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Mitochondrial DNA deletions inhibit proteasomal activity and stimulate an autophagic transcript

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Abstract

Deletions within the mitochondrial DNA (mtDNA) cause Kearns Sayre Syndrome (KSS) and Chronic Progressive External Ophthalmoplegia (CPEO). The clinical signs of KSS include muscle weakness, heart block, pigmentary retinopathy, ataxia, deafness, short stature, and dementia. The identical deletions occur and rise exponentially as humans age, particularly in substantia nigra. Deletions at >30% concentration cause deficits in basic bioenergetic parameters, including membrane potential and ATP synthesis, but it is poorly understood how these alterations cause the pathologies observed in patients. To better understand the consequences of mtDNA deletions, we microarrayed 6 cell types containing mtDNA deletions from KSS and CPEO patients. There was a prominent inhibition of transcripts encoding ubiquitin-mediated proteasome activity, and a prominent induction of transcripts involved in the AMP kinase pathway, macroautophagy, and amino acid degradation. In mutant cells, we confirmed a decrease in proteasome biochemical activity, significantly lower concentration of several amino acids, and induction of an autophagic transcript. An interpretation consistent with the data is that mtDNA deletions increase protein damage, inhibit the ubiquitin-proteasome system, decrease amino acid salvage, and activate autophagy. This provides a novel pathophysiological mechanism for these diseases, and suggests potential therapeutic strategies.

Introduction

Mitochondrial deletions cause KSS, CPEO, and PS

Mitochondrial DNA deletions (Δ mtDNA), when present at concentrations of 30% and greater in muscle tissue, can cause three disorders, Kearns-Sayre Syndrome (KSS), Chronic Progressive External Ophthalmoplegia (CPEO), and Pearson's Syndrome (PS) [1-3]. Clinical signs include muscle weakness, exercise intolerance, droopy eyelids and weakness of ocular muscles (CPEO), plus heart block, pigmentary retinopathy, ataxia, deafness, and dementia (KSS), and sideroblastic anemia (PS).

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Diagnosis is usually confirmed by muscle histochemistry, showing ragged red fibers, and mosaic cytochrome oxidase negativity.

Deletions vary in size (1.3-8 kb) and position within the mitochondrial genome and occur with higher frequency at direct repeats [4,5]. The amount of the deleted mtDNA is the most important determinant of clinical severity, the size and location of deletion are not [6-8].

Origin of mtDNA deletions is twofold

mtDNA deletions are acquired through two main mechanisms. Single, sporadic deletions of mtDNA are thought to occur somatically early in development, as spontaneous errors of the gamma polymerase, and the identical deletion is observed in multiple tissues [9]. Alternatively, patients may inherit nuclear 'mutator' alleles in the germline of the mitochondrial polymerase gamma, the mitochondrial helicase twinkle, and the adenine nucleotide translocator, which promote multiple mtDNA deletions of various lengths and cause mendelian disorders dominated by CPEO [10-12].

mtDNA deletions also occur spontaneously and rise exponentially with normal aging

Deletions also occur and accumulate exponentially with human age, particularly in non-mitotic tissues [13]. Although in most tissues the level of mtDNA deletions is in the aged is less than 5% of the total, levels reach 5% in some aged muscles, and 50% in aged substantia nigra [14-18]. These deletions occur as spontaneous errors in individual cells as a consequence of age, and rise in concentration over time, with each cell containing a different predominant deletion species. Age-dependent mtDNA deletions have also been correlated with respiratory chain defects in muscle and substantia nigra [19-24].

The pathogenetic mechanism of KSS and CPEO is unclear

Although the occurrence of mitochondrial deletions was first documented in 1988 [1], and several experiments have shown that 'thresholds' of 30% (in patients) and 60 % (in HeLa cells) have to be surpassed for a biochemical effect to become evident [25,26], it is still uncertain how mitochondrial deletions trigger the pathophysiological cascade; even with the advent of a mouse model [27,28], the pathogenetic mechanism of KSS/CPEO is still unclear.

The two main pathophysiological mechanisms proposed for the occurrence of the CPEO/KSS/PS phenotypes involve a decline in mitochondrial bioenergetics [26] plus an increased production of reactive oxygen species (ROS), which may reinforce the bioenergetic defect through oxidative damage to DNA, proteins, and lipids [29-33].

However, in addition to their well-known role in respiration and oxidative phosphorylation, mitochondria are also sites for mitochondrial protein synthesis, and for such diverse cellular functions as apoptosis, heme, urea and amino acid biosynthesis, ion homeostasis, and fatty acid oxidation, all of which might be involved in the pathophysiological mechanism [34-37].

In order to clarify the cellular response to mtDNA deletions, we investigated by oligonucleotide microarray the global gene expression profile of nuclear encoded genes in muscle biopsies, myoblasts, fibroblasts, and lymphoblast cell lines from controls and patients with CPEO or KSS. We also investigated transmitochondrial cybrids with preneuronal NT2 or 143B osteosarcoma nuclear background and harboring different mtDNA deletions. The significance of the mechanisms suggested by the microarray data is discussed.

