Biallelic *PPA2* Mutations Cause Sudden Unexpected Cardiac Arrest in Infancy

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Sudden unexpected death in infancy occurs in apparently healthy infants and remains largely unexplained despite thorough investigation. The vast majority of cases are sporadic. Here we report seven individuals from three families affected by sudden and unexpected cardiac arrest between 4 and 20 months of age. Whole-exome sequencing revealed compound heterozygous missense mutations in *PPA2* in affected infants of each family. *PPA2* encodes the mitochondrial pyrophosphatase, which hydrolyzes inorganic pyrophosphate into two phosphates. This is an essential activity for many biosynthetic reactions and for energy metabolism of the cell. We show that deletion of the orthologous gene in yeast (*ppa2Δ*) compromises cell viability due to the loss of mitochondria. Expression of wild-type human *PPA2*, but not *PPA2* containing the mutations identified in affected individuals, preserves mitochondrial function in *ppa2Δ* yeast. Using a regulatable (doxycycline-repressible) gene expression system, we found that the pathogenic *PPA2* mutations rapidly inactivate the mitochondrial energy transducing system and prevent the maintenance of a sufficient electrical potential across the inner membrane, which explains the subsequent disappearance of mitochondria from the mutant yeast cells. Altogether these data demonstrate that *PPA2* is an essential gene in yeast and that biallelic mutations in *PPA2* cause a mitochondrial disease leading to sudden cardiac arrest in infants.

Sudden unexpected death in infancy (SUDI) remains the most common cause of post-neonatal infant mortality in the developed world.[1] Although the causes of SUDI remain largely inconclusive, next-generation sequencing has been used to identify genetic factors, especially in genes associated with cardiac disease.[2] At least one in five SUDI victims carries potentially damaging variations in genes encoding cardiac ion channels and in cardiomyopathy-associated genes.[3] Mitochondrial fatty acid oxidation disorders may also present as sudden infant death.[4] The role of polymorphisms in genes involved in regulation of the immune system, serotoninergic function, or brain maturation that might predispose infants to death in critical situations is more controversial.[5] SUDI remains a diagnosis of exclusion and monogenic causes with or without recurrence in siblings are rare.

Here, we report seven children from three independent families (F1, F2, and F3) who died of cardiac arrest, despite extensive attempts at resuscitation, between 4 and 20 months of age (Table 1; Figure 1A; Supplemental Case Reports). There was recurrence of SUDI in families F1 and F2 with 4/5 and 2/2 affected children, respectively. Family F3 consisted of one simplex case diagnosed with a myopathy prior to cardiac arrest. All parents were healthy although the mother in family F1 and one of her brothers had been diagnosed with Brugada syndrome (MIM: 601144). There was no known case of SUDI in relatives. All affected children were born at term, after normal pregnancy and delivery, with normal birth parameters. In families F1 and F2, growth parameters and psychomotor milestones were in the normal range although some children in family F1 were considered clumsy (Table 1; Supplemental Case Reports). Arrhythmia was never recorded before death. The simplex case from family F3 presented with hypotonia, feeding difficulties, and failure to thrive at 3 months of age. Myopathy was suspected and a muscle biopsy was performed 1 month before she was admitted to an intensive care unit (ICU). Hypertrophic

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Table 1. Clinical Data of Affected Individuals and *PPA2* Genotypes for Families F1, F2, and F3

