shoulder flexion (15.0 ± 7.3 degrees), shoulder abduction (19.0 ± 8.8 degrees), knee flexion (10.7 ± 10.3 degrees), hip flexion (14.2 ± 5.1 degrees; $p = 0.031$), and elbow extension (8.1 ± 3.4 degrees). However, most of the changes did not achieve statistical significance. Shoulder extension (−0.3 ± 4.1 degrees), elbow flexion (0.9 ± 2.5 degrees), knee extension (0.8 ± 2.5 degrees), and hip extension (−1.3 ± 1.8 degrees) showed little change during the study. Fig. 1 shows a 23 year-old study patient with severely limited shoulder range of motion (abduction and flexion), which improved following one year treatment with idursulfase.

Oxygen desaturation index (ODI)

At baseline, the mean oxygen desaturation index (ODI) was 18.5 events/h ($n = 9$), which is moderately abnormal [23]. Three patients had a normal ODI (<5 events/h), two had a mildly abnormal ODI (5–15 events/h), and four had a moderately to severely abnormal ODI (>15 events/h). During the study, the mean ODI increased by 3.9 ± 3.5 events/h, which was largely due to a single patient with an increase of 26.8 events/h. The other seven patients had stable ODI values (changes ⩽ 10 events/h).

Safety

Idursulfase was well-tolerated over the course of the study. Adverse events were mainly mild, unrelated, and attributable to expected symptoms of MPS II disease. Fifty percent (5/10) of patients experienced a total of 11 drug-related adverse events. Urticaria was the most frequent event (five events in two patients), followed by erythema (two events in the same patient). Similarly, 50% (5/10) of patients experienced infusion-related reactions (i.e. adverse events assessed as drug-related and occurring within 24 h of the infusion). The highest patient incidence involved skin reactions, i.e. urticaria and erythema (three patients each), while dyspnea, abdominal pain, and vasovagal syncope also were observed in one patient each. Except for one patient who experienced several episodes of urticaria between 9 and 12 months, the other four patients had infusion-related reactions only once or twice during the first three months of treatment. Management of infusion-related reactions included antihistamine therapy and temporary interruption of the infusion, and all events were followed by a successful patient recovery. There were no clinical laboratory abnormalities reported as related to idursulfase.

Two patients experienced serious adverse events, including one death, in the study. A 26 year-old male experienced an infusion-related reaction involving diffuse urticaria, flushing, and numbness of the tongue 1 h after initiation of the fifth infusion. The patient was pre-medicated with antihistamines without further events. A 42 year-old male had an infusion-related reaction reported by the investigator as vasovagal syncope, which consisted of hypotension, vomiting, weak pulse, and decreased consciousness and occurred 30 min into the first infusion. Subsequent infusions were preceded by corticosteroid pre-medication administration without further infusion-related reactions. The patient had a history of cardiac valve incompetence and cardiac failure requiring medications, including furosemide. Later in the study, he experienced an increase in leg edema secondary to worsening congestive heart failure. He was depressed and attempted suicide by drug overdose (not idursulfase). Upon arrival at the hospital, the patient went into cardiac arrest. Subsequent resuscitation measures were unsuccessful, and he died due to hypoxic encephalopathy, pneumonia and renal failure.

Antibodies

Anti-idursulfase IgG antibodies were detected in 60% (6/10) of patients, two of who became seronegative later in the study. No IgE antibodies were detected in patients who underwent testing for infusion-related reactions. The mean reductions in urinary GAG levels did not differ between patients who were seropositive at any time (−80.9% ± 3.8%; $n = 5$) and those who remained seronegative throughout the study (−78.6% ± 1.8%; $n = 4$). Although hypersensitive reactions or infusion-related adverse reactions tended to occur in the antibody-positive patients (four antibody-positive patients versus one antibody-negative patient), there was no correlation between the presence of antibodies and other adverse events. Furthermore, the frequency of hypersensitivity reactions did not correlate with antibody titer.

Discussion

The most remarkable difference between this and previous clinical studies of idursulfase [19,20] relates to the patient demographics and characteristics. The purpose of the JET study was to provide access to treatment for the most seriously ill MPS II patients while awaiting regulatory approval of idursulfase in Japan, which occurred in October 2007. Patients in the JET study had a mean age of 30.1 years, all were Japanese, and all were seriously ill (mean percent predicted FVC 39.9% and mean 6MWT distance 286.0 m). By comparison, MPS II patients in the Phase 1/2 and Phase 2/3 studies of idursulfase were younger (mean ages 13.9 years and 14.2 years), predominantly Caucasian (100% and 83%, respectively), and less severely affected (mean percent predicted FVC 55.1% and 55.4%; mean 6MWT distance 397 m and 395 m) [19,20]. Despite these patient differences, the JET study has shown that idursulfase is a safe and effective (Table 1) treatment for Japanese patients with MPS II and its risk–benefit profile is similar to that reported in previous studies.

In this study, idursulfase efficiently reduced GAG storage, as evidenced by the statistically significant reductions in urinary GAG levels ($p = 0.004$) and hepatosplenomegaly ($p = 0.002$) (Fig. 2; Table 1). These pharmacodynamic changes appeared to translate into clinical benefit, as evidenced by trends towards improvement in functional capacity (mean 54.5 m increase in 6MWT), respiratory function (mean 15.0% relative increase in percent predicted FVC), joint range of motion (mean increases ranging from 8.1–19.0 degrees for several joints), and LVMI (mean −12.4% decrease). Cardiac EF and valve disease remained mostly stable, although one patient with severe congestive heart failure showed progressive worsening and one patient with a greatly elevated LVMI showed a further increase. The mean ODI increased slightly by 3.9 events/h, but importantly 89% (8/9) of patients showed no clinically significant changes.

The safety profile of idursulfase in the JET study was similar to that of previous studies with no new or unexpected adverse events despite the older and more seriously ill patient population. Most adverse events were considered by investigators to be disease-related and unrelated to idursulfase. The most common drug-related adverse events were infusion-related reactions, occurring in 50% of patients. The most common infusion-related reactions were skin reactions consisting of urticaria and erythema. There were two related serious adverse events that occurred during the infusions—one involving urticaria, flushing, and numbness of the tongue, and the other involving vasovagal syncope. The one patient death was attributed to suicide from a drug overdose and was not related to idursulfase.

MPS II is a progressive and debilitating multisystem disease that is associated with a shortened lifespan, primarily from cardiorespiratory compromise [28]. Therefore, it is noteworthy that in this one-year study, cardiac and respiratory functions were improved or stable in most patients. Decreasing lung volumes are known to be associated with increased morbidity and mortality [26];

given the low percent predicted FVC values at baseline in study patients (mean 39.9%), a relative increase of 15% is of particular importance. The American Thoracic Society defines a >15% relative change in FVC occurring over a one-year period as being clinically meaningful [26]. Similarly, the 54.5 m mean increase in 6MWT distance also is considered to be a clinically meaningful improvement, based on a study of adult men with chronic obstructive pulmonary disease [25]. The 6MWT is a sub-maximal exercise test that is a composite assessment of cardiac, respiratory, and musculoskeletal function. Because all three of these organ systems are involved in the MPS disorders, walking tests have been widely used as primary efficacy endpoints in clinical trials of enzyme replacement therapy for other MPS disorders, including MPS I [29,30] and MPS VI [31].

We observed no evidence for an effect of race on immunogenicity or safety. IgG antibodies were detected in 60% (6/10) of patients treated with idursulfase, which is similar to the 49.6% rate seen in the Phase 2/3 study that enrolled predominantly Caucasian and other non-Asian patients [20]. In addition, the adverse event profile was similar in all respects; infusion-related reactions occurred in 50% of patients in the current study compared to 69% of patients receiving weekly idursulfase in the Phase 2/3 study [20].

Limitations of this study include its open-label treatment, lack of control group, and small sample size. Other aspects of the study design, however, including the treatment dose and regimen, study duration, and efficacy and safety assessments were identical or very similar to those used in the Phase 2/3 study [20]. A placebo effect in this study cannot be excluded, especially for effort-dependent assessments such as the 6MWT and active joint range of motion. Nevertheless, the magnitude of change in the 6MWT distance was similar to those observed in previous studies of idursulfase [19,20]. Determination of FVC by spirometry is less susceptible to a placebo effect given the requirement for test–retest reproducibility at each assessment [21]. This study enrolled only 10 patients, which may not have had sufficient power to detect a statistically significant clinical response even if clinical improvements were present. On the other hand, the biomarkers of lysosomal GAG clearance, i.e. liver and spleen volumes and urinary GAG level, did have sufficiently large effect sizes (change/standard deviation of change) to show statistically significant differences. Finally, the study involved only adult males, all of whom had a substantial pre-existing disease burden. This study shows that many disease features of seriously ill patients, including diminished cardiorespiratory function, restricted joint range of motion, and hepatosplenomegaly can improve with idursulfase treatment. An even better response is expected in young children prior to final organ maturation and the development of chronic tissue damage. In this regard, a study in MPS II patients ≤5 years of age is underway.

Conclusions

Idursulfase was generally well-tolerated and produced clinical improvements in adult Japanese patients with attenuated MPS II treated with the labeled dose, 0.5 mg/kg administered intravenously once weekly. Treatment with idursulfase also resulted in substantial reductions in hepatosplenomegaly and urinary GAG excretion, indicating efficient clearance of lysosomal GAG. The safety profile and immunogenicity of idursulfase appear to be similar between Japanese and previously studied Caucasian patients.

