Supporting Online Material

Materials and Methods

Kindred 129. Kindred 129 is Caucasian and was ascertained via an index case presenting with fatigue and palpitations. We recruited all members of the extended kindred who were available and willing to participate. These included 142 blood relatives comprised of 31 children (age < 18), 111 adults, 81 females and 61 males. Informed consent was obtained from all participants and the study protocol was approved by the Yale Human Investigation Committee. Subjects provided a medical history, access to medical records, and underwent a limited physical examination. Body mass index (kg/m^2) was measured in 101 adults. Venous blood samples were collected from kindred members for DNA extraction and measurement of serum electrolytes (141 subjects). Fasting glucose, insulin, triglycerides, total LDL and HDL cholesterol was measured in 95 adults and 23 children. Seven members of the maternal lineage and 1 member of the non-maternal lineage were taking lipid lowering agents. Plasma renin activity, direct renin and aldosterone was measured after 15 minutes supine in 80 adults and 12 children. Twenty-four hour urine samples were collected from 64 kindred members for measurement of urinary electrolytes and creatinine.

Blood pressures were measured in a standardized fashion by a single physician participating in the study who was blinded to genealogy and clinical data (n = 94) or were obtained by the patient's personal physician (n = 30); a qualitative diagnosis of normotension was obtained from medical records in 10 additional subjects. Blood pressures were measured 3 times at 5 minute intervals in the recumbent position after 15

minutes rest using a mercury sphygmomanometer. The average of the last two measurements was used as the patient's quantitative blood pressure. In a subset of 40 subjects, blood pressure measurements were repeated one year after the first visit; the blood pressures of the two visits were highly correlated ($r = 0.75$ and 0.56 for systolic and diastolic blood pressures, respectively). Individuals taking medications for the treatment of hypertension or having blood pressure > 140/90 mm Hg were classified as hypertensive. Twenty-four subjects on the maternal lineage and five subjects on the non-maternal lineage were taking antihypertensive medication. For these individuals, blood pressure values prior to initiation of treatment were used where possible, but blood pressures on medication were used if these were not available.

The homeostasis model assessment (HOMA) was used as a measure of insulin resistance and was calculated as: HOMA = fasting insulin (μU/ml) X fasting glucose (mmol/l)/22.5.

Serum and urinary electrolytes, glucose and creatinine, as well as serum total cholesterol, LDL, HDL and triglycerides were measured in the clinical laboratory of Yale New Haven Hospital. Insulin was measured by radioimmunoassay in the core laboratory of the Yale General Clinical Research Center. Plasma renin activity was measured by radioimmunoassay for angiotensin I generation, direct renin was measured by immunochemiluminescence and serum aldosterone was measured by radioimmunoassay by Nichols Labs.

Statistical comparisons of qualitative traits between groups were performed by chi-square tests; quantitative traits were compared by two-tailed student's t-tests for electrolytes, which do not show significant age and sex dependence. Statistical analysis

of blood pressure, lipid levels, glucose and HOMA was performed by separately fitting regression models for the maternal and non-maternal lineages using age, sex, and BMI as explanatory variables. Based on the model parameter estimates, adjusted mean estimates for these traits and their standard errors were obtained using the fitted regression models together with the overall sample mean for age, BMI, and gender distribution. The difference between the adjusted means of the maternal and non-maternal lineages was tested using two-sided z-tests.

For hypomagnesemia, the likelihoods of mitochondrial transmission versus autosomal dominant transmission or autosomal dominant transmission with imprinting were determined using computer programs utilizing the Elston-Stewart algorithm (*1*) specifying 50% penetrance for mitochondrial transmission and 95% penetrance for autosomal transmission. The frequency of the mutant allele was specified as 0.01% and the phenocopy rate was specified as 1%. Changing estimates of these parameters had minor effects on the relative likelihoods. These programs are available upon request.

Muscle Biopsy and Analysis of Mitochondrial Function. A quadriceps muscle biopsy was taken from a 55-year-old male from the maternal lineage of K129. Specimens were sectioned and prepared for light and electron microscopy and stained as indicated. Sections were read by Dr. Jung Kim, Director of Neuropathology in the Department of Pathology at Yale.

In vivo ^{31}P and ^{13}C NMR spectroscopy was performed on the soleus muscle of the above patient and compared to control subjects studied concurrently. Controls were healthy subjects ranging in age from 18 to 81, and have been previously reported (*2*).