Families	**F1**					**F2**			**F3**	
Individuals	**II:1**	**II:3**		**II:4**	**II:5**	**II:1**		**II:2**	**II:1**	
Gender	**M**	**F**		**F**	**M**	**F**		**F**	**F**	
Milestones	walking aided at 12 months	walking aided at 13 months		sitting unaided at 9 months	sitting at 6 months, walking unaided at 10 months	walking unaided at 16 months		no initial concerns	hypotonia, failure to thrive, growth delay	
Age at death	14 months	14 months		15 months	14 months	20 months		4 months	4 months	
Diagnosis at death	cardiac arrest	asystole		bradycardia asystole	asystole	bradycardia		bradycardia asystole	heart failure	
Histology of cardiac muscle at necropsy	NA	myocarditis		massive circumferential fibrosis of myocardium, moderate inflammatory infiltrate	focal and discrete lesions of myocardic fibers and inflammatory infiltrate on the posterior wall of the left ventricle with no fibrosis	focal myocarditis with moderate inflammatory infiltrate, some necrosis in left ventricle. lesions of different ages		inflammatory infiltrate of pericardium, myocardium, and endocardium; lipid accumulation; and necrosis of myocytes (all lesions were recent)	hypertrophic cardiomyopathy, lipid accumulation	
Other findings at necropsy/biopsy	NA	none		none	none	none		fatty deposits in muscle, liver, kidney	lipid accumulation and nemaline bodies in skeletal muscle	
PPA2 variations	NA	c.182C>T	c.881A>C	c.182C>T, c.881A>C	c.182C>T, c.881A>C	c.280A>G	c.514G>A	c.280A>G, c.514G>A	c.318G>T	c.514G>A
Protein change	NA	p.Ser61Phe	p.Gln294Pro	p.Ser61Phe, p.Gln294Pro	p.Ser61Phe, p.Gln294Pro	p.Met94Val	p.Glu172Lys	p.Met94Val, p.Glu172Lys	p.Met106Ile	p.Glu172Lys
ExAC browser allele frequency (March 2016)	NA	1/120,034	absent	see F1.II:3	see F1.II:3	absent	59/120,800 (0 hom) (rs146013446)	see F2.II:1	absent	59/120,800 (0 hom) (rs146013446)
Score prediction PolyPhen2/SIFT	NA	0.956/0	0.430/0.01	see F1.II:3	see F1.II:3	0.950/0.07	0.996/0	see F2.II:1	0.930/0.05	0.996/0

Abbreviations are as follows: NA, not available; M, male; F, female; hom, homozygote.

