Pyruvate oxidation and ATP production rates in skeletal muscle

Substrate	Patient	Reference range
[1-^{14}C]pyruvate + malate	1.53 [a]	3.61 – 7.48 [a]
[1-^{14}C]pyruvate + carnitine	2.07 [a]	2.84 – 8.24 [a]
ATP+CrP from oxidation of pyruvate	13 [b]	42 – 81 [b]

CrP = phosphocreatine.

[a] nmol CO_2/hr · mU citrate synthase

[b] nmol ATP/hr · mU citrate synthase

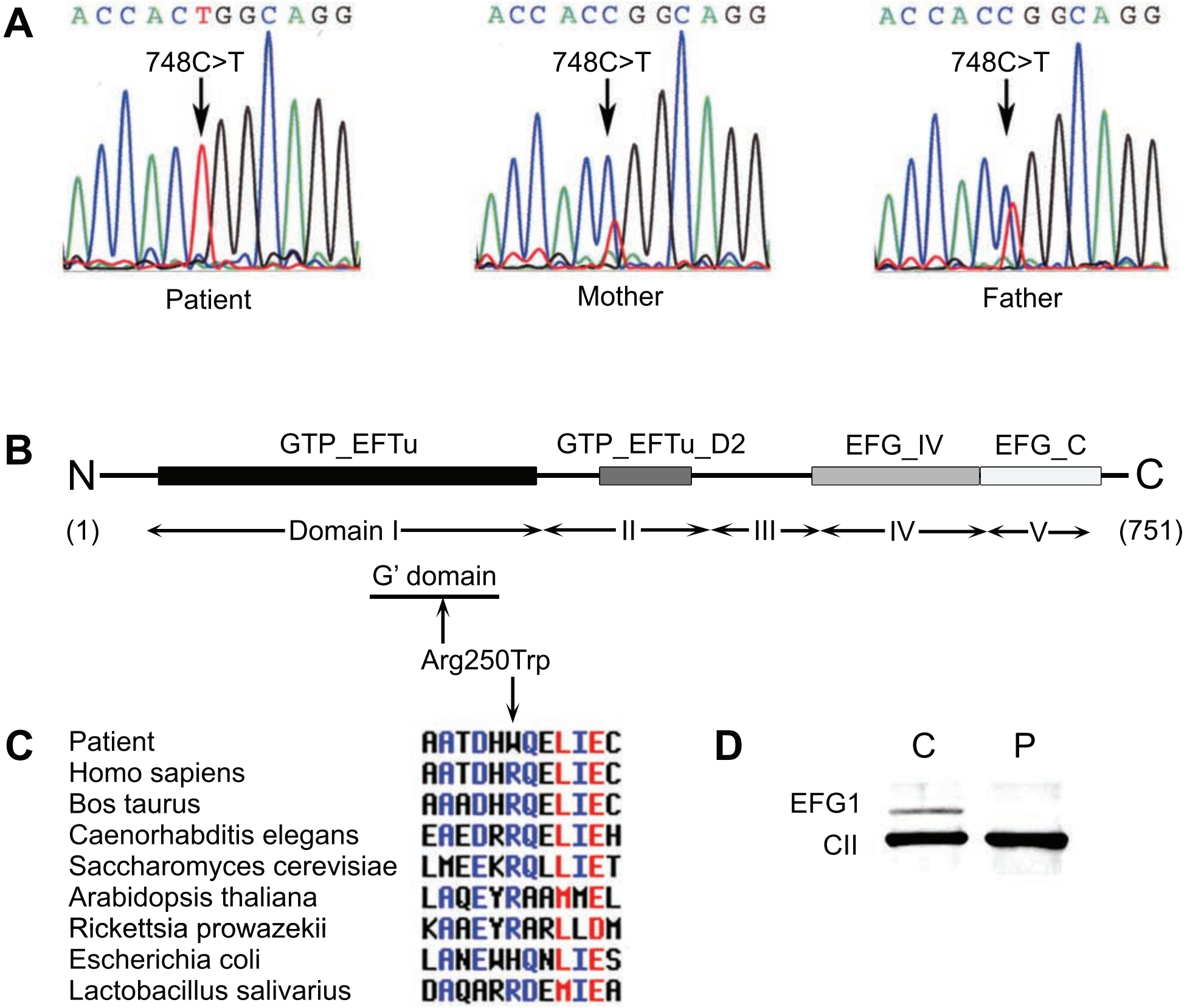
Titles and legends to figures

Figure 1: Mutational analysis of *GFM1*. (A) DNA sequence chromatogram from the patient (homozygous c.748C>T) and both parents (heterozygous). (B) EFG1 protein structure with the functional domains showing the predicted Arg250Trp substitution in the G' subdomain of domain I. (C) Protein sequence alignment from human to bacteria. The alignment shows that the mutated Arg250Trp is highly conserved among different species. (D) Immunoblot analysis in patient (P) and control (C) fibroblasts reveals the absence of the EFG1 protein. The 70 kDa subunit of complex II (CII) was used as a loading control.