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特集　臨床遺伝学の進歩と日常診療

【遺伝性疾患の臨床】

ミトコンドリア病

後　藤　雄　一

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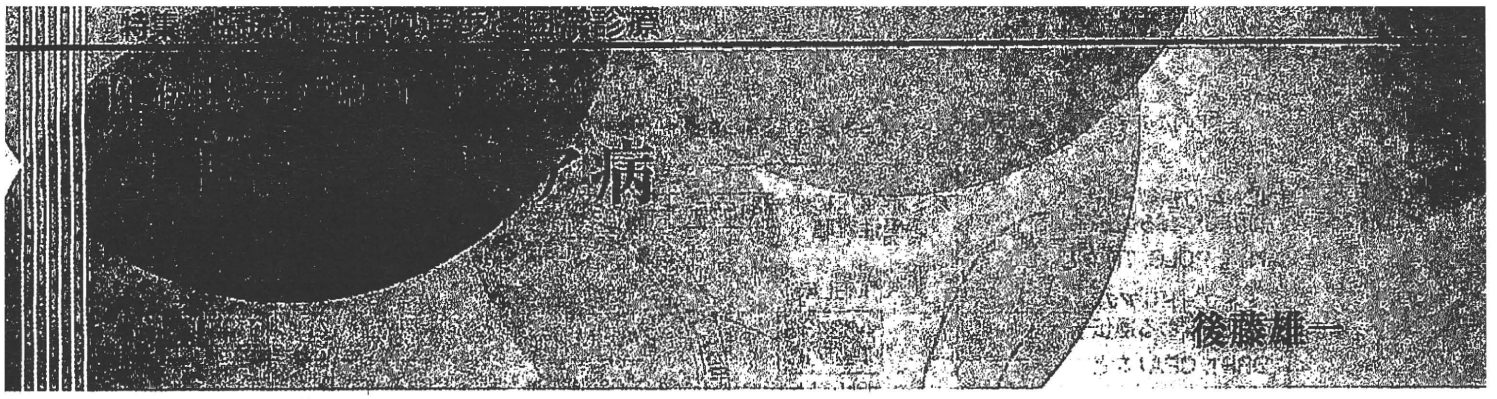

後藤雄一

キーワード●ミトコンドリア病，ミトコンドリアDNA，電子伝達系酵素欠損症

はじめに

ミトコンドリア病は，細胞内ミトコンドリアのエネルギー産生能が低下することに起因する病態であり，種々の酵素異常を基盤にしている．病因は，核DNAにコードされた遺伝子変異による場合とミトコンドリアDNA（mtDNA）の異常による場合がある．前者には，エネルギー代謝に関わる酵素や関連蛋白質の遺伝子変異，mtDNAの維持や複製に関わる蛋白の遺伝子変異の場合があり，後者には核DNA変異とmtDNA異常が同時に存在する．

精神や神経，筋，心筋など比較的頻度の高い症状以外にも，腎症状，糖尿病，内分泌症状など臨床症状は多彩である．2つ以上の臓器症状が存在し，母系遺伝，血中乳酸高値などがあれば，ミトコンドリア病を疑うことが重要である．

I 疾患概念

ミトコンドリア内には，エネルギー代謝に関する酵素が100種類以上局在している．ミトコンドリア病とは，ミトコンドリア自体およびミトコンドリア内に存在するDNAや蛋白に異常が存在し，ミトコンドリアにおけるエネルギー産生に障害を来した疾患群を総称している．

ミトコンドリア内のエネルギー代謝異常のうち最も頻度の高い電子伝達系酵素の障害は，酵素活性低下と臨床症状とが必ずしも1対1に対応せず，しかも個々の症例できわめて多彩な臨床症状が種々の障害度で認められる．また，電子伝達系酵素の一部はmtDNAにコードされており，ミトコンドリア（とmtDNA）のもつ独自な細胞生物学的特徴を色濃く反映させている．

II 病因としてのmtDNAと核DNA

mtDNAは全長約16,500塩基余りの環状2本鎖DNAであり，ミトコンドリア内で蛋白を合成するための2個のリボソームRNA，22個の転移RNAをコードしている．さらに，電子伝達系酵素群のサブユニットの一部を構成する蛋白質を計13個コードしている（図1）．

1. mtDNAの特徴

核DNAと大きく異なる特徴は，1細胞内に多数のmtDNAが存在していることである．1つの細胞内に数十～数百個存在する個々のミトコンドリア内に，mtDNAは5～10個ずつ存在しているため，1細胞では数百～数千個存在していることになる（マルチコピー性）．また，核DNAに比べて変異の起こしやすさが5～10倍程度高いとされている（易変異性）．そして，受精の際にミトコンドリアはすべて卵に由来する

Mitochondrial Disease

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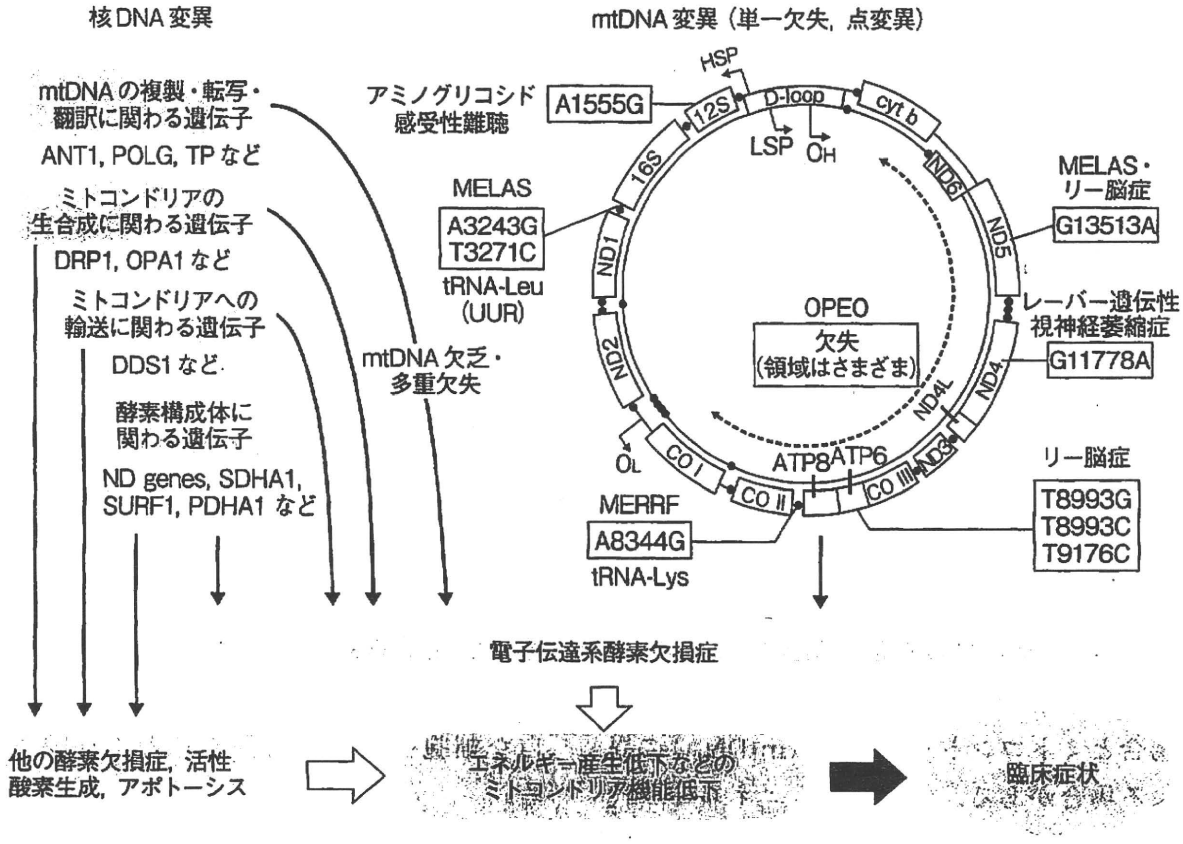

図1　ミトコンドリア病の病因

ことから，mtDNA も母からしか子に伝わらないという性質をもつ（母系遺伝形式）．

2. mtDNA の変異

mtDNA 変異には，点変異と構造異常（欠失・重複）がある．

(1) 点変異：存在する領域によって，転移 RNA 領域とそれ以外（リボソーム RNA および蛋白領域）とに分けられる．転移 RNA 領域の変異をもつ患者では，筋病理学でミトコンドリア形態異常の代表である赤色ぼろ線維（RRF）などの所見が認められる．症状が多彩で，症例ごとの差が著しいという特徴を有している．一方，転移 RNA 以外の領域の点変異は，筋病理での異常所見が乏しい．その代表はリー脳症とレーバー遺伝性視神経萎縮症で認められる点変異であり，これらの患者は比較的均一の臨床症状をとる．

(2) 構造異常（欠失・重複）：その遺伝形式は複雑である．欠失には単一欠失と多重欠失があるが，単一欠失は，ヒトの病気で発見された最初の mtDNA 異常であり，慢性進行性外眼筋麻痺症候群（CPEO）や Kearns-Sayre 症候群の臨床症状をもつ患者で認められた．一方，欠失が単一ではなく，多種類の欠失が同時に存在する家系において，adenine nucleotide translocater 1（ANT1），DNA polymerase γ（POLG）などの核 DNA 上の遺伝子変異が報告されている．

さらに，常染色体性劣性遺伝と考えられる多重欠失の例も報告され，その責任遺伝子の 1 つとして thymidine phosphorylase（TP）が同定された．これは mtDNA の維持や複製に直接影響を与える核 DNA 変異によって mtDNA が変化し，病気が発症すると考えられ，患者は核 DNA 変異と mtDNA 異常を同時にもつことになる．