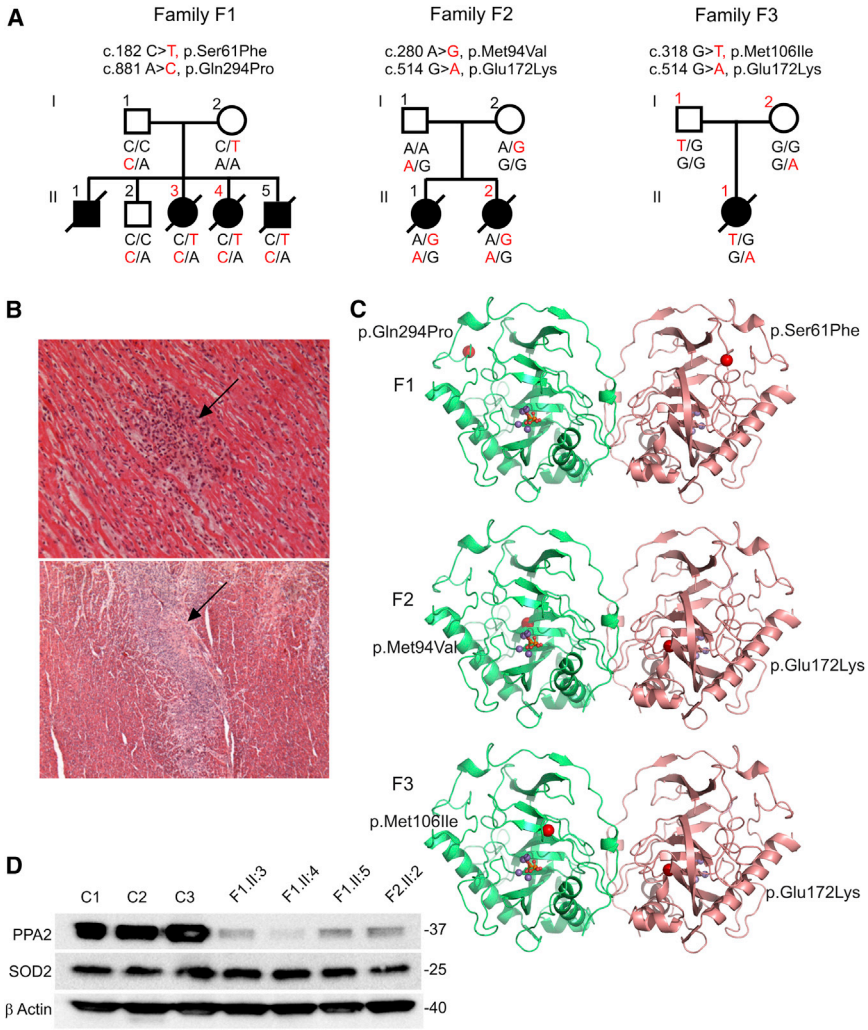

Figure 1. *PPA2* Mutations Identified in Affected Individuals from Three Families

(A) Pedigrees of families F1, F2, and F3. *PPA2* variants are reported according to GenBank: NM_176869.2. Individuals submitted to exome sequencing are indicated in red.

(B) Cardiac muscle histology (HES staining) of affected individuals. Top: F2.II:1. Magnification ×10. Arrow shows focal infiltrate of inflammatory cells and cardiomyocyte necrosis. Bottom: F2.II:2. Magnification ×2.5. Arrow shows fibrosis and inflammatory cells.

(C) Dimeric hPPA2 protein structure homology model. Views of the dimer drawn as a ribbon for each family (F1, F2, and F3). Each mutated residue is shown as a red sphere and is located on different monomers colored in green and pink. The phosphate groups (orange and red) and the four Mg^{2+} ions (purple) are drawn in ball and stick mode in the active sites. The homology model of dimeric hPPA2 is based on the 2.3 Å resolution structure of the inorganic pyrophosphatase from *Schistosoma japonicum* (PDB: 4QLZ) that shares 55% identity with hPPA2. It was built using Phyre2[25] and drawn using Pymol (DeLano WL, DeLano Scientific).

(D) hPPA2 protein steady-state level is decreased in affected individuals' fibroblasts compared to control subjects. Western blotting analysis of total protein extracts from cultured skin fibroblasts of three different age-matched control subjects (C1, C2, and C3) and affected individuals, using antibodies directed against PPA2 (Abcam cat# ab177935, 1/2,000), SOD2 (a mitochondrial matrix protein, Abcam cat#125702, 1/100,000), and β Actin (Santa Cruz Biotechnology cat# sc-81178; RRID: AB_2223230) as loading control.

cardiomyopathy with heart failure was then diagnosed and individual F3.II:1 died of cardiac arrest in the ICU (Table 1; Supplemental Case Reports). Bacterial and viral screening and testing for fatty acid oxidation deficiency in fibroblasts and/or by acylcarnitine profiling failed to provide a diagnosis for all affected infants.

Histology of the heart muscle (performed for all individuals except F1.II:1) showed features of myocarditis with polymorphic and non-specific inflammatory infiltrate (consisting of mononuclear cells, predominantly T lymphocytes), necrosis, and/or fibrosis of variable severity (Figure 1B; Table 1). In addition, fat deposits were observed in the heart, skeletal muscle, and kidney of individual F2.II:2. Lipid accumulation was also noted in heart muscle of individual F3.II:1, associated with nemaline rods in the skeletal muscle biopsy (Table 1).

