BRIEF REPORT

Congenital Hypermetabolism and Uncoupled Oxidative Phosphorylation

Rebecca D. Ganetzky, M.D., Andrew L. Markhard, B.A., Irene Yee, B.S., Sheila Clever, M.Sc., Alan Cahill, Ph.D., Hardik Shah, M.S., Zenon Grabarek, Ph.D., Tsz-Leung To, Ph.D., and Vamsi K. Mootha, M.D.

SUMMARY

We describe the case of identical twin boys who presented with low body weight despite excessive caloric intake. An evaluation of their fibroblasts showed elevated oxygen consumption and decreased mitochondrial membrane potential. Exome analysis revealed a de novo heterozygous variant in *ATP5F1B*, which encodes the β subunit of mitochondrial ATP synthase (also called complex V). In yeast, mutations affecting the same region loosen coupling between the proton motive force and ATP synthesis, resulting in high rates of mitochondrial respiration. Expression of the mutant allele in human cell lines recapitulates this phenotype. These data support an autosomal dominant mitochondrial uncoupling syndrome with hypermetabolism. (Funded by the National Institutes of Health.)

From the Mitochondrial Medicine Frontier Program, Division of Human Genetics, Children's Hospital of Philadelphia (R.D.G., I.Y., S.C., A.C.), and the Department of Pediatrics, University of Pennsylvania Perelman School of Medicine (R.D.G.) — both in Philadelphia; and Howard Hughes Medical Institute and Department of Molecular Biology, Massachusetts General Hospital, Boston (A.L.M., H.S., Z.G., T.-L.T., V.K.M.), and the Metabolism Program, Broad Institute, Cambridge (A.L.M., H.S., Z.G., T.L.T., V.K.M.) — both in Massachusetts. Dr. Ganetzky can be contacted at ganetzkyr@chop.edu or at Children's Hospital of Philadelphia, 3615 Civic Center Blvd., ARC 1002G, Philadelphia, PA 19104. Dr. Mootha can be contacted at vamsi@hms.harvard.edu or at Massachusetts General Hospital, Simches Research Bldg., 185 Cambridge St., 6th Fl., Boston, MA 02114.

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Mitochondrial oxidative phosphorylation is the major pathway for ATP production in eukaryotic cells (Fig. 1). During this process, cellular redox energy from the breakdown of food is converted to a proton gradient across the inner mitochondrial membrane (called the proton motive force), which is dissipated to catalyze the formation of ATP. Respiratory chain complexes I, III, and IV pump protons across the inner mitochondrial membrane while consuming oxygen. Complex V, the central enzyme in energy conversion, dissipates the proton gradient across the membrane, catalyzing the phosphorylation of ADP to ATP. In normally functioning mitochondria, mitochondrial respiration is tightly coupled to the formation of the proton motive force, and the dissipation of the proton motive force is closely linked to the synthesis of ATP. This tight chemiosmotic coupling between respiration and ATP synthesis ensures efficient energy transformations.

To date, more than 300 molecular genetic forms of mitochondrial disease have been reported.[1] These disorders result from pathogenic variants in either the nuclear or mitochondrial DNA (mtDNA). Most are recessive conditions with decreased respiration. In contrast, the first described mitochondrial disease, Luft syndrome, results in a euthyroid, hypermetabolic state characterized by high rates of mitochondrial respiration uncoupled from ATP synthesis.[2,3] Only one related case has been reported,[4-6] but the molecular bases are unknown.

Two domains make up complex V: transmembrane F_O, which includes a proton pore, and catalytic F_1.[7,8] The catalytic core of F_1 contains three α and three β subunits arranged around the γ subunit of the central stalk. Protons passing through F_O (the pore) cause the γ stalk to rotate, inducing conformational transitions in the β subunit that are required for the synthesis of ATP from ADP.[9,10] The synthesis of ATP is thus dependent on the rotation of the γ stalk, which, in turn, is dependent on the passage of protons through F_O.