Briefly, the rate of ATP synthesis was determined with ^{31}P NMR saturation transfer experiments by measuring the exchange between inorganic phosphate (P_i) and ATP following selective irradiation of the gamma phosphate of ATP. TCA cycle flux was measured by ^{13}C NMR spectroscopy during (2-^{13}C) acetate infusion at a rate of 2.9 mg/kg/min. The rate of the TCA cycle was determined by measuring the incorporation of ^{13}C into glutamate as previously described (*2*). Measurements were performed in the resting state following an overnight fast on a 2.1T whole body magnet connected to a modified Bruker AVANCE spectrometer.

mtDNA Mutation Detection Analysis. Total cellular DNA was prepared from venous blood by standard methods. Mitochondrial DNA deletions were sought by Southern blotting; mitochondrial DNA was digested with enzyme *Pvu* II, which linearizes the mitochondrial genome, followed by Southern blotting using probes derived from 6-1519 bp and 9313-10148 bp in the mitochondrial genome (*3*). Only products of the size expected for the wild-type mitochondrial genome were detected. Eighty-seven overlapping segments (mean size 270 bp) of the mitochondrial genome were amplified by polymerase chain reaction (PCR). Mutations were sought by single-strand conformational polymorphism (SSCP) analysis as described (*4*), and direct DNA sequencing. In addition, mitochondrial DNA sequence was also determined from renal epithelial cells isolated from urine collections obtained from two hypomagnesemic family members (*5*); the results from the two sources were indistinguishable.

After identification of the $tRNA^{Ile}$ mutation, PCR and restriction fragment length polymorphism (RFLP) analysis were performed to determine if the nucleotide change is

heteroplasmic or homoplasmic. A 263 base pair fragment spanning the mutation was amplified from blood leukocytes of maternal and non-maternal members of K129. These products were then re-amplified in the presence of ^{32}P-dCTP and digested with *Taq*I. Fragments were fractionated on 12% denaturing polyacrylamide gel and detected with a Storm 860 PhosphorImager (Amersham). To determine the sensitivity of the assay to detect heteroplasmy, varying amounts of non-maternal DNA were added to maternal DNA and used as a template for PCR with ^{32}P-dCTP, followed by *Taq*I digestion and gel electrophoresis.

Analysis of linkage. Twenty-four members of K129 were included in a genome-wide analysis of linkage. Sixteen of these family members were hypomagnesemic and classified as affected, while 8 had normal magnesium levels and were classified as unaffected. 372 di- and tetranucleotide polymorphic markers spaced at approximately 10 cM intervals were genotyped by PCR amplification with fluorescent end-labeled primers as previously described (*6*). Hypomagnesemia was specified as an autosomal dominant trait with 95% penetrance and 1% phenocopy rate. Pairwise and multipoint analysis of linkage was performed using LINKAGE 5.1 (*7*) on a Sun Sparcstation 20. The expected lod score were there a dominant locus was 5.9. No interval gave a lod score greater than 1.5 in multipoint linkage.

Supporting Online Text

Hypertension. Because the oldest generations of K129 are enriched for the maternal lineage (Fig. 1), we reanalyzed the prevalence of hypertension after excluding subjects over age 60. In this group, 19 of 41 members of the maternal lineage and 7 of 52 from the non-maternal lineage had hypertension ($\chi^2 = 12.3$, $p = 0.0005$). The prevalence of hypertension on the maternal lineage is increased compared with the general population. In the large NHANES III cohort, the prevalence of hypertension between the ages of 35 and 64 was 25% (*8*); in K129 the prevalence of hypertension on the maternal lineage in this same age group was 64% (16 of 25 subjects).

Other phenotypes. In addition to phenotypes reported in the text and figures, additional mitochondrial traits were evaluated. Clinical features of myopathy, ophthalmoplegia, and seizures were absent by physical examination and medical history. To rule out Fanconi's syndrome as a factor contributing to hypomagnesemia, we measured urinary amino acid levels in 9 hypomagnesemic subjects which were all normal, and glycosuria was absent in 16 normoglycemic subjects. Plasma lactic acid was measured in 9 subjects and in each case was normal.