Owing to the rarity of recurrence of SUDI and similarities in the clinical presentation in families F1 and F2, autosomal-recessive inheritance with mutations in the same gene was hypothesized and whole-exome sequencing (WES) was performed on individuals II:3 and II:4 from family F1 and on II:2 from family F2, in accordance with approved institutional protocols (Comité de Protection des Personnes Ile-de-France II) and with informed consent from the parents for genetic testing. DNA was extracted from leucocytes. Agilent SureSelect libraries were prepared from 3 μg of genomic DNA sheared with a Covaris S2 Ultrasonicator as recommended by the manufacturer. Exome capture was performed with the 51 Mb SureSelect Human All Exon kit V4 (Agilent Technologies). Sequencing of the SureSelect exome libraries was carried out with the SOLiD5500 instrument (Life Technologies). 75+35 paired-end reads were mapped on the human genome reference (NCBI build37/hg19 build) using LifeScope (Life Technologies). SNPs and indel calling was made using GATK tools. The mean depth of coverage obtained for each sample was >83× with >87% of the exome covered at least by 15×. An in-house software (PolyWeb) was used to annotate and filter the variants. Variant filtering strategies (Table S1) led to the identification of

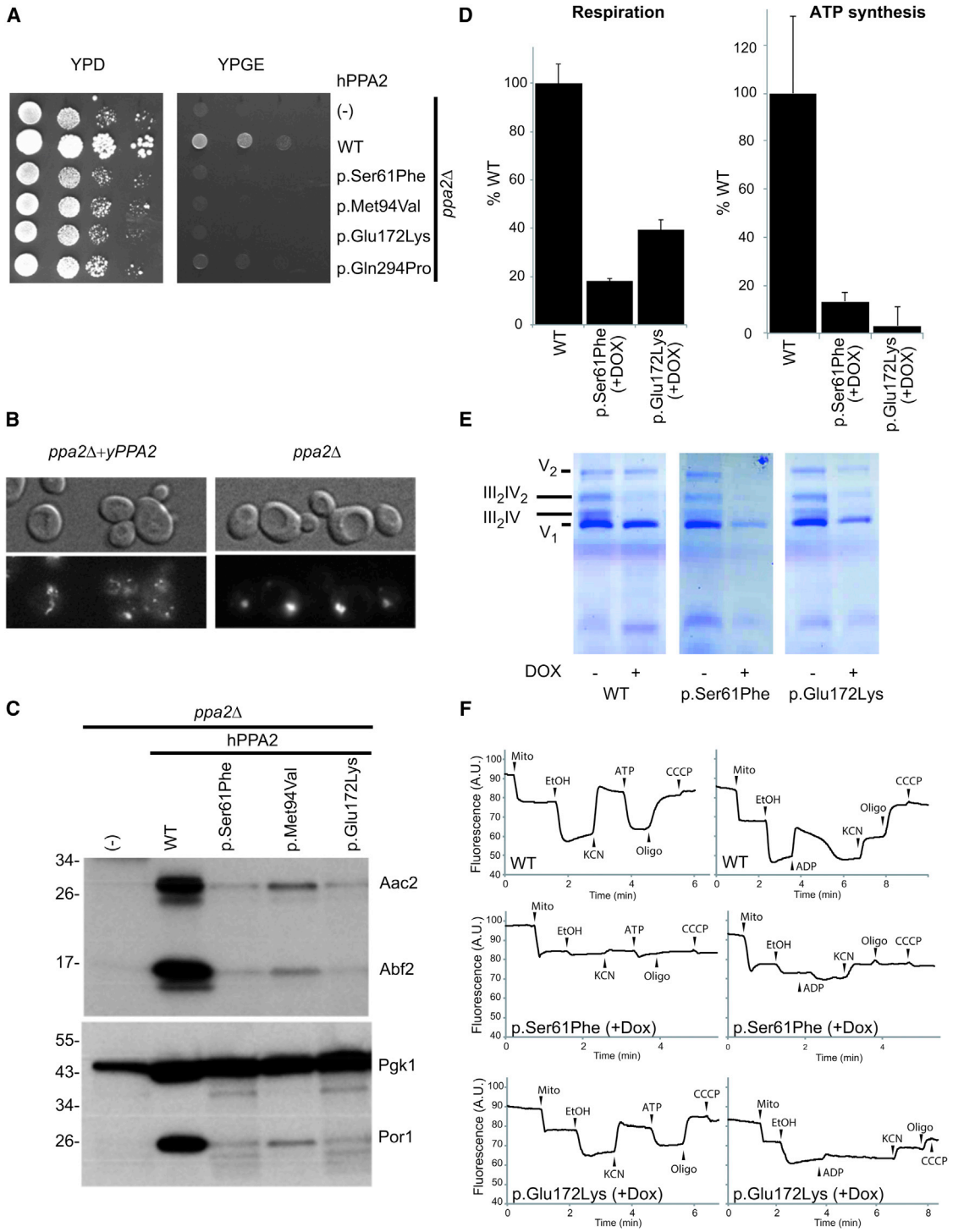

Figure 2. Functional Complementation Assays and Primary Consequences of the *PPA2* Mutations in Yeast

(A–C) Functional complementation assay of a yeast *ppa2Δ*-null mutant.

(A) Growth of *ppa2Δ* was tested either on glucose (YPD) or glycerol + ethanol medium (YPGE). The *ppa2Δ* strain containing a plasmid expressing wild-type y*PPA2* from its own promoter was transformed with either wild-type *hPPA2* (WT), *hPPA2* mutant cDNAs, or the corresponding empty plasmid (−), under the control of the constitutive *TPI* promoter. After 5-FOA treatment (as previously described[14]) to eliminate the plasmid encoding yPPA2, cells expressing only wild-type (WT) or mutant hPPA2 were spotted onto YPD or YPGE plates. Drop dilution growth tests were performed at 1/10 dilution steps and yeast were incubated on YPD or YPGE plates for 2 days at 28°C.

(B) DAPI staining of *ppa2Δ* cells transformed either with *yPPA2* or the corresponding empty plasmid.