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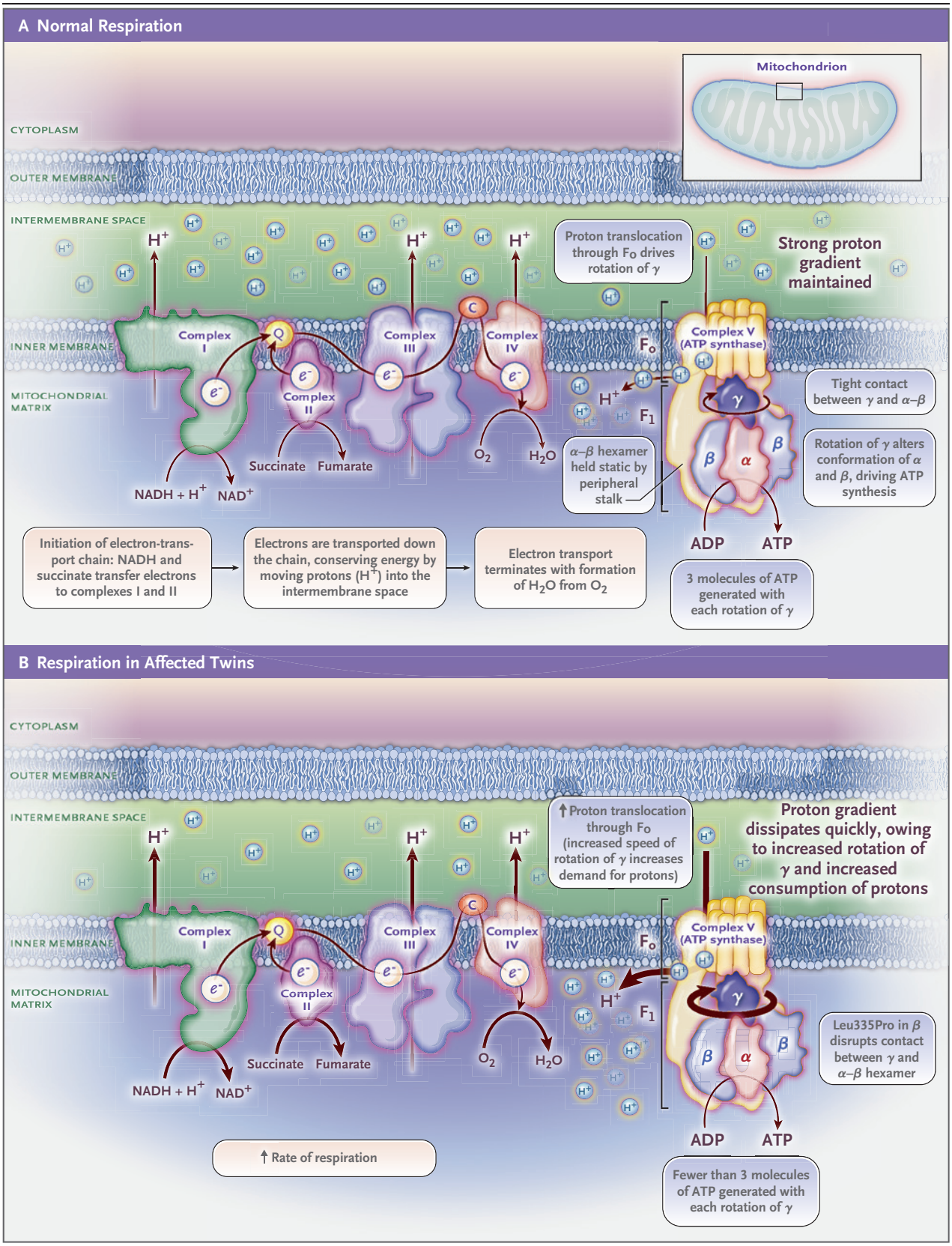

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Figure 1 (facing page). Oxidative Phosphorylation in Normal and Variant Cells.

The oxidative phosphorylation pathway — which consists of mitochondrial complexes I, II, III, IV, and V — is a means of generating energy (in the form of ATP) by oxidizing nutrients that are normally obtained from food. Intermediary metabolism transfers electrons from nutrients to electron carriers such as NADH that feed into the respiratory chain. The respiratory chain, which consists of complexes I through IV and the electron carriers coenzyme Q and cytochrome c, transfers electrons to oxygen and in the process pumps protons across the inner membrane. This creates a proton motive force consisting primarily of a membrane potential that supplies complex V with protons. Complex V is made up of several proteins arranged into two domains: the membrane-embedded F_O domain, which forms the base of the complex and is connected to the γ subunit, and the matrix-located catalytic F_1 domain. The flux of protons through the F_O domain causes the γ subunit to rotate. Since this subunit abuts the α–β hexamer (which remains stationary), the rotation alters the affinity of the α–β interface for nucleotides, which in turn drives the synthesis of ATP from ADP (Panel A). The Leu335Pro variant in *ATP5F1B* in Patients 1 and 2 is predicted to interfere with the interaction between the γ subunit and the α–β interface, diminishing the otherwise normal coupling between proton flux through the base of complex V and ATP synthesis (Panel B).