一方，重複は欠失 mtDNA と正常 mtDNA がつながったもので，一部の CPEO 患者で認められている．

表1　主な病型のまとめ

病型	慢性進行性外眼筋麻痺症候群	ミトコンドリア脳筋症・乳酸アシドーシス・脳卒中様発作症候群	赤色ぼろ線維・ミオクローヌスてんかん症候群	リー脳症	レーバー遺伝性視神経萎縮症
英文略語	CPEO	MELAS	MERRF		
英文名	chronic progressive external ophthalmoplegia	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes	myoclonic epilepsy associated with ragged-red fibers	Leigh encephalopathy	Leber's hereditary optic neuropathy
mtDNA変異	単一欠失，多重欠失	3243，3271，13513変異など	8344変異など	8993，9176，13513変異など	11778変異など
核DNA変異	ANT1，POLG，TPなど	—	—	SURF1，PDHA1など	—
遺伝形式	さまざま	母系遺伝	母系遺伝	さまざま	母系遺伝
発症年齢	小児～成人	小児～成人	小児～成人	乳児～小児	若年成人
主な症状	眼瞼下垂，全方向性眼球運動障害，嚥下障害，白質脳症など（網膜色素変性，心伝動障害を伴ったものをKearns-Sayre症候群という）	脳卒中様症状（けいれん，意識障害，半盲・視野狭窄，運動麻痺など），繰り返す頭痛・嘔吐発作，精神症状	ミオクローヌス，てんかん，小脳症状	精神運動発達遅滞，けいれん，嚥下困難など	夜盲から始まって急速に視力障害が進行
その他の症状	糖尿病・難聴，低身長，副甲状腺機能低下症など	低身長，筋力低下，糖尿病・難聴，心筋症，糸球体病変，多毛など	筋力低下，心筋症など	典型的な症例では早期に呼吸不全に至る	時に，ジストニアなどの神経症状を合併
血中乳酸値	軽度上昇	中等度～高度に上昇	中等度～高度に上昇	高度に上昇	正常～軽度上昇
筋病理所見	特徴的変化あり	特徴的変化あり	特徴的変化あり	特徴的変化なし	特徴的変化なし
特記事項	ミトコンドリア病で唯一のマウスモデルが存在	脳卒中様発作の予防にアルギニン投与が有効という報告あり	多発性脂肪腫を合併する症例あり（May-White症候群）	予後不良な例が多い	自然軽快する症例あり

III 臨床症状

ミトコンドリア病の臨床症状は多彩である．その理由は，ミトコンドリアが個体のあらゆる細胞に存在しているために，その障害は種々の機能異常を引き起こすからである．ただし，エネルギー代謝障害であるミトコンドリア病では，エネルギー依存度の高い組織や細胞が障害されやすく，したがって中枢神経，骨格筋，心筋などがミトコンドリア病の主な罹患臓器である．

しかし，全糖尿病患者の約1％がmtDNA変異をもつことが報告されており，特に難聴を合併している母系遺伝が疑われる糖尿病患者はミトコンドリア病である可能性を考えるべきであ

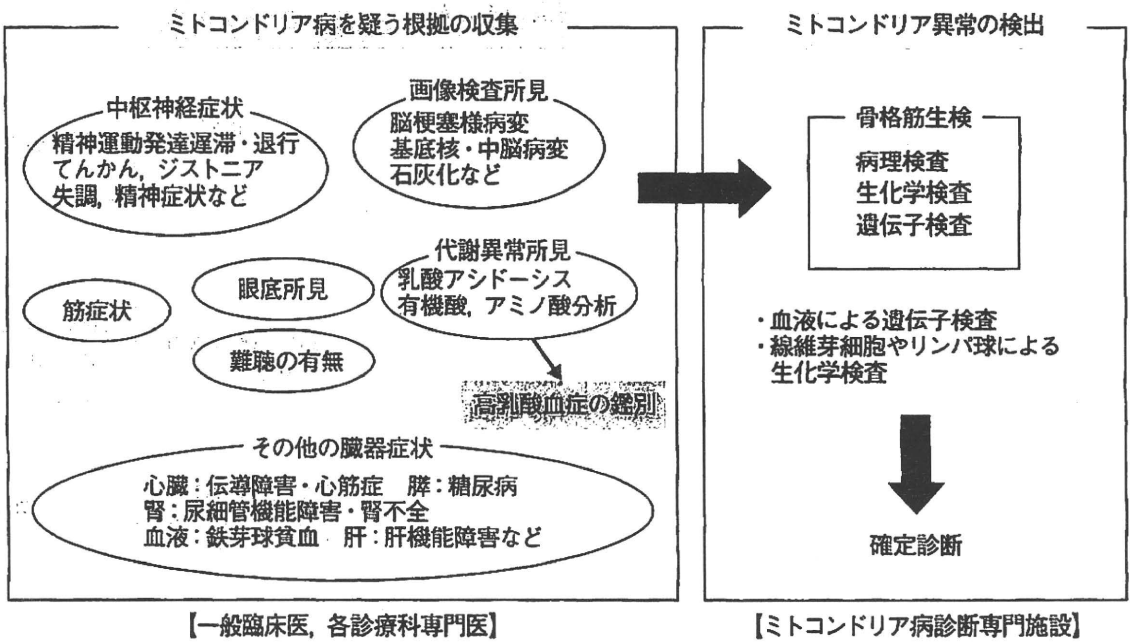

図2　ミトコンドリア病の診断手順

る．また，糸球体硬化症や腎尿細管機能障害，精神症状，網膜色素変性，低身長（成長ホルモン低下）なども比較的頻度の高い症状である．

また，mtDNA上の1555変異をもつ家系では，アミノグリコシド系抗生物質の服用によって高度難聴を来すことが知られている．このような薬剤を投与する際には，難聴の家族歴を聴取し，できれば投与前に1555変異の検査を実施することが望ましい．

表1に，代表的な病型である慢性進行性外眼筋麻痺症候群（CPEO），ミトコンドリア脳筋症・乳酸アシドーシス・脳卒中様発作症候群（MELAS），赤色ぼろ線維・ミオクローヌスてんかん症候群（MERRF），リー脳症，レーバー遺伝性視神経萎縮症についてまとめておく．

Ⅳ 診断

患者は小児科，内科，神経内科，循環器内科などを受診することが多いものの，臨床症状を考えるとあらゆる診療科を受診する可能性がある．説明のつきにくい2つ以上の臓器症状を合併している患者，母系遺伝が疑われる患者では，まずミトコンドリア病を疑うことが重要である．

診断手順を図2に示す．ミトコンドリア病を疑った場合は，多くの例で血中乳酸値が高いので，その測定が診断の契機になる．確定診断には骨格筋生検が必要になることがほとんどであり，その実施と評価のできる専門施設へ紹介することが望ましい．

Ⅴ 治療

根本的な治療法は確立していない．バランスの良い食事，疲れ過ぎない生活習慣などを基本にし，ミトコンドリア内で補酵素として働く各種ビタミン剤の投与を行う．てんかん，糖尿病などは積極的に対症療法を行うことが重要である．

■ おわりに

ミトコンドリア病は平成21年10月1日に国の特定疾患として認定された．ミトコンドリア病はその臨床症状の多様性からあらゆる診療科に関係する．診察にあたる医師がその存在を知り，各診療科専門医と協力することで診断率が上がるものと考えられる．

Mitochondrial Lon protease regulates mitochondrial DNA copy number and transcription by selective degradation of mitochondrial transcription factor A (TFAM)

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Lon is the major protease in the mitochondrial matrix in eukaryotes, and is well conserved among species. Although a role for Lon in mitochondrial biogenesis has been proposed, the mechanistic basis is unclear. Here, we demonstrate a role for Lon in mtDNA metabolism. An RNA interference (RNAi) construct was designed that reduces Lon to less than 10% of its normal level in *Drosophila* Schneider cells. RNAi knockdown of Lon results in increased abundance of mitochondrial transcription factor A (TFAM) and mtDNA copy number. In a corollary manner, overexpression of Lon reduces TFAM levels and mtDNA copy number. Notably, induction of mtDNA depletion in Lon knockdown cells does not result in degradation of TFAM, thereby causing a dramatic increase in the TFAM:mtDNA ratio. The increased TFAM:mtDNA ratio in turn causes inhibition of mitochondrial transcription. We conclude that Lon regulates mitochondrial transcription by stabilizing the mitochondrial TFAM:mtDNA ratio via selective degradation of TFAM.

AAA+ protease | mtDNA maintenance | quality control

Lon is the major protease in the mitochondrial matrix and is well conserved among species (1–4). Lon is a member of the super family of ATPases associated with diverse cellular activities (AAA^+ ATPases)[1], and forms a homooligomeric, ring-shaped structure (5, 6). Lon contributes to protein quality control surveillance in mitochondria by degrading preferentially oxidatively-modified or misfolded proteins before they aggregate (7–9). In bacteria, in addition to proteolysis of damaged proteins, Lon also plays a key role in turnover of specific unstable proteins involved in a variety of biological processes (3, 4). Similarly, the steroidogenic acute regulatory protein StAR, several subunits of cytochrome *c* oxidase, and oxidized mitochondrial aconitase are known to be Lon substrates in animal mitochondria (10–14).

In addition to its proteolytic function, mitochondrial Lon has the ability to bind DNA in vitro (15–17), and has been shown to interact with mtDNA in human cultured cells (18). However, the physiological role of DNA binding by Lon is not clear. In yeast, loss of PIM1, which is the ortholog of animal Lon protease, causes mtDNA deletion, impairs mitochondrial gene expression and results in respiratory deficiency (19, 20). A role for Lon has been postulated in mtDNA replication, transcription, and/or maintenance, but this remains to be validated. Lon was demonstrated to be a component of mitochondrial nucleoids, which are protein: DNA complexes formed to package mtDNA (21, 22). The major protein component of mtDNA nucleoids is mitochondrial transcription factor A (TFAM or mtTFA) (23, 24). TFAM contains two high mobility group (HMG) amino acid sequence boxes; it binds to mtDNA both specifically and nonspecifically (25). TFAM is essential for mtDNA transcription and for mtDNA packaging in mtDNA maintenance (26–30). Interestingly, mtDNA and TFAM levels are interdependent, such that knockdown of TFAM results in mtDNA depletion, and reduction of mtDNA copy number causes reduction of TFAM levels (26, 29, 31).

In this study, we investigated the role of Lon protease in regulating mtDNA maintenance and transcription, and the protein components of mitochondrial nucleoids in cultured cells. Our results argue strongly that Lon modulates mtDNA biogenesis by the selective degradation of TFAM.

Results

Overexpression of Lon Reduces TFAM Levels and mtDNA Copy Number. Mitochondrial localization of the *Drosophila Lon* gene product (CG8798) was confirmed in Schneider cells by fluorescence microscopy (Fig. S1). Next, *Drosophila Lon* was subcloned into the inducible expression vector pMt/Hy under the control of the metallothionein promoter. The resulting expression vector, pMt/Lon/Hy, was introduced into Schneider cells, and stable cell lines harboring this plasmid were cultured in media with or without 0.2 mM $CuSO_4$. After 10 d of incubation in the presence of copper, immunoblot analysis indicated a fivefold increase in Lon relative to that in the uninduced control cells (Fig. 1*A*). In contrast, expression of β-tubulin, used as a control protein, was unchanged. Levels of protein components of the mitochondrial nucleoid were measured by immunoblotting of cells carrying no plasmid, pMt/Hy, or pMt/Lon/Hy.