(C) Western blots demonstrating expression of Por1 (mitochondrial outer membrane protein, monoclonal anti-porine [Invitrogen]), Aac2 (mitochondrial inner membrane ADP/ATP translocator, polyclonal anti-Aac2), Abf2 (mitochondrial matrix DNA-binding protein, polyclonal anti-Abf2), and the cytosolic protein Pgk1 (cytosolic phosphoglycerate kinase, monoclonal anti-Pgk1 [Invitrogen]) in total

(legend continued on next page)

rare, compound heterozygous missense variations in *PPA2* (pyrophosphatase [inorganic] 2 [MIM: 609988, GenBank: NM_176869.2]) in the affected individuals from families F1 and F2 (c.182C>T [p.Ser61Phe] and c.881A>C [p.Gln294Pro] in family F1; c.280A>G [p.Met94Val] and c.514G>A [p.Glu172Lys] in family F2) (Figure 1A; Table 1). The c.182C>T (p.Ser61Phe) variant has been reported at a frequency of 1/120,034 alleles in the Exome Aggregation Consortium (ExAC) database and c.514G>A (p.Glu172Lys) corresponds to rs146013446 with an allele frequency of 0.0005 in ExAC. Neither has been reported in the homozygous state. The other two variants have not been reported in public databases. WES was performed independently on the family F3 trio as described previously.[6] After filtering, 854 rare variants remained in the proband. Because no variants were identified in any of the known nemaline myopathy (MIM: 609284) genes, a trio analysis was subsequently performed and revealed three genes with compound heterozygous variants inherited in *trans*: *ABCA4* (MIM: 601691), *DNAH1* (MIM: 603332), and *PPA2*. Mutations of *ABCA4* cause cone-rod dystrophy 3 and retinal dystrophy, so *ABCA4* was not a good candidate for the disease in this family. *DNAH1* was also discarded because one of the variants is rs112505934 with a frequency of 0.006 and four homozygous individuals have been reported in ExAC. The GeneMatcher website was used to identify *PPA2* as the disease-associated gene in common between families F1, F2, and F3. *PPA2* variants identified in family F3 were rs146013446 (c.514G>A [p.Glu172Lys]) previously found in family F2 and c.318G>T (p.Met106Ile) variant absent from ExAC. All variations were confirmed by Sanger sequencing (Figure S1) and co-segregated as expected for autosomal-recessive inheritance (Figure 1A). Each of the five missense variants led to a substitution that was predicted damaging or deleterious by PolyPhen-2 and/or SIFT (Table 1) and 3/5 of the affected amino acids were conserved in the yeast *PPA2* ortholog (Figure S2). The p.Met94Val, p.Met106Ile, and p.Glu172Lys substitutions fall within the pyrophosphatase domain (Figures 1C and S2).