Here, we report the case of monozygotic twin boys who underwent their first biochemical genetics evaluation in the neonatal intensive care unit at the age of 2 months after euthyroid hypermetabolism, failure to thrive despite excessive caloric intake, intermittent hyperthermia, and developmental delay had been observed. Genetic and biochemical studies in samples containing the patients' fibroblasts and in engineered cellular models implicated a dominant-acting, de novo pathogenic variant affecting complex V that loosens coupling between the passage of protons (generated by mitochondrial respiration) through F_O and the synthesis of ATP by complex V.

METHODS

PATIENT PHENOTYPES

Patients 1 and 2 are monozygotic twins born at 34 weeks' gestation with intrauterine growth restriction. Failure to thrive had developed by 2 months of age and persisted despite caloric intake in excess of the calculated need for catch-up growth.

The two infants had hyperphagia, with increasing caloric intake over time. At the last follow-up at 21 months of age, Patient 1 was consuming 1280 kcal per day (152 kcal per kilogram) and Patient 2 was consuming 1480 kcal per day (175 kcal per kilogram) (2.8 and 3.2 times the estimated resting energy expenditure, respectively[11]) (Fig. 2A). Their body-weight z score was −2.5 and −3.2, respectively (Fig. 2B). They had persistent tachypnea (50 to 70 breaths per minute) without increased work of breathing, apnea, oxygen desaturation, or pulmonary disease. Both twins had recurrent unexplained hyperthermia (38.0 to 39.0°C). Their baseline temperatures ranged from 37.0 to 37.8°C with persistent diaphoresis and tactile warmth. Patient 1 underwent chordee repair, during which he had an unexplained temperature of 40°C.

Laboratory results were notable for elevated levels of branched-chain amino acids, mild hyperammonemia and uremia, and elevated triglyceride levels (Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). Both twins had mild developmental delay (Table S2). Their nonconsanguineous parents and their older brother were healthy (Fig. 2C).

CREATION OF PRIMARY CELL LINE

Protocols for characterization of the patients and the creation of a primary cell line were developed in accordance with the requirements of the institutional review board at Children's Hospital of Philadelphia. The twins' parents provided written informed consent.

MOLECULAR AND BIOCHEMICAL STUDIES

Details regarding the molecular and biochemical studies are provided in the Supplementary Appendix. Briefly, primary dermoid fibroblast lines were established from the foreskin of each boy; a primary dermoid fibroblast line was used as a control. Knock-in heterozygous clones of ATP5B Leu335Pro mutant were generated with the use of clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 gene editing, and an isogenic control (also treated with CRISPR-Cas9) was generated in A375 melanoma cells (Synthego). HeLa cells were engineered for heterologous expression of either ATP5B-FLAG or ATP5B-Leu335Pro-FLAG by means of lentiviral expression. For the analysis of primary fibro-