Figure 2: Computational modeling based on the crystal structure of the *T. thermophilus* EFG protein. The left panel shows the structure of the entire human EFG1 protein and the right panel is a close-up of the outlined region containing the mutation. The wild-type Arg 250 residue is depicted in green and the mutant Trp is depicted in red (bound GDP is depicted in yellow). The right panel shows that the Trp residue fits in the structure, however, the hydrogen bond that Arg forms with Glu 153 is lost.

Figure 3: Rescue of the mitochondrial translation defect and combined OXPHOS deficiency in patient fibroblasts by expression of *GFM1*. Control (C) and patient (P) fibroblasts were transduced with a retroviral vector expressing the wild-type *GFM1* cDNA and analyzed by a mitochondrial translation assay (A) and BN-PAGE (B). The mitochondrial translation products are indicated on the left: seven subunits of complex I

(ND), one subunit of complex III (cyt b), three subunits of complex IV (COX) and two subunits of complex V (ATP). Co I – Co V = complex I through V.


A
ACCACTGGCAGG
748C>T
Patient
ACCACCGGCAGG
748C>T
Mother
ACCACCGGCAGG
748C>T
Father
B
GTP_EFTu
GTP_EFTu_D2
EFG_IV
EFG_C
N
C
(1)
Domain I
II
III
IV
V
(751)
G' domain
Arg250Trp
C
Patient AATDHWQELIEC
Homo sapiens AATDHRQELIEC
Bos taurus AAADHRQELIEC
Caenorhabditis elegans EAEDRRQELIEH
Saccharomyces cerevisiae LMEEKRQLLIET
Arabidopsis thaliana LAQEYRAAMMEL
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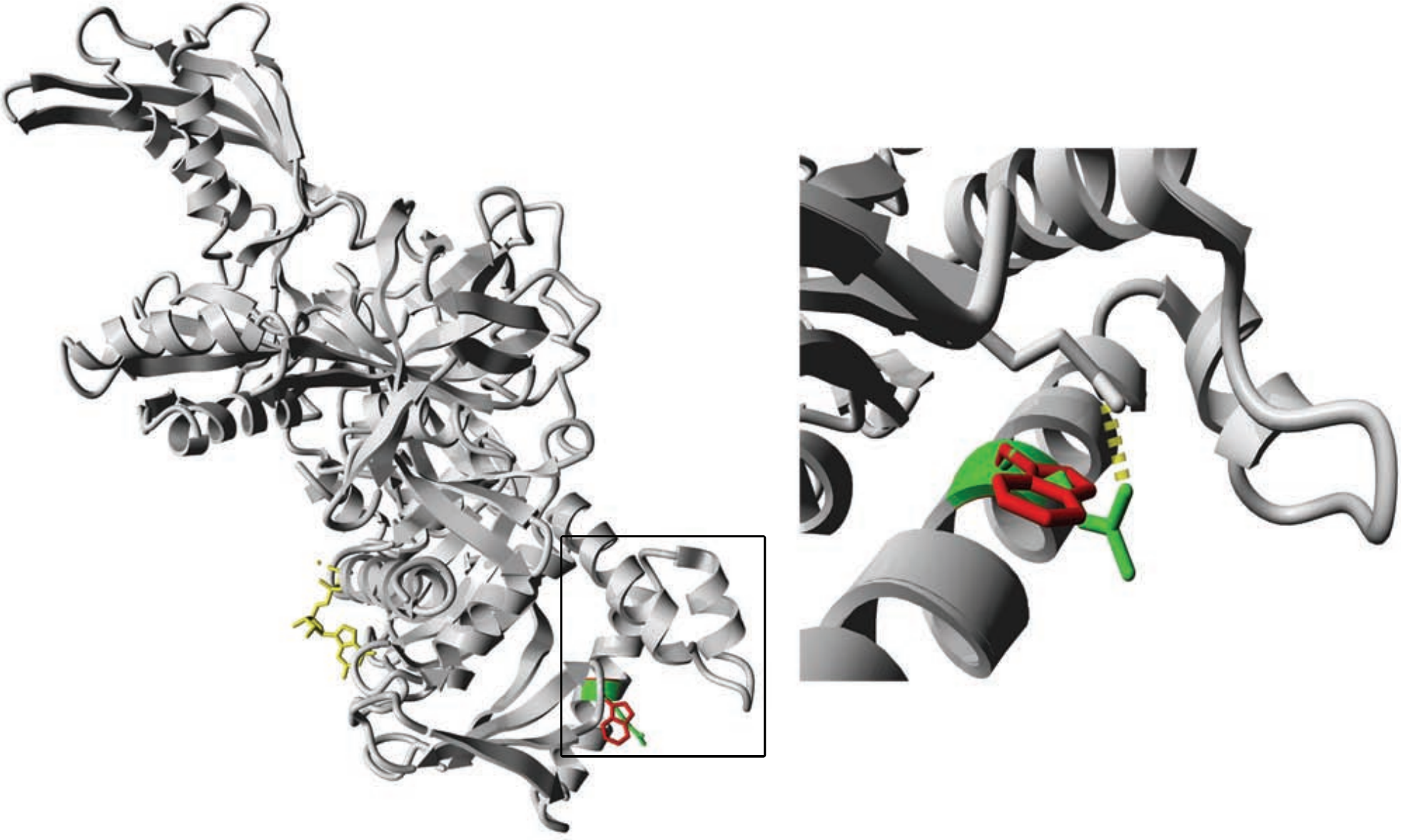