Inorganic pyrophosphatases (PPases) are ubiquitous enzymes catalyzing the hydrolysis of inorganic pyrophosphate (PPi) into two molecules of orthophosphate (Pi). This is a highly exergonic enzymatic reaction that provides a thermodynamic pull for many biosynthetic reactions, such as DNA, RNA, protein, polysaccharide, and lipid synthesis, and also has an important role in energy metabolism.[7,8] In humans, the mitochondrial PPase (hPPA2) shares 62% amino acid identity with the cytosolic hPPA1 protein and has an N-terminal 31-amino acid mitochondrial import signal[9] (Figure S2). We built a structural homology model of hPPA2 based on the crystal structure of the most homologous PPase (from *Schistosoma japonicum*), which exists as a dimeric unit as for other PPases[10] (Figures 1C and S2). The altered residues in families F2 and F3 map close to the PPi processing site, whereas those in F1 are more remotely located, near the surface of the protein. All the substitutions are predicted to generate unfavorable steric or electrostatic interactions leading to destabilization/misfolding of hPPA2. Consistent with this, we observed a large decrease of hPPA2 protein steady-state levels in affected individuals' fibroblasts compared to control subjects, whereas the amount of SOD2, a mitochondrial matrix protein, was similar to control subjects (Figure 1D).

The effect of the four missense mutations identified in families F1 and F2 were subsequently studied in yeast, which is a convenient system for modeling mitochondrial disease mechanisms.[11] The mitochondrial yeast Ppa2 protein shares 44% identity with hPPA2 (Figure S2). Consistent with previous studies,[12] we observed that a null mutation in the corresponding gene (*ppa2Δ*) abolished the capacity of yeast to grow on non-fermentable substrates (e.g., glycerol and ethanol) (Figures 2A and S3) and led rapidly to cell populations mostly lacking mitochondrial DNA (rho^0 cells) (Figure 2B). Additionally, we found that even though *ppa2Δ* cells grew on fermentable medium,

extracts of *ppa2Δ* cells transformed with empty plasmid (−) or wild-type (WT) or mutant *hPPA2* cDNA constructs. Protein extraction and western blotting were performed as previously described.[26]

(D–F) Bioenergetic investigations. To overcome the problem of mitochondrial DNA (mtDNA) instability in *ppa2Δ* yeast (B), we constructed *ppa2Δ* yeast cells co-expressing wild-type *hPPA2* under the control of a doxycycline-repressible (Tet-Off) promoter (pCM189)[15] and one of the mutated forms: p.Ser61Phe or p.Glu172Lys under the control of the constitutive promoter *TPI* (pYX142 plasmid). After blocking the expression of the wild-type *hPPA2* with doxycycline, only the mutated form is expressed. The strain referred to as WT contains only the regulatable wild-type *hPPA2*. The consequences of the mutations on mitochondrial function can then be assessed while the cells still contain functional mtDNA (Table S3). The cells were grown in 2 L of rich glycerol/ethanol medium until OD = 1 and then supplemented with 5 mM doxycycline. 12 hr later, by which time the source of newly synthesized PPA2 was totally drained or supplied by only one of the *hPPA2* mutants, the cells were harvested and their mitochondria isolated.[27]

(D) State 3 respiration (in the presence of an excess of external ADP, phosphorylating conditions) and ATP synthesis, expressed as percent of the WT (see Figure S4 and Table S3 for details). Values represent mean ± SD.