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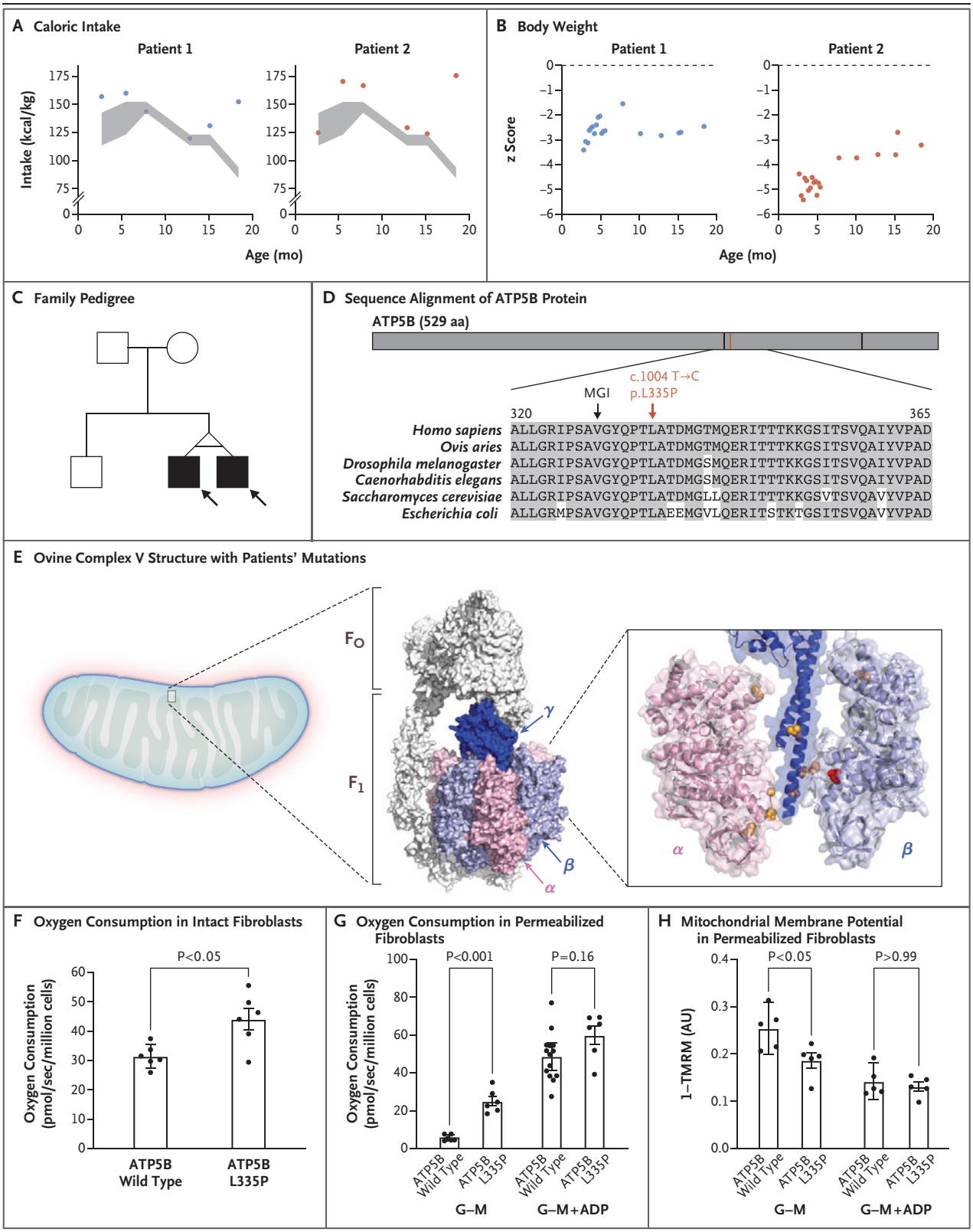

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Figure 2 (facing page). Infant Twins with Evidence of Hypermetabolism and Uncoupled Respiration.

Panel A shows the caloric intake of Patient 1 and Patient 2 at representative time points and the calculated caloric need for catch-up growth (shaded area). Panel B shows the twins' weights that were calculated as a z score on the basis of weight-for-age standards of the World Health Organization. Panel C shows the family pedigree of the twin boys, which included no known history of hereditary metabolic conditions. Panel D shows multiple sequence alignment of the ATP5B protein, indicating evolutionarily conserved residues (shaded area), as well as the Leu335Pro mutation. The aligned region includes a residue that corresponds to the mitochondrial genome integrity (MGI) mutation in yeast, which is associated with uncoupled respiration. Panel E shows an overview of the ovine complex V structure (Protein Data Bank identifier, 6TT7) with a focus on the mutation site (in red) in the β subunit in Patients 1 and 2 and the additional MGI mutations that are known to have uncoupling activity in yeast (yellow spheres). Panel F shows baseline oxygen consumption in intact fibroblasts. Panel G shows oxygen consumption in permeabilized fibroblasts in the presence of complex I substrates glutamate–malate (G–M) before the addition of ADP and in the presence of saturating ADP. Panel H shows the mitochondrial membrane potential in response to G–M, followed by ADP, which was calculated as 1 minus the change in fluorescence intensity of tetramethylrhodamine methyl ester perchlorate (TMRM). AU denotes arbitrary units. In Panels F through H, columns indicate means, and I bars indicate 95% confidence intervals; individual data points represent biologic replicates.