Overexpression of Lon reduced the level of TFAM to 75% of that in the control cells (Fig. 1 *A*, *B*). Levels of other proteins localized in mitochondrial nucleoids, including mtTFB2, mtDNA helicase, pol γ-α, and mtSSB, were not changed. We used Southern blots to quantify relative mtDNA copy number in the Lon overexpression cells. We found that the relative mtDNA copy number in the overexpression cells was ~0.7-fold of that in the control cells (Fig. 1*C*). Northern blots were used to quantify the relative expression of the *Cytb, ND4, and 12S rRNA* genes in cells grown for 10 d in the presence or absence of copper. Overexpression of Lon did not show any significant changes on these mitochondrial transcript levels as compared to the control cells (Fig. 1*D*). Furthermore, TFAM mRNA levels were unchanged by Lon overexpression, indicating that the reduction in TFAM protein levels in the knockdown cells does not result from the reduction of TFAM mRNA.

RNAi-Dependent Knockdown of Lon Increases TFAM, Mitochondrial DNA Copy Number, and Mitochondrial Transcription. We reduced the abundance of Lon by expressing a metallothionein-inducible *Lon*-targeted RNAi species from the plasmid pMt/invLon/Hy. The RNA species produced a form of dsRNA hairpin homolo-

Author contributions: Y.M. and L.S.K. designed research; Y.M. performed research; Y.M., Y.G., and L.S.K. analyzed data; and Y.M. and L.S.K. wrote the paper.

The authors declare no conflict of interest.

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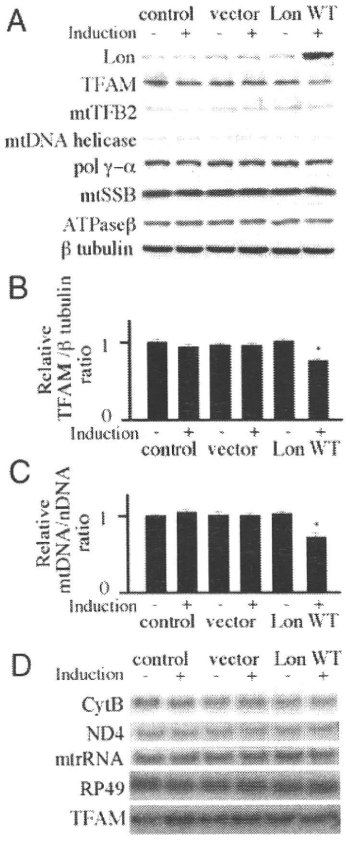

Fig. 1. Expression of *Drosophila* Lon protease in Schneider cells. Schneider cells with no plasmid (control) or carrying pMt/Hy (vector) or pMt/Lon/Hy (Lon WT) were cultured for 10 d in the presence or absence of 0.2 mM CuSO4. (*A*) Protein extracts (20 μg) were fractionated by 7.5%, 10.5%, or 13.5% SDS-PAGE, transferred to nitrocellulose filters and probed with antibodies against Lon protease, TFAM, mtTFB2, mtDNA helicase, pol γ-α, mtSSB, ATPase β, or β tubulin as indicated. (*B*) The TFAM/ β tubulin ratio was quantitated by normalizing TFAM protein levels to β tubulin protein levels as described under *Materials and Methods*. Error bars indicate means ± standard error of three independent experiments. The asterisk indicates $P < 0.05$ in comparison to control. (*C*) Total DNA (10 μg) was extracted from Schneider cells or Schneider cells carrying pMt/Hy or pMt/Lon/Hy that were cultured for 10 d in the presence of 0.2 mM CuSO4. DNA was digested with XhoI, fractionated in a 0.7% agarose/ TBE gel, and then blotted to a nylon membrane. The membrane was hybridized with a radiolabeled probe for CytB, and then stripped and rehybridized with radiolabeled probe for the histone gene cluster as a control. The relative mtDNA copy number was quantitated as described under *Materials and Methods*. Error bars indicate means ± standard error of three independent experiments. The asterisk indicates $P < 0.05$ in comparison to control. (*D*) Total RNA (10 μg) was extracted from Schneider cells or Schneider cells carrying pMt/Hy or pMt/Lon/Hy after 10 d of culture in the presence or absence of 0.2 mM CuSO4. RNA was fractionated in a 1.2% agarose/formaldehyde gel, blotted to nylon membrane, and hybridized with radiolabeled probes for the mitochondrial transcripts *12S rRNA, ND4*, and *Cytb*, the nuclear transcript *RP49*, and *TFAM*.

gous to *Lon*. Schneider cells stably expressing pMt/invLon/Hy showed the accumulation of oxidized proteins in mitochondria (Fig. S2), but the knockdown cells could be maintained for at least 6 mo under normal culture conditions. Schneider cells stably expressing pMt/invLon/Hy were cultured for 10 d in the absence or presence of 0.2 mM $CuSO_4$. Immunoblot analysis of copper-treated cells showed that cells carrying pMt/invLon/Hy expressed >10-fold less Lon than cells carrying the control vector (Fig. 2*A*). Even in the uninduced condition, the cells carrying pMt/invLon/Hy suppressed expression of Lon by >10-fold, most likely due to leaky expression (32–35). In contrast, expression of β-tubulin was unchanged. Again levels of mitochondrial nucleoid proteins were measured by immunoblotting of cells carrying no plasmid, pMt/Hy, or pMt/invLon/Hy. Depletion of Lon increased the protein levels of TFAM and mtTFB2 ~1.4-fold relative to their levels in the control cells (Fig. 2*A*, *B*). At the same time, the levels of other mitochondrial nucleoid proteins were not changed significantly. Next, relative mtDNA copy number was measured in the knockdown cells in the presence or absence of copper and found to be ~1.3-fold higher than in the control cells (Fig. 2*C*). These results suggest that the increase in mtDNA copy number results from the increased TFAM levels.

Northern blots were used to quantitate relative expression of the *Cytb, ND4*, and *12S rRNA* genes in cells grown for 10 d in the presence or absence of copper. Basal expression of Lon-targeted RNAi increased the transcript levels of *Cytb, ND4*, and *12S rRNA* to ~1.4-fold that of the control cells (Fig. 2*D*). This increase in the mitochondrial transcripts may result from either the increase in mtTFB2 or mtDNA copy number, or both. In contrast, the level of transcripts from the nuclear gene *RP49* was unchanged by Lon knockdown. Similar to that observed upon the overexpression of Lon, TFAM mRNA levels were unchanged in the Lon knockdown cells (Fig. 2*D*), indicating that the increase in TFAM pro-

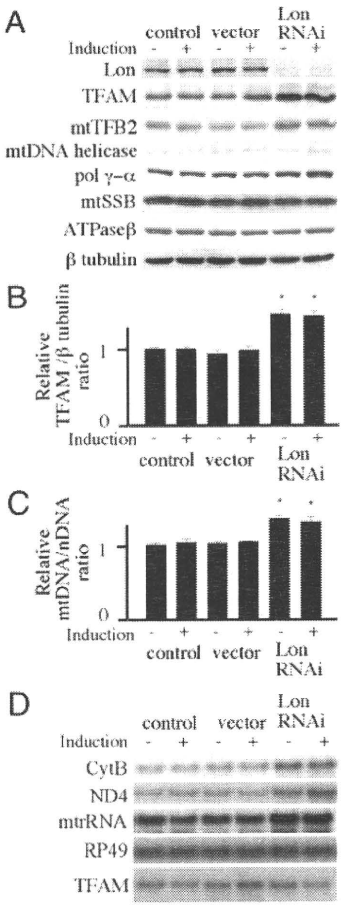

Fig. 2. Expression of *Drosophila* Lon-targeted RNAi in Schneider cells. Schneider cells with no plasmid (control) or carrying pMt/Hy (vector) or pMt/invLon/Hy (Lon RNAi) were cultured for 10 d in the presence or absence of 0.2 mM CuSO4. (*A*) Immunoblot analysis was carried out as described in the legend to Fig. 1*A*. (*B*) The TFAM/β tubulin ratio was quantitated by normalizing TFAM protein levels to β tubulin protein levels as described under *Materials and Methods*. Error bars indicate means ± standard error of three independent experiments. The asterisk indicates $P < 0.05$ in comparison to control. (*C*) Relative mtDNA copy number was determined as described in the figure legend to Fig. 1*C*. Error bars indicate means ± standard error of three independent experiments. The asterisk indicates $P < 0.05$ in comparison to control. (*D*) Northern blot analysis using *12S rRNA, ND4, Cytb, RP49*, and *TFAM* was carried out as described in the legend to Fig. 1*D*.

tein in the knockdown cells does not result from an increase in the steady-state level of TFAM transcripts.

Lon Regulates mtDNA-Dependent TFAM Degradation in Schneider Cells. Our data show that Lon regulates TFAM levels and mtDNA copy number. Because of the interdependent relationship of TFAM and mtDNA, we asked whether or not Lon degrades TFAM directly. To do so, we cultured the cell lines in the presence of ethidium bromide (EtBr), an inhibitor of mtDNA replication. In the control cells, EtBr treatment results in a rapid reduction of mtDNA copy number, and TFAM protein levels were also reduced, albeit more slowly than that of mtDNA (Fig. 3 *A*, *B*). At the same time, TFAM mRNA levels were unchanged (Fig. S3). Because the levels of other mitochondrial nucleoid proteins were not affected by mtDNA depletion with EtBr, we conclude that TFAM is depleted selectively following mtDNA reduction. In the Lon overexpression cells, EtBr treatment also resulted in the reduction of mtDNA (Fig. 3*B*). Interestingly, here the reduction of TFAM was faster than in the control cells (Fig. 3*A*). EtBr treatment also caused mtDNA depletion in the Lon knockdown cells. However in this case, the relative level of TFAM protein was increased 1.2-fold (Fig. 3*A*), while again TFAM mRNA levels were unchanged (Fig. S3). Similar to the control cells, the levels of other mitochondrial nucleoid proteins were unchanged in the EtBr treated Lon knockdown cells.