Three manifestations of mitochondrial disease were more prevalent among mutation carriers. Migraine headache was present in 10 members of the maternal lineage compared with 1 member of the non-maternal lineage ($p = 0.003$, Fisher's exact test). Sensorineural hearing loss was increased among members of the maternal lineage (13 vs. 3 on non-maternal lineage; $p = 0.002$). Finally, three siblings on the maternal lineage had severe hypertrophic cardiomyopathy with congestive heart failure resulting in death of two of these siblings at 1 and 12 years of age; this phenotype has been previously

described in patients with other mutations in $tRNA^{Ile}$ (*9*). Although there was a trend toward increased type 2 diabetes mellitus on the maternal lineage, this was not significant.

Supporting Figures

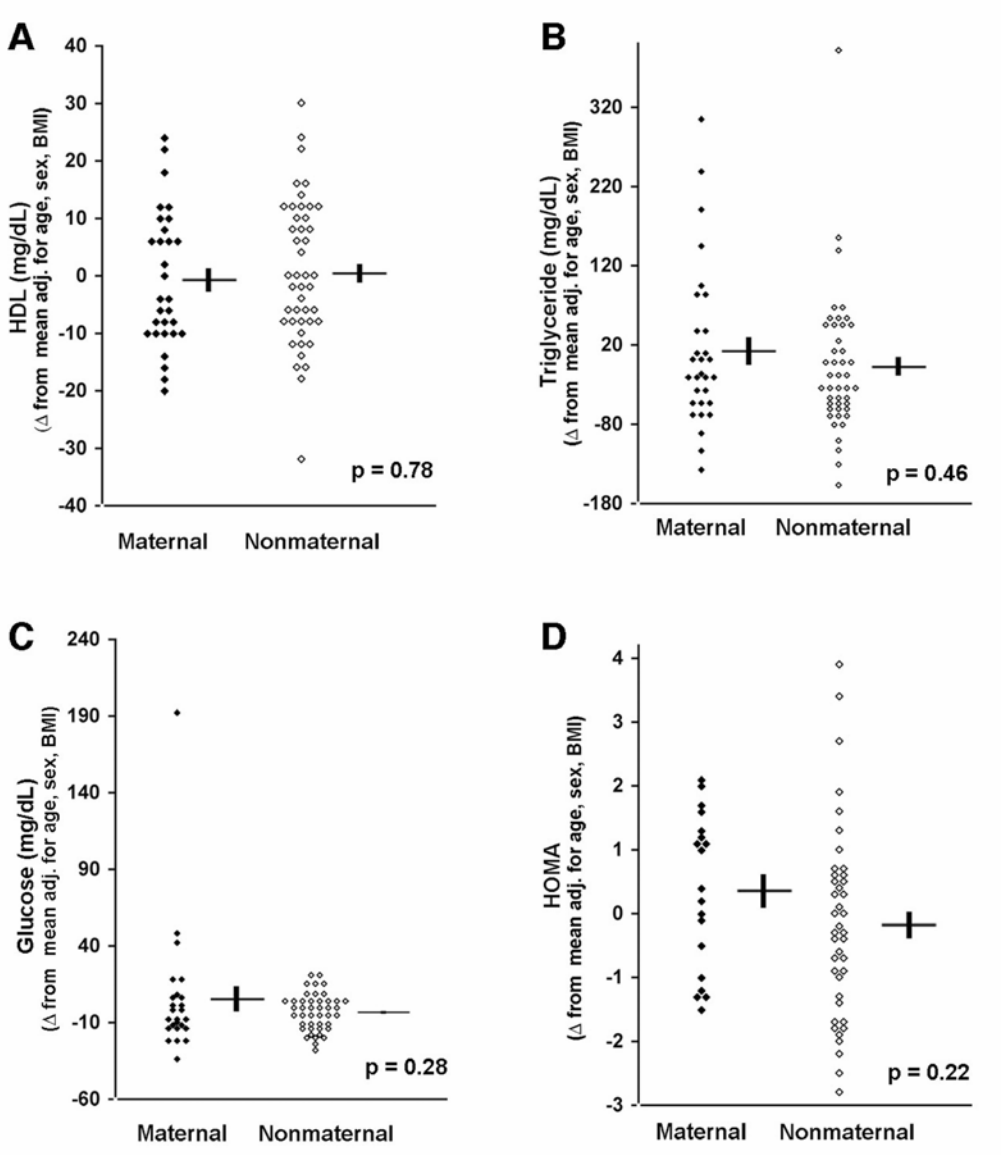

Fig. S1. Quantitative fasting HDL, triglyceride, glucose and HOMA values in K129. HDL (**A**) triglycerides (**B**), glucose (**C**) and HOMA (**D**) in maternal and non-maternal family members after adjustment for age, sex, and BMI are shown. Mean and SEM values are indicated for maternal and non-maternal groups.