blasts, we measured the oxygen consumption rate and mitochondrial membrane potential simultaneously using an O2k-FluoRespirometer (Oroboros Instruments). The plasma membrane of cells was selectively permeabilized with digitonin, and a substrate–uncoupler–inhibitor titration (SUIT) protocol was performed. For engineered A375 and HeLa cells, we determined the oxygen consumption rate in intact or permeabilized cells and the extracellular acidification rate using a Seahorse XFe96 Analyzer (Agilent), both at baseline and after the addition of oxidative phosphorylation modulators. The mitochondrial membrane potential in intact cells was measured with the use of tetramethylrhodamine methyl ester perchlorate (TMRM) with a BioTek Cytation 5 microplate reader (Agilent). We performed sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Blue native PAGE, and Western blotting using standard methods with commercially available antibodies.

STRUCTURAL MODELING

We used the cryo-EM structure of ovine complex V (Protein Data Bank [PDB] identifier, 6TT7)[12] to model the Leu335Pro variant of human ATP5B. We identified positions that corresponded to yeast mitochondrial genome integrity (MGI) variants and human Leu335Pro both by multiple sequence alignment and by structural alignment of yeast (PDB identifier, 6CP6)[13] and ovine complex V. Structures were rendered with the PyMOL molecular visualization system.[14]

STATISTICAL ANALYSIS

We performed Mann–Whitney testing on the results of pulse oximetry (Fig. 2F and 2G) and Wilcoxon testing for TMRM measurements (Fig. 2H). We performed ordinary two-way analysis of variance comparing the mean value in each sample with that in every other sample, followed by Tukey's multiple comparisons test, with a family-wise alpha threshold of 0.05 and with individual variances computed for each comparison (Fig. 3). We report P values for specified comparisons after adjustment for multiple comparisons. All the statistical analyses were performed with the use of RStudio software, version 1.1.456, and Prism software, version 9.

RESULTS

MOLECULAR GENETIC ANALYSIS

Exome analysis in the twins revealed an apparently de novo heterozygous variant in *ATP5F1B* (NM_001686.3:c.1004 T→C), which predicted a p.(Leu335Pro) change in the β subunit of complex V. Both Leu335 and the surrounding region were highly conserved (Fig. 2D). According to the gnomAD database,[15] *ATP5F1B* is highly evolutionarily constrained and the Leu335Pro variant has not been observed in any of approximately 200,000 samples.[15]

Leu335 lies close to the hydrophobic sleeve in the $\alpha_3\beta_3$ assembly that holds the tip of the γ subunit of the central stalk (Fig. 2E and Fig. S1). Mutations in this region in the genes encoding the α, β, or γ subunits in yeast are MGI variants and result in uncoupling between ATP synthesis and mitochondrial respiration.[13] Leu335Pro is close to several such variants in the yeast equivalent of β.