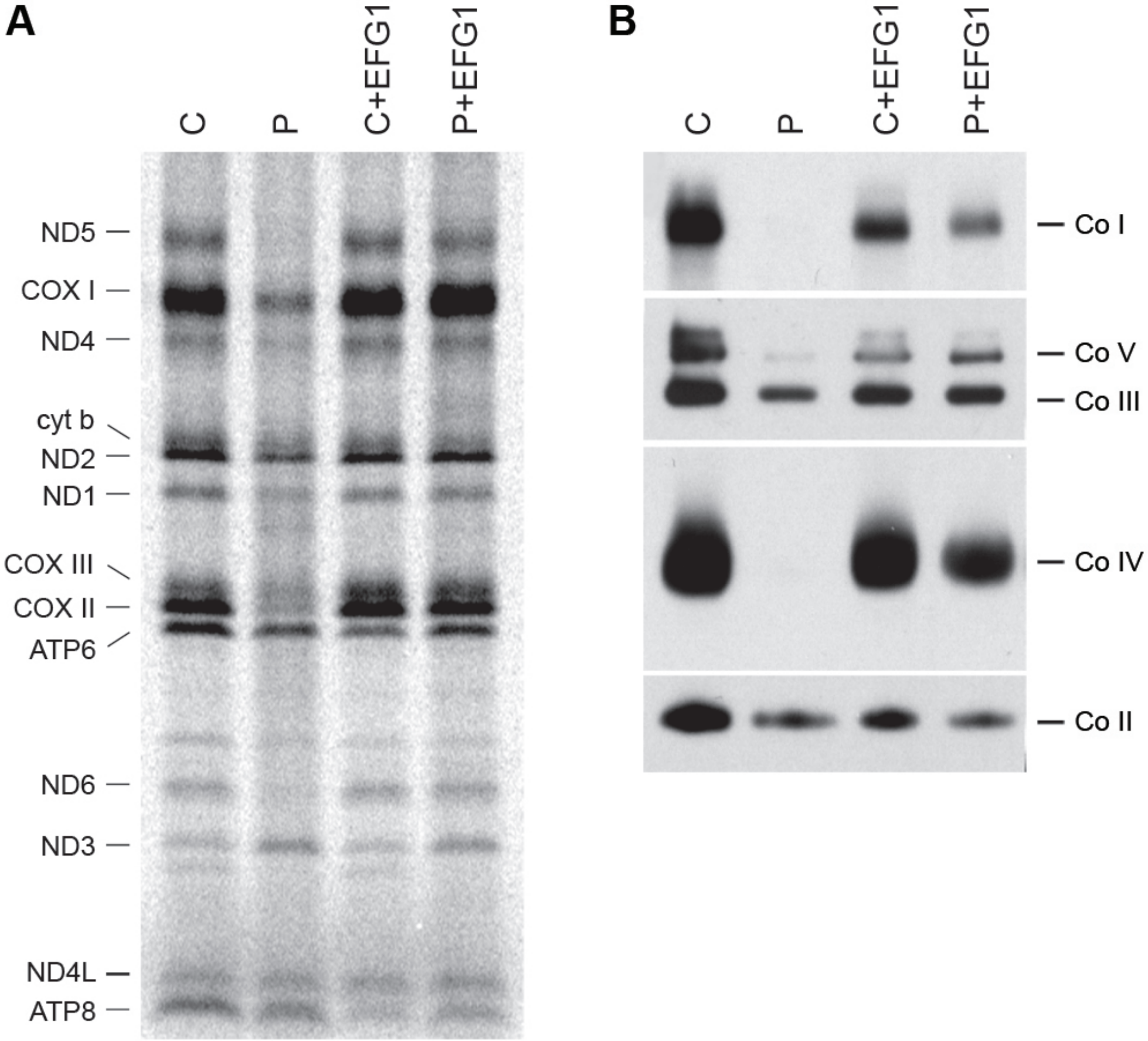
A
C
P
C+EFG1
P+EFG1
ND5
COX I
ND4
cyt b
ND2
ND1
COX III
COX II
ATP6
ND6
ND3
ND4L
ATP8
B
C
P
C+EFG1
P+EFG1
Co I
Co V
Co III
Co IV
Co II