To demonstrate conclusively the proteolytic role of Lon in mtDNA-dependent TFAM degradation, we established a cell line expressing a Lon mutant carrying a S880A amino acid substitution, in which the conserved serine in the proteolytic active site

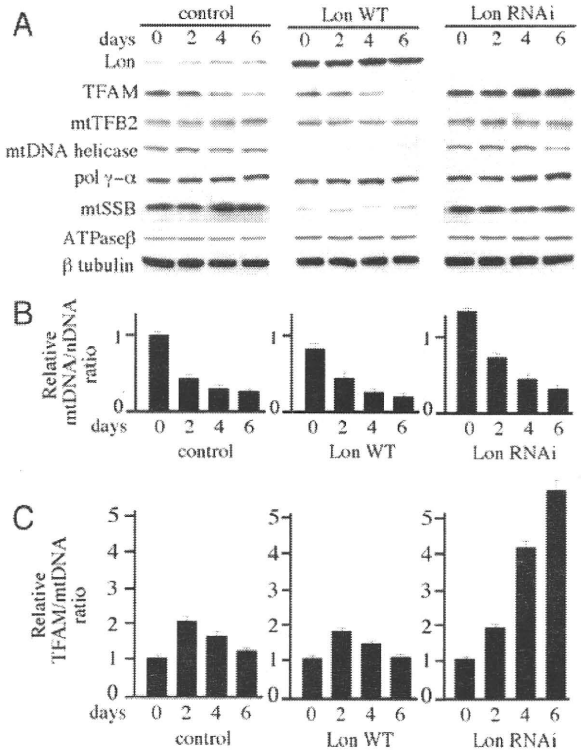

Fig. 3. Dynamics of mitochondrial nucleoid proteins during mtDNA depletion in Lon overexpressing or knockdown Schneider cells. Schneider cells with no plasmid (control) or carrying pMt/Lon/Hy (Lon WT) or pMt/invLon/Hy (Lon RNAi) were cultured for 6 d in the presence of 200 ng/mL EtBr. The cells were harvested prior to EtBr treatment (0 d) and after 2, 4, and 6 days of EtBr treatment. (*A*) Immunoblot analysis was carried out as described in the legend to Fig. 1*A*. (*B*) Relative mtDNA copy number was determined as described in the legend to Fig. 1*C*. Error bars indicate means ± standard error of two independent experiments. (*C*) The TFAM/ mtDNA ratio was quantitated by normalizing TFAM levels to relative mtDNA copy number. Error bars indicate means ± standard error of two independent experiments.

was replaced by alanine. The cell line expressing Lon S880A showed severe retardation of TFAM degradation following mtDNA depletion, likely because overexpression of Lon S880A results in a dominant negative phenotype that is caused by the formation of mixed oligomeric forms (Fig. S4). Taken together, our data show clearly that Lon is responsible for specific degradation of TFAM.

What Is the Physiological Role of Lon Degradation of TFAM? We sought to investigate the functional significance of the specific degradation of TFAM in mtDNA-depleted cells. In control cells treated with EtBr, the TFAM:mtDNA ratio was raised transiently, and then reverted to normal levels within 6 d (Fig. 3*C*). However, Lon knockdown cells did not recover a normal TFAM:mtDNA ratio. In mammalian cells, overexpression of TFAM causes suppression of mitochondrial transcription (36). We confirmed this phenomenon in Schneider cells overexpressing TFAM under the control of the metallothionein promoter (Fig. S5), and find that suppression occurs in cells containing a TFAM:mtDNA ratio >2 (Fig. 4). Interestingly, the highest overexpression level showed depletion of mtDNA copy number in addition to transcriptional suppression. We thus hypothesized that Lon regulates mtDNA transcription by stabilizing the cellular TFAM:mtDNA ratio. To document this hypothesis, we measured mitochondrial transcript levels in mtDNA-depleted cells. Because EtBr also inhibits mtDNA transcription, we instead induced mtDNA depletion by knockdown of mtDNA replication factors and in particular, the catalytic subunit of mitochondrial DNA polymerase and the mtDNA helicase. Control and Lon knockdown cells were cultured for 10 d with dsRNA targeted against these proteins or GFP as a control, and the relevant protein levels were then evaluated by immunoblotting (Fig. 5*A*). The cells cultured with GFP dsRNA showed no change in these protein levels, whereas in the cell lines cultured with the mtDNA helicase or mtDNA polymerase dsRNAs, mtDNA copy number was reduced to ~60% of that in the control cells (Fig. 5*B*). After the dsRNA treatments, the TFAM levels decreased to 75% in control cells, but increased 1.2-fold in the Lon knockdown cells (Fig. 5*A*). After the dsRNA treatments, the TFAM:mtDNA ratio in the control cells was increased ~1.3-fold relative to the control cells alone, and the ratio in the Lon RNAi cells was increased ~2.5-fold (Fig. 5*C*). Mitochondrial transcripts in the control cells were unchanged with or without dsRNA treatment (Fig. 5*D*). However, the transcript levels in mtDNA-depleted Lon RNAi cells were reduced to 47%–60% of those in the mtDNA-depleted cells that showed transcript levels equivalent to 66%–84% of the

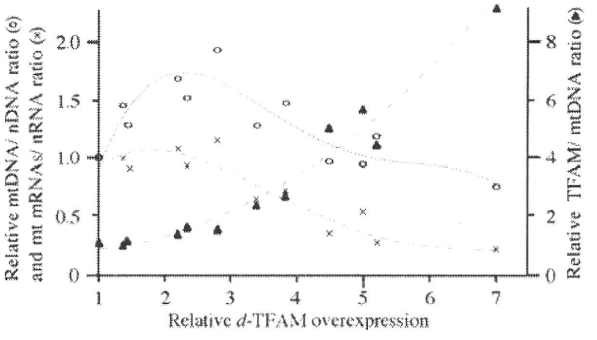

Fig. 4. Expression of TFAM in Schneider cells. Relative ratio of mtDNA copy number (*open circles, solid line*), mt mRNAs/nRNA (*crosses, dotted line*), and TFAM/mtDNA (*filled triangles, dashed line*) were measured at different overexpression levels of TFAM in Schneider cells as indicated. Relative mtDNA copy number was determined as described in the legend to Fig. 1*C*. The mt mRNAs/nRNA ratio was quantitated by normalizing mitochondrial transcript abundance (*ND4* and *Cyt b*) to that of nuclear *Rp49*. TFAM/mtDNA ratio was quantitated by normalizing TFAM protein levels to relative mtDNA copy number.

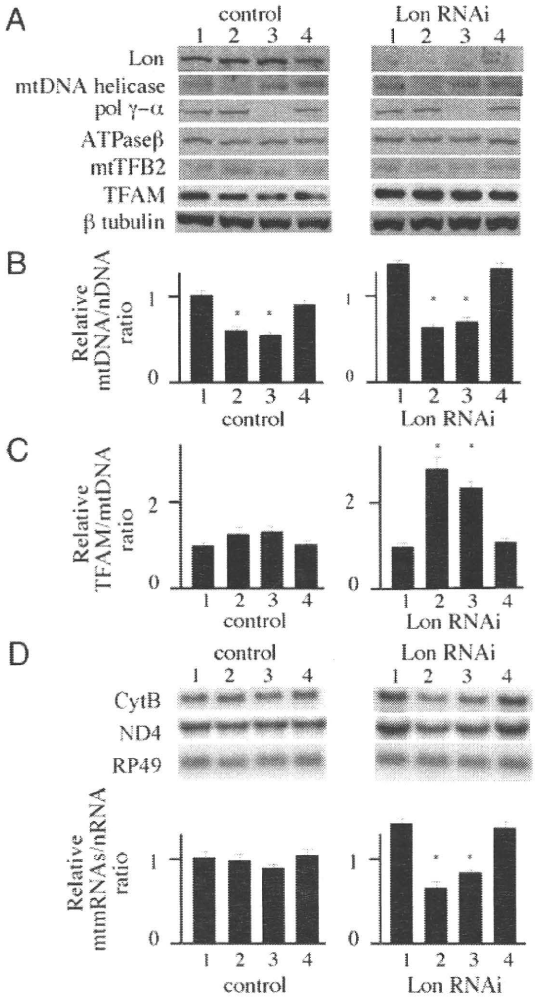

Fig. 5. Effects of Lon knockdown on mitochondrial transcript levels after mtDNA depletion in Schneider cells. Schneider cells carrying no plasmid (control) and or pMt/invLon/Hy (Lon RNAi) were cultured for 10 d in the presence or absence of dsRNA of mtDNA helicase, pol γ-α, or GFP as control. (*A*) Immunoblot analysis was carried out as described in the legend to Fig. 1*A*. (*B*) Relative mtDNA copy number was determined as described in the legend to Fig. 1*C*. Error bars indicate means ± standard error of two independent experiments. The asterisk indicates $P < 0.05$ in comparison to the control. (*C*) The ratio of TFAM/mtDNA was determined as described in the legend to Fig. 3*C*. Error bars indicate means ± standard error of two independent experiments. The asterisk indicates $P < 0.05$ in comparison to each cell that was cultured in the absence of dsRNA. (*D*) Northern blot analysis using *ND4, Cytb, and RP49* was carried out as described in the legend to Fig. 1*D*. Relative mitochondrial mRNA levels were quantitated by normalizing *ND4* and *Cytb* abundance to that of RP49. Error bars indicate means ± SE of two in dependent experiments. The asterisk indicates $P < 0.05$ in comparison to the control.

control cells. Interestingly, the reduced mitochondrial transcript levels were lower than that of control cells. Together, these results indicate that Lon regulates mitochondrial transcription by controlling the TFAM:mtDNA ratio.