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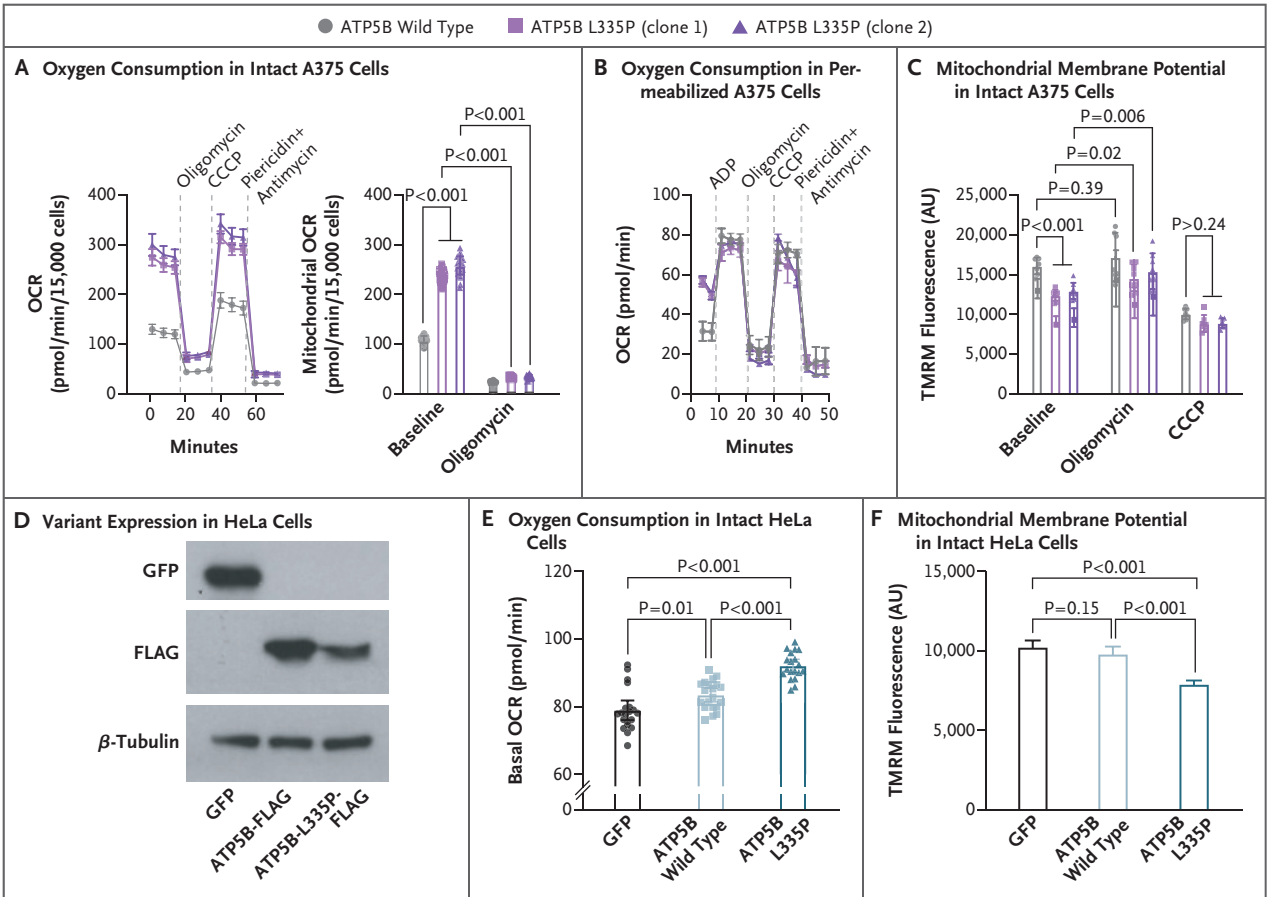

Figure 3. Bioenergetic Characterization of Engineered Human Cell Lines.

CRISPR (clustered regularly interspaced short palindromic repeats) was used to insert the observed heterozygous L335P mutation into the endogenous genomic locus of A375 cell lines (Panels A, B, and C). In Panel A, the graph at left shows the oxygen consumption rate (OCR) in intact cells with sequential additions of oligomycin (complex V inhibitor), carbonyl cyanide m-chlorophenylhydrazone (CCCP) (protonophore), and piericidin plus antimycin (CI–CIII inhibitors). The graph at right shows baseline mitochondrial respiration and oligomycin-insensitive mitochondrial respiration that were estimated by subtracting the nonmitochondrial OCR (after the addition of piericidin plus antimycin) from the OCR at baseline and the rate after the addition of oligomycin, respectively. In the CRISPR-engineered isogenic cell lines, the OCR in permeabilized cells was evaluated with sequential additions of ADP, oligomycin, CCCP, and piericidin plus antimycin (Panel B), and the mitochondrial membrane potential in intact cells was evaluated with TMRM under baseline, oligomycin, and CCCP conditions (Panel C). HeLa cells were used for heterologous expression of the wild-type and Leu335Pro variants (Panels D through F), as shown on Western blot analysis of the GFP control, FLAG epitope-tagged wild-type ATP5B, and the FLAG epitope-tagged Leu335Pro variant (Panel D). In the Hela cell lines with heterologous expression under baseline conditions, OCR was measured in intact cells (Panel E), and mitochondrial membrane potential was measured in intact cells with TMRM (Panel F). In Panels A, C, and E, mean values for biologic replicates are shown; I bars indicate standard deviations. In Panel F, mean values for more than 300 single-cell measurements are shown; I bars indicate 95% confidence intervals. AU denotes arbitrary units.