(E) BN-PAGE analyses. Experiments were performed as previously described.[28] Mitochondrial proteins were solubilized with digitonin (2 g/g protein), separated in a non-denaturing 3%–13% gradient polyacrylamide gel, and stained with Coomassie blue. The figure shows the results obtained with mitochondria extracted from cells grown in the absence (−) or presence (+) of 5 mM doxycycline (DOX).

(F) Mitochondrial membrane potential (ΔΨ) analyses. Energization of the mitochondrial inner membrane was monitored by rhodamine 123 fluorescence quenching with intact mitochondria using a SAFAS (Monte Carlo, Monaco) fluorescence spectrophotometer.[29] The additions were 0.5 μg/mL rhodamine 123, 0.15 mg/mL mitochondrial proteins (Mito), 1% (V/V) of ethanol (EtOH) (electron donor), 6 μg/mL oligomycin (oligo) (ATP synthase proton channel inhibitor), 0.2 mM potassium cyanide (KCN) (complex IV inhibitor), 50 μM ADP, 1 mM ATP, and 3 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone, membrane uncoupler).

they did so rather poorly and eventually died quite rapidly (Figures 2A and S3). Furthermore, they were largely depleted in proteins from different mitochondrial compartments (Figure 2C) and showed very poor staining with DASPMI (2-(4-dimethylaminostyryl)-1-methylpyridinium iodide), a fluorescence probe sensitive to the mitochondrial membrane potential (Figure S3). These data indicate that yeast *PPA2* (*yPPA2*) is crucial for the maintenance of mitochondria.

Subsequently, *hPPA2* cDNA was cloned into pCRII-topo using primers PPA2-cDNA-Fwd and PPA2-cDNA-Rev (Table S2) and point mutations were generated in pCRII-topo-wt-hPPA2 by site directed mutagenesis using primers: PPA2-c.182C>T-S and PPA2-c.182C>T-AS, PPA2-c.280A>G-S and PPA2-c.280A>G-AS, PPA2-c.514G>A-S and PPA2-c.514G>A-AS and PPA2-c.881A>C-S and PPA2-c.881A>C-AS (Table S2). Direct Sanger sequencing confirmed that the mutations were introduced successfully. Human wild-type and mutant *PPA2* cDNAs were cloned into the yeast expression vector pYX142, with expression driven by the constitutive *TPI* promoter.[13] Using a plasmid shuffling system,[14] we showed that wild-type *hPPA2* was able to rescue the growth defect of *ppa2Δ* yeast, demonstrating that it is a true functional ortholog of *yPPA2* (Figures 2A and S3). In contrast, none of the tested mutant *hPPA2* forms was able to rescue growth and mitochondrial function in *ppa2Δ* yeast (Figures 2A, 2C, and S3), indicating that each mutation disrupts the function of hPPA2.

To determine very early consequences of the *hPPA2* mutations in yeast (i.e., after 3–4 generations, well before the loss of the mitochondrial genome), we generated *ppa2Δ* yeast co-expressing *hPPA2* under the control of a doxycycline repressible (Tet-off) promoter[15] and one of the *hPPA2* mutants expressed constitutively (Figures 2D–2F). Using this system, 12 hr after blocking expression of wild-type *hPPA2*, we observed that expression of the mutant forms resulted in a significant decrease (60%–80%) in oxygen consumption (Figures 2D and S4, Table S3), owing to a reduced content in complexes III and IV (Figure 2E). As a result, the rate of mitochondrial ATP synthesis was also decreased (Figure 2D). We additionally observed that the mutants prevented the maintenance of an electrical potential ($\Delta\Psi$) across the mitochondrial inner membrane (Figures 2F and S5). This latter defect explains why the mutant cells failed to maintain mitochondria upon long-term culturing, since a minimal $\Delta\Psi$ is required for mitochondrial biogenesis.[16,17]