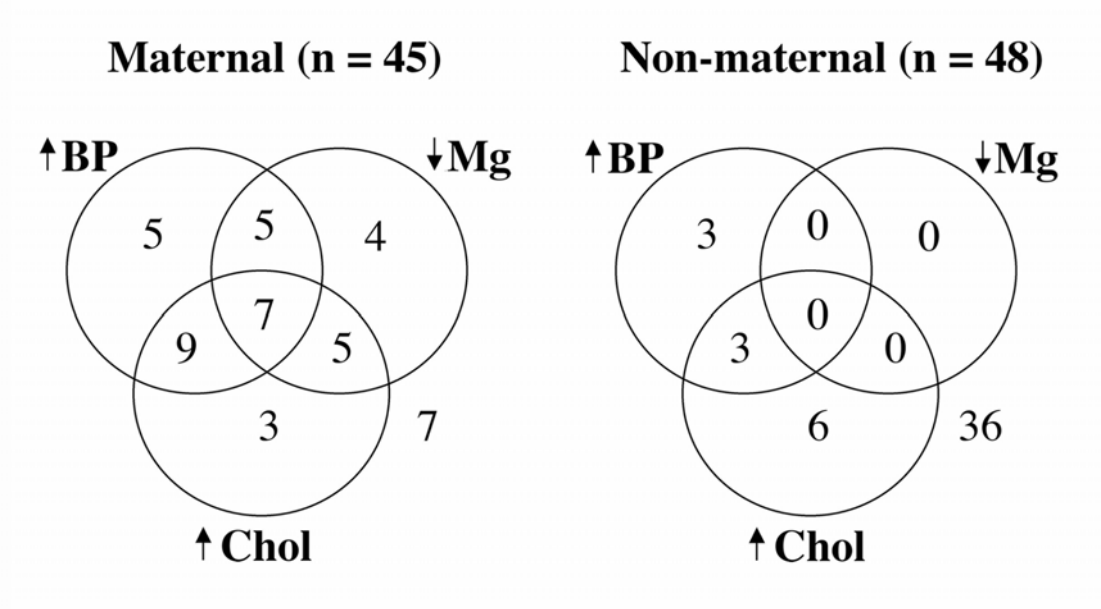

Fig. S2. Clustering of hypertension, hypercholesterolemia, and hypomagnesemia on maternal and non-maternal lineages in K129. The intersections of these three traits are shown for the 93 adults in whom all 3 traits were measured. Almost all individuals with two or more of these phenotypes are on the maternal lineage.