BIOENERGETIC ANALYSIS OF PATIENTS' FIBROBLASTS

We observed a higher rate of basal oxygen consumption in intact fibroblasts than in control fibroblasts (Fig. 2F and Fig. S2). This difference was more pronounced in permeabilized cells after the addition of the complex I-linked substrates glutamate–malate but normalized after the addition of saturating ADP (Fig. 2G). In human mitochondria, the membrane potential is the dominant component of the proton motive force. A cell line derived from Patient 2 showed comparatively diminished inner membrane polarization after the addition of glutamate–malate, which suggests higher basal leak across the membrane (Fig. 2H). The membrane potential after the addition of ADP was similar to that in the control cell line. Overall, these results sug-

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gest that the increased oxygen consumption rate was caused by the dissipation of the proton motive force without a corresponding increase in ATP synthesis — in other words, the dissipation of the proton motive force had become less coupled to the synthesis of ATP. In such circumstances, more energy is theoretically lost in the form of heat and less is being captured in the form of ATP.

BIOENERGETIC ANALYSIS OF ENGINEERED CELL LINES

To further corroborate these findings, we next used CRISPR-Cas9 to engineer two independent clones with the heterozygous Leu335Pro mutation and compared them with an isogenic control. As in the patient-derived fibroblasts, we observed an elevated rate of basal mitochondrial oxygen consumption in intact mutant cells (Fig. 3A) and in permeabilized mutant cells (Fig. 3B), elevations that were associated with a lower baseline mitochondrial membrane potential (Fig. 3C). Both the increased oxygen consumption rate and lower mitochondrial membrane potential normalized after treatment with oligomycin, which inhibits complex V. After treatment with a chemical protonophore (which allows protons to move freely across the inner mitochondrial membrane) in permeabilized cells, the oxygen consumption rate was similar in the wild-type and mutant cells, which corresponded to the complete uncoupling and depolarization of the mitochondrial membrane in both samples. The oxygen consumption rate remained higher in intact mutant cells than in intact wild-type cells after protonophore treatment, which suggested the occurrence of secondary changes in cytosolic metabolism. Indeed, we observed an elevated baseline rate of extracellular acidification, increased glucose consumption, and increased lactate release in the mutant cells, which suggests that secondary cellular adaptations include increased glycolysis (Fig. S4). These studies in engineered knock-in cell lines support the identification of a mitochondrial uncoupling phenotype that biochemically originates from a variant affecting complex V.

The Leu335Pro variant appears to have a dominant effect on the basis of the familial pattern of inheritance. To distinguish between haploinsufficiency (insufficient wild-type β subunit) and dominant negativity (variant protein suppressing function of the wild-type protein), we heterologously expressed either an epitope-tagged wild-type or Leu335Pro variant in HeLa cells using lentiviral transduction. Cells tolerated the expression of either allele. Although the expression of the Leu335Pro variant was consistently lower than that of the wild-type variant (Fig. 3D), it assembled into macromolecular complex V (Fig. S5A and S5B). Despite these lower levels of expression, the baseline oxygen consumption rate (Fig. 3E) and glycolysis (Fig. S5C) were higher and the basal membrane potential was lower than the values in the wild-type variant (Fig. 3F). Although MGI mutations in yeast can cause mtDNA depletion, we did not observe changes in the mtDNA copy number in our cell lines (Fig. S5D). The data support the findings that heterologous expression of the Leu335Pro variant is sufficient to decrease basal mitochondrial membrane potential and increase respiration, consistent with a dominant-negative effect of the mutation on coupling.

Collectively, our analyses provide strong support for the pathogenicity of the Leu335Pro variant. We confirmed that this was a de novo variant in the twin boys, since it had not been identified in either of their parents or in controls. In addition, the variant affects an amino acid residue that is evolutionarily conserved. Leu335 is in the critical hydrophobic sleeve region; mutations in yeast affecting nearby residues cause an uncoupling phenotype. Oxygen consumption and membrane potential studies in fibroblasts and cells with genetic introduction of the Leu335Pro variant showed loosened coupling between the proton motive force (generated by mitochondrial respiration) and ATP synthesis due to intrinsic dysfunction of complex V. Furthermore, heterologous expression of the Leu335Pro variant on a wild-type background recapitulates these biochemical findings, which is also consistent with a dominant-negative effect on chemiosmotic coupling. On the basis of these findings, we classified the variant as pathogenic according to the criteria of the American College of Medical Genetics and Genomics[16] (ClinVar accession number, SCV002555570) (Table S4).