Discussion

We have established that Lon knockdown cell lines express < 10% of endogenous Lon protein levels, yet these cells grow for at least 6 mo. In human fibroblast cells, depletion of Lon over 4 d resulted in apoptotic cell death (37, 38), whereas Lon knockdown in human colon carcinoma cells allows survival for at least 15 d (18). Thus, the effects of Lon depletion may be species- or cell type- specific. As in a recent report with human rhabdomyosarcoma cells (39), depletion of Lon protease in *Drosophila* Schneider cells results in an increase in the levels of oxidized proteins in mitochondria, indicating that Lon is responsible for degradation of oxidized mitochondrial proteins, and suggesting that variations in cell viability as a result of Lon depletion may reflect varying cellular tolerances for oxidative damage to mitochondrial proteins.

It is known that TFAM protein levels are reduced coincident with mtDNA depletion in animal cells, but the mechanism for this phenomenon is unclear (31). Here, we show that Lon is responsible for degradation of TFAM upon mtDNA depletion. Moreover, Lon may be also responsible for TFAM degradation under normal conditions, because the cellular level of TFAM varies in concert with Lon levels. These findings imply that TFAM turnover is strongly dependent on Lon protease function. In addition to TFAM, we found that the mtTFB2 level is increased in Lon knockdown cells, so it may also be a specific substrate for the Lon protease. Other mitochondrial biogenesis proteins tested were unchanged appreciably in either Lon knockdown or overexpression cells. In *Escherichia coli*, some substrates for Lon overlap those of other AAA^+ proteases such as the ClpP protease; notably, the ClpP ortholog in animal cells comprises another major protease in the mitochondrial matrix space (2, 3, 40). Therefore, the depletion of mitochondrial Lon may be compensated partially by mitochondrial ClpP protease. Alternatively, the residual Lon in knockdown cells may be sufficient to degrade its protein targets.

We found that mitochondrial transcripts in Lon knockdown cells are increased moderately in association with an increase of TFAM and mtTFB2 levels. Previous studies showed that the relative levels of mtDNA and TFAM are not critical to observe stimulation of mitochondrial transcription in *Drosophila* Kc167, and Schneider cells (29, 35). We suggest that in contrast, increase of mtTFB2, which is essential for mitochondrial transcription, may be responsible for up-regulation of mitochondrial transcription.

We show that degradation of TFAM by Lon protease is facilitated by mtDNA depletion. Interestingly, a similar phenomenon was reported in *Bacillus subtilis*. *B. subtilis* LonA, which is the ortholog of Lon, is involved in degradation of the structural maintenance of chromosomes protein (SMC), and the degradation of SMC is facilitated by DNAase treatment (41). Although the mechanisms by which Lon recognizes its target proteins are not well understood, a recent report showed that *E. coli* Lon recognizes specific aromatic residue-rich sequences that are hidden in the hydrophobic cores of native structures, but are accessible in unfolded structures (42). Interestingly, the HMG boxes in TFAM contain four conserved aromatic residues within a hydrophobic core, and these residues may be masked when TFAM binds DNA (25, 27, 29). Another possible explanation is that TFAM not bound to mtDNA becomes exposed to oxidative stress, whereas TFAM bound to mtDNA comprises part of the core of the mitochondrial nucleoids, and is thus surrounded by other proteins (43). Mitochondrial Lon has the ability to bind DNA and localizes in mitochondrial nucleoids (15–18). Our current hypothesis is that excess, free TFAM is degraded by Lon before the TFAM binds DNA. Alternatively, it seems possible, albeit more complicated, that excessive DNA compaction resulting from binding of high TFAM levels signals the degradation of TFAM by DNA-bound Lon. Further experiments are warranted to address these and other possibilities, and to clarify the link between the DNA-binding activity of Lon and TFAM turnover.

Suzuki and colleagues showed that mtDNA copy number was unchanged after 15 d of Lon knockdown in human colon carcinoma cells (18). This model differs from ours, in which we observe an increase in mtDNA copy number. We established our Lon knockdown cells over a period of 8 wks and because of leaky expression from the inducible promoter in the RNAi vector, Lon depletion was already ongoing prior to the 10 d induction

period. Thus, one possible explanation for the difference we observe is that TFAM accumulation may be slower in their model and consequently, increased mtDNA copy number might not have been apparent. Another possible explanation is that compensation by other proteases such as ClpP (2, 40) might effect TFAM degradation in the Lon-depleted human colon carcinoma cells.

We found that upon EtBr treatment, the TFAM:mtDNA ratio is nearly restored within 6 d in both control and Lon overexpressing cells, whereas restoration did not occur in Lon knockdown cells. Moreover, TFAM turnover resulting from mtDNA depletion by EtBr treatment is strongly reduced in cells overexpressing a protease-deficient Lon variant, which shows a dominant negative effect. Similar results involving restoration of normal TFAM/ mtDNA ratios were produced in the case of knockdowns of mtDNA helicase and mtDNA polymerase, though there the reduction of mtDNA is lower as compared to that upon EtBr treatment. Until stabilization occurs, excess overexpression of TFAM results in reduction of mitochondrial transcript levels; notably, in TFAM overexpressing cells, ratios of TFAM:mtDNA >2 show inhibitory effects on mitochondrial transcription. In the case of knockdown of mtDNA helicase or mtDNA polymerase, mitochondrial transcription levels are unchanged in control cells upon mtDNA depletion, whereas Lon knockdown cells showed a similar reduction in mitochondrial transcripts. Upon knockdown of mtDNA helicase or mtDNA polymerase, the TFAM:mtDNA is ~1.3 in control cells and >2 in Lon knockdown cells, consistent with the observations in TFAM overexpression cells. Because there are environmental conditions under which mtDNA copy number is known to vary, such as during development and upon drug treatment (44, 45), transient Lon degradation of excess TFAM would be important to maintain normal mitochondrial transcription levels. We conclude that the TFAM:mtDNA ratio is crucial for both mtDNA biogenesis and homeostasis, and that Lon stabilizes the TFAM:mtDNA ratio by degradation of excess TFAM. With the example of TFAM, we provide direct evidence of the physiological role of Lon protease activity in mtDNA maintenance in animal cells, warranting future study of its potential involvement in the surveillance and turnover of other proteins involved in mtDNA replication, transcription, and translation.

Materials and Methods

Preparation of Lon Antibody. A recombinant protein corresponding to amino acids Asp613 to Ser838 of *Drosophila* Lon (CG8798) was used to immunize rabbits to obtain polyclonal antibody.

Preparation of Inducible Plasmids Expressing Lon, TFAM, and Lon-Targeted RNAi. The plasmid pMt/Lon/Hy, in which Lon is regulated by the metallothionein promoter and the plasmid pMt/invLon/Hy, which carries an inverted repeat of a nucleotide sequence from *Lon* cDNA that is transcribed from the metallothionein promoter were constructed as described in the *SI Text*. Plasmid pMt/TFAM/Hy is as described previously (35).

Generation and Induction of Stable Cell Lines. *Drosophila* Schneider S2 cells were cultured at 25 °C in *Drosophila* Schneider Medium (Invitrogen) supplemented with 10% FBS. Cells were subcultured to 3×10^6 cells/mL every fifth day. Cells were transfected using Effecten (QIAGEN). Hygromycin-resistant cells were selected with 200 μg/mL hygromycin. Cells were passaged for 8 wks in hygromycin-containing medium and then cultured in standard medium. The cell lines were grown to a density of 3×10^6/mL and then treated with 0.2 mM $CuSO_4$ to induce expression from the metallothionein promoter.

Immunoblotting. Total cellular protein (20 μg per lane) was fractionated by 13.5%, 10.5%, or 7.5% SDS-PAGE and transferred to nitrocellulose filters. Immunoblotting was performed as described previously (46). Protein bands were visualized using ECL Western blotting reagents (Amersham). ECL luminescence was quantified on a Kodak Image Station 4000R. Antibodies against *Drosophila* mtSSB (47), TFAM (29), Pol γ-α (48), mtDNA helicase (32), ATPase β (32), mtTFB2 (35), and β tubulin (E7) (Developmental Studies Hybridoma Bank) were prepared and used as described. The ratio of the signals for TFAM and β tubulin was used to estimate the relative protein levels of TFAM. The TFAM immunoblotting experiments shown in Figs. 1, 2, 3, and 5 were performed two or thee times with each of the two or three independent cell lines carrying each plasmid construct, including control (no plasmid). The data presented represent one such experiment, and the quantitation is provided for the duplicate or triplicate experiments from one of two or thee cell lines.

Northern and Southern Blotting. Northern blotting was performed as described previously (46), and the data were analyzed using a PhosphoImager (Molecular Dynamics). The signal for *RP49* was used to normalize mitochondrial transcripts.

Southern blotting was performed as described previously (32), and the data were analyzed using a PhosphoImager (Molecular Dynamics). Blots were probed with radiolabeled DNAs for the mitochondrial gene *Cytb* and the nuclear histone gene cluster. The ratio of the signals for these two genes was used to estimate the relative copy number of mtDNA. The Northern and Southern blot experiments shown in Figs. 1, 2, 3, 4, and 5 were performed two or thee times with each of the two or three independent cell lines carrying each plasmid construct, including control (no plasmid). The data presented represent one such experiment, and the quantitation is provided for the duplicate or triplicate experiments from one of two or thee cell lines.

RNA Interference. To generate double-stranded RNA (dsRNA) for RNAi, sequences directed against the protein to be silenced were amplified by PCR from each cDNA. Each primer used in the PCR contained a T7 RNA promoter followed by sequences specific for the targeted genes. The following primer sets were used for each protein: mtDNA helicase for GTAATACGACTCACTATAGGGCATGGAAAATGAGACGCGC and GTAATACGACTCACTATAGGGGATTGCTGACTAGAACCGC; DNA polymerase γ-α for TAATACGACTCACTATAGGGTGCCTACGCCTGCGGTGAGC and TAATACGACTCACTATAGGGCTCCAATGCTCGACTAAGAC; GFP for GTAATACGACTCACTATAGGGGGAGAAGAACTTTTCACTGG and GTAATACGACTCACTATAGGGTCTGCTAGTTGAACGCTTC. PCR products were used as templates for in vitro transcription using the T7 Megascript RNAi kit (Ambion). 3×10^7 S2 *Drosophila* tissue culture cells were plated into a T25 flask in 5 mL of medium without FBS. 100 μg of dsRNA was added and mixed by swirling. After 30 min, 5 mL of media containing 20% FBS was added. The cells were collected after 5 d culture and repeat dsRNA treatment. After 5 d after second dsRNA treatment, the cells were harvested for the analysis.