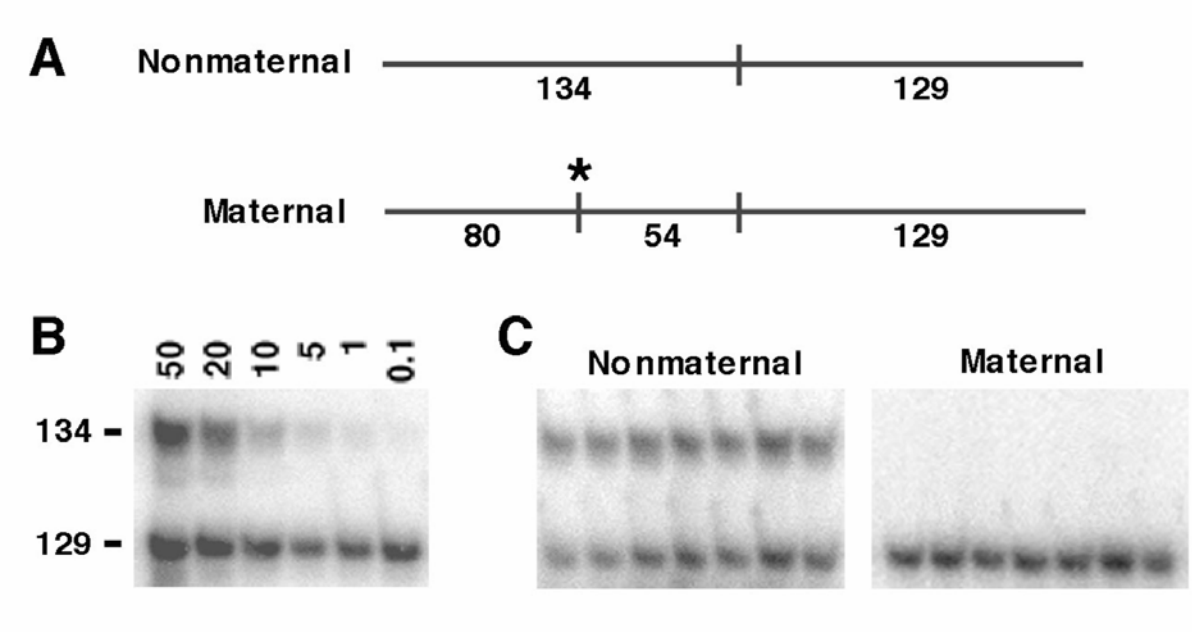

Fig. S3. Mitochondrial tRNA^{Ile} mutation is homoplasmic in K129. The T→C transition in mitochondrial tRNA^{Ile} creates a new *Taq*I cleavage site (CT**TCGA**TA). Specific primers were used to direct PCR amplification of the segment containing this mutation as described in *Methods*. Products were digested with *Taq*I and fractionated on polyacrylamide gel. (**A**). A schematic representation of the amplified segment with the mutation (maternal) and without (non-maternal) is shown. *Taq*I cleavage sites are indicated with vertical lines. The new *Taq*I site created by the tRNA^{Ile} mutation is denoted with an asterisk. Fragment sizes resulting from *Taq*I digestion are shown in base pairs. (**B**). Sensitivity of PCR-RFLP analysis to detect heteroplasmy. PCR-RFLP analysis was performed using template containing a mixture of maternal and non-maternal mitochondrial DNA as described in *Methods*. The percentage of non-maternal mitochondrial DNA in each template is indicated across the top of the panel. The results indicate that 1% heteroplasmy can be detected. Sizes of digested fragments are indicated

in base pairs. (**C**). The $tRNA^{Ile}$ mutation appears homoplasmic in blood leukocytes. PCR-RFLP analysis of mitochondrial DNA from members of the maternal and non-maternal lineages is shown. The wild-type 134 bp restriction fragment is not detected in maternal DNA; these members of the maternal lineage included members with hypomagnesemia and normal Mg^{2+} levels. Similar results were seen for all members of the maternal lineage (data not shown).