DISCUSSION

Here we describe monozygotic twin boys with euthyroid hypermetabolism characterized by excessive caloric intake, inability to gain weight,

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and tachypnea with a pathogenic variant (Leu-335Pro) in *ATP5F1B* that results in a loosened coupling between dissipation of the proton motive force and the generation of ATP. At the time of this report, the twins continued to have persistent symptoms despite excess calorie provision, supplemental creatine to support muscle-energy metabolism, and supplemental folinic acid. Mitochondria in the patients' fibroblasts and in engineered heterozygous mutant cells had decreased mitochondrial membrane potential, presumably resulting from a greater flux of protons through complex V, which has resulted in less efficient ATP production. The twins also have had increased mitochondrial oxygen consumption.

Complex V uses the proton motive force that is generated by the respiratory chain for the synthesis of ATP. The catalytic F_1 domain of complex V consists of a trimer of α–β dimers and a central $\gamma\delta\varepsilon$ stalk.[17] The nucleotide-binding sites are located at the interface between each α and β subunit. Protons pass through the pore of F_O, which is connected to the central stalk, driving its rotation within the $\alpha_3\beta_3$ assembly. This rotation alters the affinity of the α–β interface for nucleotides, driving the catalytic cycle for ATP formation. Normally, during every 360° rotation, eight protons are translocated into the matrix and three molecules of ATP are produced. Tight contacts between the γ subunit and the $\alpha_3\beta_3$ assembly maximize efficient powering of ATP production by the translocation of protons.[17] The C-terminal tip of the γ subunit fits snugly in a hydrophobic sleeve made of amino acids 287 to 294 of the α subunits and amino acids 274 to 281 of the β subunits.[17]

Our measurements of oxygen consumption and membrane potential show that if we chemically eliminate complex V activity or, alternatively, activate it maximally (saturating ADP), there are no residual differences in mitochondrial leak, which shows that uncoupling arises from a defect intrinsic to complex V. Arsenieva and colleagues[13] identified two categories of uncoupling by MGI mutations in complex V in yeast. Group 1 mutations affect amino acids that make up the hydrophobic sleeve of the α–β hexamer and allow the γ subunit to rotate without tightly engaging the α–β regions. Group 2 mutations alter the matrix-facing interface of the α and β subunits. Given the proximity of Leu335 to the hydrophobic sleeve and the effect of certain group 1 mutations in yeast, we propose that Leu335Pro is a group 1 mutation, because it probably impairs engagement of the γ subunit by the α–β hexamer by disrupting contact between the β and γ subunits. It thereby disrupts the efficiency of ATP synthesis, even with normal translocation of protons through F_O.

We propose that the current condition being evaluated in the twins belongs to a category of mitochondrial uncoupling syndromes (including Luft syndrome) that are characterized by elevated mitochondrial respiration uncoupled from mitochondrial ATP synthesis. However, Luft syndrome appears to be caused by uncoupled respiration that originates outside complex V.[3] Both conditions are characterized by high caloric intake, low body mass, and recurrent hyperthermia. Distinct clinical features in the twin brothers were presentation in infancy, developmental delay, and episodic hyperthermia.[4] The occurrence of hyperthermia was consistent with the observation that uncoupling respiration from ATP synthesis results in heat generation in a variety of systems.[18,19] Although it is reasonable to assume that the hypermetabolism and hyperthermia are directly attributable to the effect of Leu335Pro on complex V, it remains unclear why hyperthermia is episodic. Future challenges lie in delineating how the uncoupling defect within complex V leads to secondary changes within mitochondria, the cell, and the patient.

We anticipate that mitochondrial uncoupling syndromes may have diverse molecular and biochemical causes. For example, uncoupling may be mediated by genetic variants that make the mitochondrial inner membrane leaky to protons. Alternatively, variants in the genes encoding the α, β, or γ subunits of complex V may loosen coupling between the proton motive force and ATP synthesis.[13,20] In the future, it will be important to determine whether inherited variation in these pathways may contribute to differences in energy metabolism in the broader population.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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