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Supporting Information

Matsushima et al. 10.1073/pnas.1008924107

SI Text

SI Materials and Methods. ***Preparation of inducible plasmids expressing cMyc-Lon and the Lon S880A mutant.*** The plasmid pMt/Lon/Hy, in which Lon is regulated by the metallothionein promoter, was constructed as follows: a fragment of *Drosophila* Lon cDNA was amplified by PCR using as 5′-primer 5′- GGGCTCGAGTGCGAGTGGATATTGCTTTC -3′ and as 3′-primer 5′- GCGCACTAGTATTACAAGTCTTCTTCAGAAATAAGCTTTTGAGAATAAGGCCACGTCTC -3′. The PCR fragment was cleaved by XhoI and SpeI and subcloned.

pMt/S880A/Hy was constructed from pMt/Lon/Hy by site directed mutagenesis using the following pair of primers: 5′-AGATGGCCCCGCTGCGGGCATC -3′ and 5′- GATGCCCGCAGCGGGGCCATCT -3′.

Detection of carbonylated proteins. The OxyBlot procedure (Millipore) was used to perform immunoblot detection of oxidatively modified proteins by the generation of carbonyl groups. Ten micrograms of protein were used for each reaction. Carbonyl groups in mitochondrial protein samples (10 μg) were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine. Carbonylated proteins were detected by immunoblot analysis using an anti-DNP antibody.

Indirect immunofluorescence. Indirect immunofluorescence was performed as described (1) with anti-c-Myc monoclonal antibody (Sigma) and Alexa Fluor 488 anti-mouse IgG (Molecular Probes).

Preparation of inducible plasmids expressing Lon, TFAM, and Lon-Targeted RNAi. The plasmid pMt/Lon/Hy, in which Lon is regulated by the metallothionein promoter, was constructed as follows: a fragment of *Drosophila* Lon cDNA was amplified by PCR using as 5′-primer 5′- GGGCTCGAGTGCGAGTGGATATTGCTTTC -3′ and as 3′-primer 5′- GCGCACTAGTCTAAGAATAAGGCCACGTCTC -3′. The PCR fragment was cleaved by XhoI and SpeI and subcloned. The plasmid pMt/TFAM/Hy was as described previously (2). The plasmid pMt/invLon/Hy carries an inverted repeat of a nucleotide sequence from *Lon* cDNA that is transcribed from the metallothionein promoter. The insert in pMt/invLon/Hy was generated from two PCR-amplified fragments of *Lon* cDNA. One fragment has terminal XhoI and EcoRI sites and was prepared using the following pair of primers: 5′- GCGCCTCGAGACTAGTGGGATGATTCCAACGGGGAT -3′ (forward) and 5′- GCGCGAATTCGGGATCGATTCCGCTTGATCAGTGCTTTG -3′(reverse). A second fragment has terminal SpeI and EcoRI sites and was prepared using the primers 5′- GCGCCTCGAGACTAGTGGGATGATTCCAACGGGGAT -3′ (forward) and 5′- GCGCGAATTCAAAAAGCTTTCCGCTTGATCAGTGCTTTG -3′ (reverse). The two PCR products were ligated and cloned into the pMt/Hy vector cleaved with XhoI and SpeI.

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2. Matsushima Y, Garesse R, Kaguni LS (2004) *Drosophila* mitochondrial transcription factor B2 regulates mitochondrial DNA copy number and transcription in schneider cells. *J Biol Chem* 279:26900–26905

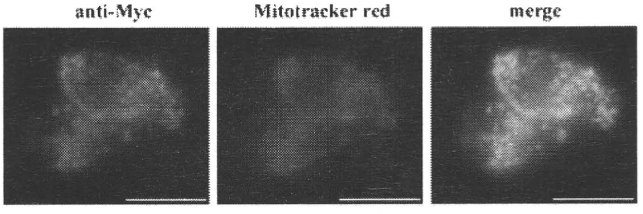

Fig. S1. Overexpression of *Drosophila* Lon in Schneider cells. Immunocytochemistry was performed on Schneider cells that were transiently transfected with pMK/Lon-Myc/Hy using anti-c-Myc monoclonal antibody (Sigma), and counterstained with Mitotracker Red.

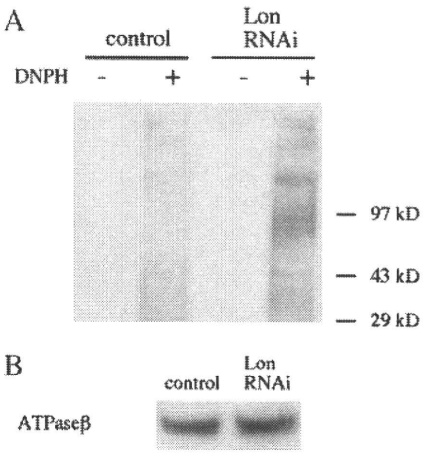

Fig. S2. Protein oxidation status in mitochondria from Lon knockdown cells. Mitochondrial fractions were prepared from Schneider cells carrying no vector (control) or pMt/invLon/Hy (Lon RNAi). (*A*) Detection of oxidized protein was performed using the Oxyblot protocol (Millipore). Mitochondrial protein extracts (10 μg) were incubated in the presence or absence of 2,4-dinitrophenylhydrazine (DNPH) to derivatize protein carbonyl groups. After fractionation by 4%–10% gradient SDS-PAGE, the proteins were transferred to a nitrocellulose filter, and oxidized proteins were detected with antibody against DNP. (*B*) Mitochondrial protein extracts (10 μg) were fractionated by 12% SDS-PAGE, transferred a nitrocellulose filter and probed with affinity-purified rabbit antiserum against ATPase β as a control.

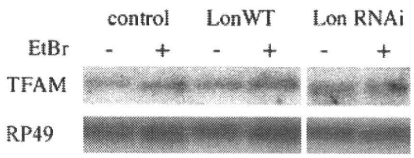

Fig. S3. Unchanged TFAM mRNA levels in Schneider cells upon EtBr treatment. After 6 d culture, Total RNA was isolated from Schneider cells with no plasmid (control) or carrying pMt/Lon/Hy (Lon) or pMt/invLon/Hy (Lon RNAi) that were cultured for 6 d in the presence or absence of 200 ng/mL EtBr. Northern blot analysis using *TFAM* and *RP49* probes was carried out as described in the legend to Fig. 1*D*.

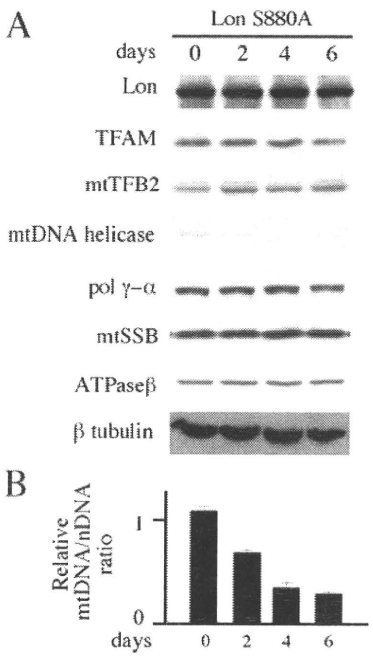

Fig. S4. Steady-state levels of mitochondrial nucleoid proteins during mtDNA depletion in Lon S880A- overexpressing Schneider cells. Schneider cells carrying pMt/S880A/Hy (S880A) were cultured for 6 d in the presence of 200 ng/mL EtBr. The cells were harvested prior to and after EtBr treatment at 0, 2, 4, and 6 days. (*A*) Immunoblot analysis was carried out as described in the legend to Fig. 1*A*. (*B*) Relative mtDNA copy number was determined as described in the legend to Fig. 1*C*.

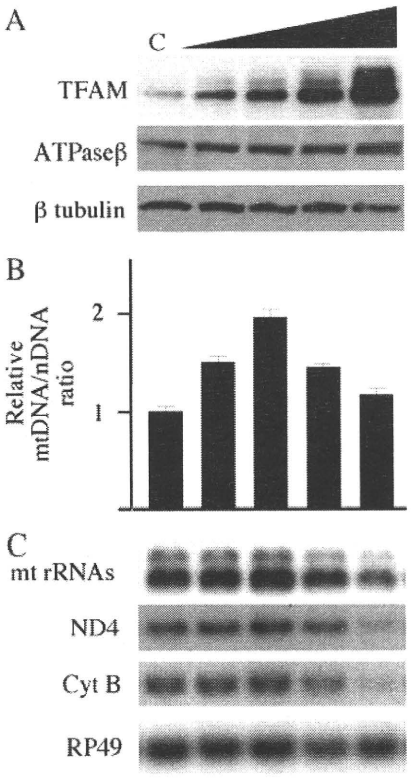

Fig. S5. Expression of *Drosophila* TFAM in Schneider cells. Schneider cells (*C*) or cells carring pMt/TFAM/Hy were grown for 10 d in the presence of 0, 0.05, 0.1, or 0.4 mM CuSO4. (*A*) Immunoblot analysis was carried out as described in the legend to Fig. 1*A*. (*B*) Relative mtDNA copy number was determined as described in the legend to Fig. 1*C*. Error bars indicate means ± standard error of two independent experiments. (*C*) Northern blot analysis using *12S rRNA, ND4, Cytb*, and *RP49* was carried out as described in the legend to Fig. 1*D*.