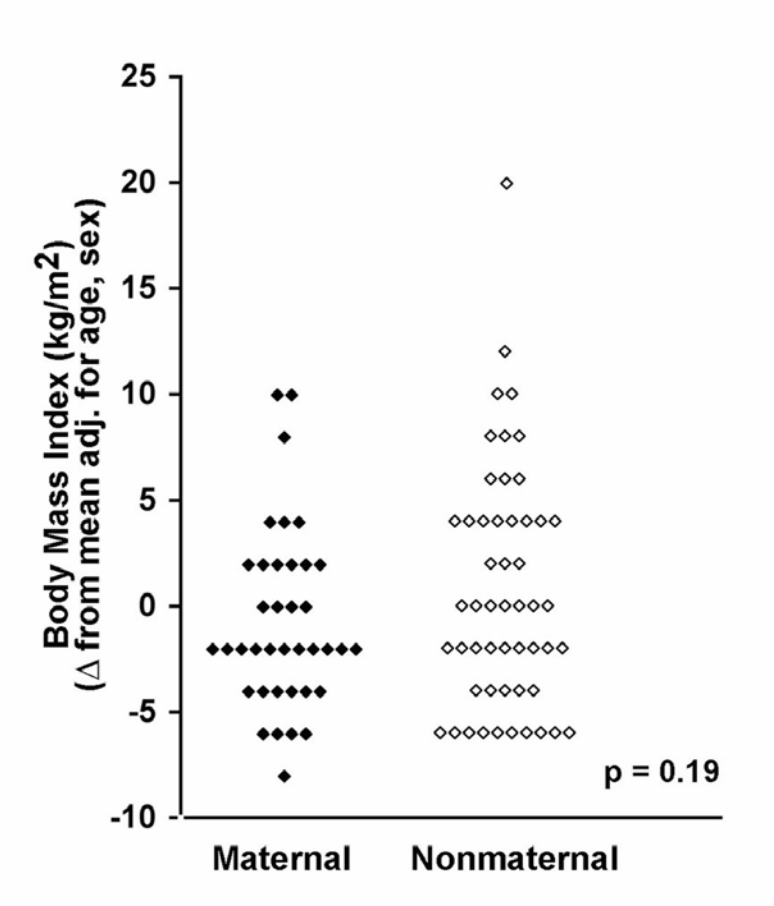

Fig. S4. Body mass index in members of the maternal and non-maternal lineages in K129. The BMI for subjects age 18-60 from the maternal and non-maternal lineages of K129 were adjusted for age and sex and are expressed as the deviation from the mean value. There is no significant difference in BMI between members of these lineages.