The present study reports recessive missense mutations in *PPA2* encoding the mitochondrial inorganic pyrophosphatase as a cause of sudden unexpected cardiac arrest in seven infants from three independent families. A mitochondrial disease was unexpected in F1 and F2 because there were no clinical or biological signs of mitochondrial dysfunction. Only individual II:1 from family F3 presented with myopathy and further developed a cardiomyopathy rapidly followed by cardiac arrest at 4 months of age. Nemaline rods were found in her skeletal muscle but no mutations were identified in any known nemaline myopathy-associated gene (Supplemental Case Reports). Such skeletal muscle inclusions also occur under conditions of stress, e.g., in individuals with mitochondrial disease, haemodialysis, or HIV infection.[18,19]

Using the yeast model, we provide strong evidence that the identified mutations are extremely detrimental to mitochondrial maintenance and function. Our data indicate that the loss of mitochondria in *ppa2Δ* yeast, which became obvious after 20–30 generations, most likely resulted from an inability of the cell to maintain a sufficient electrical potential across the inner mitochondrial membrane owing to combined deficiencies in respiration and adenine nucleotide exchange across this membrane. Indeed, simultaneous inhibition of both activities has been shown to be sufficient to collapse $\Delta\Psi$ and induce cell death.[20–22] Mitochondrial function was much less compromised in fibroblasts from affected individuals, as evidenced by SOD2 levels (Figure 1D) and respiratory chain enzyme activity measurements (Table S4). However, citrate synthase activity, considered as a marker of mitochondrial mass in cells, appeared decreased, as did complex III and IV activities (Table S4), suggesting that mitochondrial proliferation was not optimal. It is worth noting that the partitioning of mitochondria during mitosis is very different in human and yeast. Indeed, whereas a newly formed human cell inherits about half of the mitochondria present in its mother cell, a yeast bud receives only a tiny portion of mitochondria, which may explain why the loss of the organelle was more rapid in yeast than in human *PPA2*-deficient cells. Considering the high-energy demands of cardiomyocytes,[23] our results may explain why cardiac arrest was unresponsive to early and optimal resuscitation in these infants. Though further study will be required to fully understand the pathogenesis of *PPA2* mutations, we suggest that a progressive poisoning of the heart by PPi beyond a certain threshold could be at the origin of the sudden cardiac arrest. Indeed, abnormally high concentrations of PPi result in the competitive inhibition of the mitochondrial ADP/ATP translocase.[24] As a result, mitochondria can no longer provide energy to the cell. The inability of the yeast strains expressing the mutant hPPA2 to energize properly the mitochondrial inner membrane is consistent with the hypothesis of a defective exchange of adenine nucleotides across this membrane.

In conclusion, we have shown that *PPA2* is an essential gene and that biallelic loss-of-function mutations lead to sudden cardiac arrest with or without preexisting signs of skeletal/cardiac muscle dysfunction. Sudden death by cardiac arrest is an undescribed clinical presentation of mitochondrial disease for which genetic counselling in sibships may be particularly difficult. The extent to which unexplained sudden cardiac arrest results from energetic metabolism anomalies remains to be determined.

Supplemental Data

Supplemental Data include case reports, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.06.021.

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Web Resources

Clustal W and Clustal X, http://www.clustal.org/clustal2/
ESPript 3, http://espript.ibcp.fr/ESPript/ESPript/
ExAC Browser, http://exac.broadinstitute.org/
GenBank, http://www.ncbi.nlm.nih.gov/genbank/
GeneMatcher, https://genematcher.org/
OMIM, http://www.omim.org/
Phyre2, http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
RCSB Protein Data Bank, http://www.rcsb.org/pdb/home/home.do
SIFT, http://sift.bii.a-star.edu.sg/

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