Reversible Infantile Respiratory Chain Deficiency: A Clinical and Molecular Study

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Objective: To characterize the clinical features and clarify the pathogenicity of "benign cytochrome *c* oxidase deficiency myopathy."
Methods: The study included 8 patients with the phenotype of this disease. Six patients underwent muscle biopsies and all the 8 underwent mitochondrial DNA analyses. To confirm the pathogenicity of the detected mitochondrial DNA mutation, we performed northern blot analysis, using muscle specimens, and blue native polyacrylamide gel electrophoresis and respiratory chain enzyme activity assay of transmitochondrial cell lines (cybrids).
Results: Clinical symptoms were limited to skeletal muscle and improved spontaneously in all cases; however, 2 siblings had basal ganglia lesions. In all patients, we identified a homoplasmic m.14674T>C or m.14674T>G mitochondrial transfer RNA-glutamate mutation. Northern blot analysis revealed decreased levels of mitochondrial transfer RNA-glutamate molecules. Muscle specimens and cybrids derived from patients showed decreased activity of respiratory complexes IV, and/or I, III; however, this was normal in naive myoblasts.
Interpretation: Identification of a novel m.14674T>G mutation in addition to m.14674T>C indicated the importance of this site for disease causation. Analyses of cybrids revealed the pathogenicity of m.14674T>C mutation, which resulted in defects of cytochrome *c* oxidase and multiple respiratory chain enzymes. Furthermore, patients with basal ganglia lesions provided new insights into this disease, in which only skeletal muscle was thought to be affected. Normal respiratory chain enzyme activities in naive myoblasts suggested the compensatory influence of nuclear factors, which may be a clue to understanding the mechanisms of spontaneous recovery and low penetrance in families carrying the mutation.

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Mitochondrial cytochrome *c* oxidase (COX) a multisubunit assembly present in the inner membrane. is responsible for the terminal event in electron transport.[1] Deficiency of this enzyme is one of the most frequent and clinically heterogeneous causes of respiratory chain deficiency.[1–3] An increasing number of genetic defects have been identified in both mitochondrial DNA (mtDNA) and nuclear DNA.[4–12] The onset of COX deficiency is variable, and most patients show progressive clinical symptoms.

Benign infantile COX deficiency, known as "benign COX deficiency myopathy," is very rare and distinct because of its unusual disease course, which is characterized by clinical and pathological improvement. These patients have lactic acidosis, hypotonia, hyporeflexia, and generalized muscle weakness from early infancy—features similar to those seen in the classical fatal infantile form.[13,14] The symptoms, however, improve spontaneously after 1 year of age.[15–20] The molecular defect in this disease was previously unknown; however, Horvath et al.[21] have recently reported a single homoplasmic m.14674T>C mutation.

In this study, we investigated 8 patients with benign infantile COX deficiency to evaluate its clinical and pathological characteristics and disease outcome. We detected

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Additional Supporting Information can be found in the online version of this article.

the reported homoplasmic mutation in 6 patients and identified a novel T to G base change at the same nucleotide point (np) in 2 patients. Furthermore, we performed biochemical analyses on the transmitochondrial cell lines (cybrids) to confirm the pathogenicity of the mutation and influence of nuclear factors.

Patients and Methods

Case Reports

Subjects of this study were 8 (3 male and 5 female) benign infantile COX deficiency patients (Table 1). Patient 7 and 8 were siblings. Patient 4 has been described previously in a Japanese report.[20] Six patients underwent muscle biopsies and the muscle specimens for patients 7 and 8 were not available. We obtained written informed consent to perform muscle biopsies, biochemical studies, and/or molecular analyses from parents of all patients.

None of the patients' parents were consanguineous. All patients were born uneventfully after a normal pregnancy. All patients presented as "floppy infants" with muscle weakness and hypotonia from early infancy, but symptoms improved with age, particularly after 1 year of age. The follow-up period ranged from 18 months to 15 years. We documented developmental milestones, history of tube feeding and respirator management, complications (short stature, hearing loss, renal dysfunction, liver dysfunction, cardiac failure, diabetes mellitus, seizure, and mental status), laboratory data (lactate levels in blood and cerebrospinal fluid [CSF], and creatinine kinase [CK] in blood), and neuroimaging.

Histopathological Studies

Muscle biopsy from biceps brachii was performed at mean age of 5.6 months (range, 3–9 months). Serial frozen sections were stained with hematoxylin and eosin, modified Gomori trichrome, succinate dehydrogenase (SDH), COX, and ATPase stains.

mtDNA Analysis

For mtDNA analysis, total DNA was extracted from muscle specimens of 6 patients (patients 1–6) and lymphocytes of patients 7 and 8. Southern blot and long polymerase chain reaction (PCR) analyses were performed to detect mtDNA rearrangements, as described previously.[22,23] Total mtDNA sequencing was performed as described previously.[24] The sequence data were compared with the Human Mitochondrial DNA Revised Cambridge Reference Sequence (MITOMAP; http://www.mitomap.org). To confirm m.14674T>C and m.14674T>G mutations by restriction fragment length polymorphism (RFLP) analysis, PCR fragments were digested by *BclI* and *NlaIII*, respectively (see the section on RFLP analysis in Supporting Information Methods).

Mitochondrial Transfer RNA Analysis

For transfer RNA (tRNA) analysis, total RNA was extracted by Isogen (Nippon Gene, Toyama, Japan) from muscle specimens, naive myoblasts, and cybrids obtained by fusing enucleated myoblasts of patient 6 carrying m.14674T>C mutation with human osteosarcoma 143B/TK-cells lacking mtDNA as described previously.[25]

To evaluate the molecular size and quantity of tRNA-glutamate ($tRNA^{Glu}$), we performed northern blot analysis using total RNA extracted from muscle specimens of patients 3 and 4, and normal infants. Patients 3 and 4 had the m.14674T>G mutation and m.14674T>C mutation, respectively. Samples of total RNA were electrophoresed and northern hybridization was performed using a DNA probe specific for mitochondrial $tRNA^{Glu}$. $tRNA^{Glu}$ amounts were normalized by the amount of mitochondrial tRNA-Leu (UUR) and nuclear-encoded 5S ribosomal RNA (rRNA)[26] (see the section on Quantification of $tRNA^{Glu}$ in Supporting Information Methods).

To evaluate aminoacylation levels of tRNA, we performed acid polyacrylamide gel electrophoresis (PAGE). Total RNA samples were extracted from cybrids and 143B/TK-cells (normal control), prepared to be aminoacyl-tRNAs or forcibly deacylated tRNAs according to the literature,[27] and electrophoresed to separate aminoacyl-$tRNA^{Glu}$ and uncharged $tRNA^{Glu}$ (see the section on acid PAGE in Supporting Information Methods).

Enzymatic Activity of Respiratory Chain Complexes of Naive Myoblasts, Cybrids, and Muscle Specimens

Enzymatic activity of individual mitochondrial respiratory complexes was determined using isolated mitochondria obtained from cultured primary myoblasts and cybrids derived from patient 6, according to Trounce et al.[28] with modifications. Skeletal muscle specimens from patients 3, 4, and 6 were also available. The activities of complexes I, II, III, and IV and citrate synthase (CS) were measured using spectrophotometric assays. All samples were measured at least in duplicate and averaged.

Blue Native PAGE and Western Blot for Immunodetection

Mitochondrial proteins were isolated from cultured 143B/TK cells and cybrids derived from patient 6.[29] The mitochondrial proteins (100μg) were solubilized in sample buffer containing 0.5% (w/v)*n*-dodecyl-β-D-maltoside. Electrophoresis was performed on 3% to 12% polyacrylamide gels (Invitrogen, Carlsbad, CA).[29,30] Following blue native PAGE (BN-PAGE), gels were blotted on polyvinylidene fluoride membranes using the iBlot transfer system (Invitrogen). Subunit-specific mouse monoclonal antibodies (Molecular Probes, Eugene, OR) were used to immunodetect protein complexes. The cocktail of primary antibodies included 39kDa (complex I) (0.5 μg/mL), 70kDa (complex II) (0.5μg/ml), core II (complex III) (0.5 μg/ml), subunit I (complex IV) (2.5 μg/ml), and subunit β(complex V) (0.5 μg/ml). After removing the cocktail of primary antibodies, alkaline phosphatase-conjugated anti-mouse secondary antibody was reacted and nitroblue tetrazolium chloride–derived chromogenic detection was performed.

TABLE 1: Clinical and Laboratory Findings at Muscle Biopsy or DNA Analysis

Patient	Age at Muscle Biopsy (mo)	Sex	Clinical Diagnosis before Muscle Biopsy	Main Complaints	High Arched Palate	Laboratory Findings			Muscle Pathology							Respiratory Chain Enzyme Activities									
						Serum CK (U/L) (n = 180)	Blood lactate (mg/ml) (n = 5–20)	CSF lactate (mg/ml) (n = 5–15)	RRF	SSV	COX Deficiency					Complex I		Complex II		Complex III		Complex IV		CS
											RRF	non-RRF Type 1	Small Artery	Muscle Spindle	Nerve	/mg Protein (%)	/CS (%)	/mg Protein (%)	/CS (%)	/mg Protein (%)	/CS (%)	/mg Protein (%)	/CS (%)	/mg Protein (%)
1	8	F	CMP	Muscle weakness	–	ND	12.5	8.3	+	–	All	+	–	NP	NP	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	5	F	CMP	Poor sucking	+	226	24	ND	+	–	All	+	–	NP	–	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	5	F	MMP	Failure to thrive	?	422	25.2	21.3	+	–	All	+	–	NP	–	105.7	50.2	106.6	52.5	108.8	52.5	44.6	23.1	193.2
4	3	F	MMP	Failure to thrive	–	203	149.3	50	+	±	All	+	–	NP	NP	72.4	39.7	145.6	82.7	118.6	66	54.1	32.3	167.5
5	4	M	MMP	Failure to thrive	–	604	23.5	11.1	+	–	All	+	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	9	M	CMD	Floppy infant	+	644	18.7	ND	+	–	All	+	–	–	–	68.5	39.1	125.6	74.4	124	71.9	63.7	39.7	160.7
7	ND	F	MMP	Poor sucking	?	279	81.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
8	ND	M	MMP	Floppy infant	?	182	24.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

F/M = female/male; CMD = congenital muscular dystrophy; CMP = congenital myopathy; COX = cytochrome c oxidase; MMP = mitochondrial myopathy; ND = not determined; NP = not present in sections; RRF = ragged-red fiber; SSV = strongly succinate-dehydrogenase–reactive blood vessels.