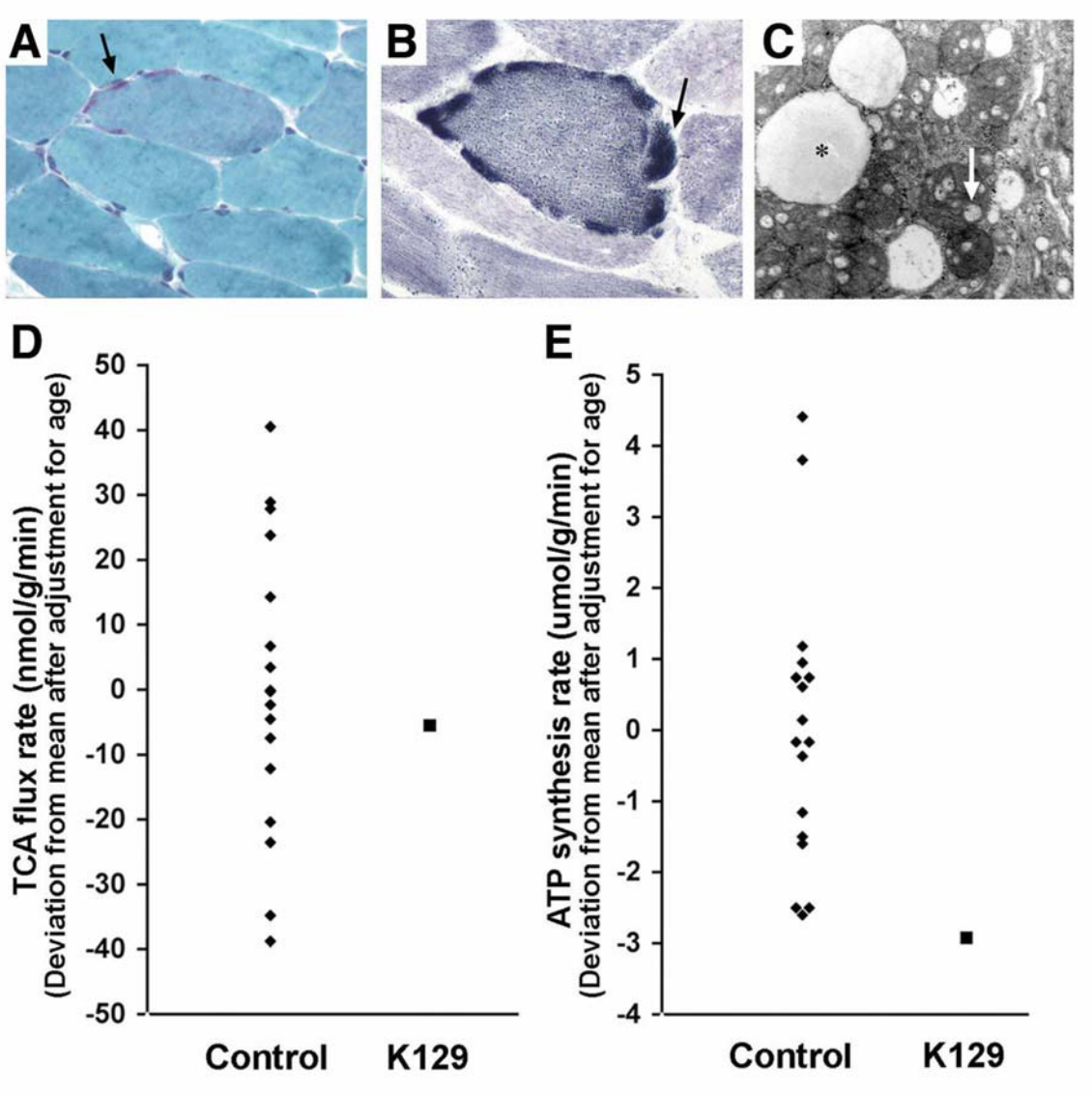

Fig. S5. Abnormal mitochondrial function in K129. Panels (**A-C**): Muscle biopsy of a member of the maternal lineage of K129. (**A**) Gomori's modified trichrome staining of a quadricep muscle biopsy specimen reveals a ragged red fiber (indicated by arrow), consistent with mitochondrial DNA accumulation due to mitochondrial dysfunction. (**B**) Succinate dehydrogenase histochemistry demonstrates enhanced focal subsarcolemmal activity (arrow), a finding characteristic of pathologic mitochondrial accumulation. (**C**) Electron microscopy reveals cytoplasmic fat accumulation (asterisk) and increased

glycogen storage (small black spots) as a result of mitochondrial dysfunction. Mitochondria are dysmorphic with enlarged vacuoles (arrow) due to clustering of cristae. These findings are typical of mitochondrial myopathy. Panels **D**, **E**: *In vivo* NMR spectroscopy of the soleus muscle of a member of the maternal lineage of K129 was performed and compared to values in healthy control subjects. Values are expressed as deviation from the mean after adjustment for age. (**D**) TCA cycle flux. The rate in the subject from K129 is near the mean value seen in controls. (**E**) Rate of ATP synthesis. The rate in the member of K129 is lower than any seen in control subjects.

Table S1. Serum and 24-hour urinary electrolyte values in K129

	Nonmaternal	Maternal	P
Serum			
Mg^{2+} (mg/dL)	2.06 ± 0.20	1.72 ± 0.05	$2x10^{-9}$
Na^{+} (mmol/L)	140 ± 0.3	140 ± 0.4	0.45
K^{+} (mmol/L)	3.98 ± 0.05	3.76 ± 0.04	0.001
Cl^{-} (mmol/L)	104.6 ± 0.4	104.1 ± 0.5	0.38
Ca^{2+} (mg/dL)	9.5 ± 0.1	9.7 ± 0.1	0.19
Uric Acid (mg/dL)	4.7 ± 0.2	4.7 ± 0.2	0.99
24h-Urine			
$FEMg^{2+}$ (%)	3.6 ± 0.2	6.3 ± 0.6	0.0001
Ca^{2+}/Cr (mg/mg)	0.45 ± 0.05	0.25 ± 0.04	0.0005
K^{+} (mmol)	61 ± 5	73 ± 6	0.13
Na^{+}(mmol)	192 ± 20	188 ± 16	0.86

Values are mean ± SEM. $FEMg^{2+}$, Fractional excretion of Mg^{2+}

Table S2. Renin and aldosterone levels in K129

	Plasma Renin Activity (ng/ml/h)	Direct Renin (μU/ml)	Aldosterone (ng/dl)
Maternal (n=28)	1.4 ± 0.2	15 ± 3	11 ± 3
Maternal HTN off medication(n=6)	1.3 ± 0.4	11 ± 3	6 ± 1
Nonmaternal (n=52)	1.1 ± 0.1	11 ± 1	10 ± 1
P-value	0.32	0.24	0.53

Values are mean ± SEM. Samples were collected after 15 minutes in the supine position. The total number of individuals examined in each group is indicated. Kindred members taking medications that alter renin and aldosterone levels (e.g., diuretics, β-blockers, angiotensin-converting enzyme inhibitors) were excluded. No differences are observed between the maternal and nonmaternal groups.

Table S3. Mitochondrial polymorphisms on maternal lineage of K129

No.	Nucleotide Position	Gene	Nucleotide Change
1	204	D-Loop	T→C
2	239	D-Loop	T→C
3	263	D-Loop	A→G
4	310	D-Loop	C insertion
5	315	D-Loop	C insertion
6	3915	ND1	G→A
7	4727	ND2	A→G
8	9380	ND2	G→A
9	10589	ND4L	G→A
10	16139	D-Loop	C→T
11	16219	D-Loop	A→G
12	16362	D-Loop	T→C
13	16482	D-Loop	A→G

Nucleotide positions in the mitochondrial genome are numbered according to the revised Cambridge reference sequence (*3*).

References

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