Materials and Methods

Cell lines and Culture conditions

We studied different cell types coming from patients harbouring a single 4.9 kb mtDNA deletion and from age and sex matched healthy subjects. Muscle cells were snapfrozen and kept at -80°C until RNA isolation. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 mM pyruvate, 5mg/ml uridine and 50 mg/ml gentamycin. Lymphoblasts were grown in RPMI-1640 medium (Life Technologies) supplemented with 15% FBS, 50mM sodium pyruvate, 0.5 mM uridine, 100 U/ml penicillin and 100 ug/ml streptomycin. Myoblasts were cultured in skeletal muscle basal medium (SkBM) supplemented with hEGF, 10% FBS and L-glutamine (Cambrex Bio Science Walkersville, Inc.). Transmitochondrial cybrids containing either NT2 or 143B osteosarcoma nuclear backgrounds were created as previously described [38]. Briefly, enucleation of mitochondria donor cells (lymphoblasts for NT2 cybrids and fibroblasts 143B cybrids) were carried out using the treatment of 15×10^6 cells with 10 ug/ml Cytochalasin-B for one hour at 37°C . Enucleated cells were mixed with mitochondrial-depleted cells (NT2 rho-zero and 143B rho-zero) and then treated with 0.2 ml 40% Poly Ethylene Glycol (PEG) for 45 seconds, suspended in DMEM media and plated. Two weeks later the visible colonies were expanded and checked for mtDNA deletion and nuclear background with at least three microsatellites. In order to exclude fusion specific effects on gene transcriptions, we used fusion control cell lines (FC) created by fusing rho-zero cells with mitochondria donor cells (lymphoblasts and fibroblasts, for NT2 and 143B cybrids respectively) bearing mtDNA with no disease-related mutations. 2 NT2 control cybrids and three 143B control cybrids (HGA13, HPC7 and H1959, kindly provided by A. Martinuzzi) were utilized, as described elsewhere [39]. 2 NT2 mutant cybrids harbouring the 4.9 kb 'common deletion', 3 143B mutant cybrids harbouring the 4.9 kb deletion (14.6, 51-18 kind gifts of Y.H. Wei and BH.10.59 from C. Moraes), 2 143B mutant cybrids harbouring a 7.5 kb deletion (16.3 and $\Delta\text{H.2.1}$ kind gifts from C. Moraes) and one 143B rho-zero cell line (kindly donated by M. Zeviani) were used. All cybrid cell lines were cultured in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM uridine, 100 U/ml penicillin and 100ug/ml streptomycin. Parental osteosarcoma 143B cells (1 line purchased from ATCC and 1 line kind gift from G. Attardi) were cultured in the same medium. All the cultured cells were kept in a humidified atmosphere containing 5% CO_2 at 37°C and frequently tested for mycoplasma infection using the mycosensor PCR testing protocol (Stratagene).

Oligonucleotide Microarray Analysis

Microarray analysis was performed as previously described [40]. Briefly, Total RNA was isolated from cell lines and frozen muscle tissues using a Qiagen RNeasy mini kit (Valencia, CA). Double-stranded cDNA was synthesized from 10 μg of purified total RNA using the SuperScript Choice System (Invitrogen, Gaithersburg, MD) and T₇-(dt)₂₄ primer (Genset, La Jolla, CA). The cRNA was prepared and biotin-labeled using the cDNA as a template for an in vitro transcription (ENZO Farmingdale, NY). The labeled cRNA was randomly fragmented by heating at 95°C for 35 minutes in a fragmentation buffer (40mM Tris Acetate, PH8.1, 100mM potassium acetate, and 30mM magnesium acetate) prior to hybridization to the chips. The chips were then washed, stained with avidin-labelled phycoerytherin and scanned with an Affymetrix GeneChip scanner. Raw data were processed by Microarray Suite 4.0 (Affymetrix) and microarray data files analyzed using DNA-chip Analyzer (dChip) software [41].

54 Affymetrix Hu95Av2GeneChips (Affymetrix, Santa Clara, CA) were utilized: 11 for muscle biopsies (7 patients and 4 controls), 4 for lymphoblasts (2 patients and 2 controls), 7 for fibroblasts (4 patients and 3 controls), 6 for NT2 cybrids (2 mutants in duplicate and 2 fusion

controls), 9 for 143B cybrids (2 mutants with 5kb deletion in duplicate, 2 mutants with 7.5kb deletion in duplicate and 3 fusion controls, two of them in duplicate) and 8 for 143B cells (2 for 143B rho zero and 6 for 2 parental 143B cell lines in triplicate). Differentially expressed genes were identified by comparing GeneChips designated as baseline (muscles, myoblasts, fibroblasts and lymphoblasts from healthy subjects, fusion control cell lines and parental 143B cells) with the ones that represent the experimental parameters (muscles, myoblasts, fibroblasts and lymphoblasts from patients, mutant cybrids and 143B rho zero cells) using a difference in mean fluorescence ≥ 30 and a p value < 0.05 (Table 1). To determine whether the significant altered genes deviated from random expectation with respect to biochemical pathway, cellular compartment or molecular function, we used the program Onto-Express (OE) [42].

Quantitative Real Time Reverse Transcriptase PCR (QRT-PCR)

The same total RNA utilized for microarray analysis was used to create cDNA for quantitative RT-PCR examination of 6 selected genes. Superscript reverse transcriptase II (Invitrogen, Gaithersburg, MD) was used and PCR was performed with Roche LightCycler (Roche Diagnostic, Indianapolis, IN), annealing at 58°C for 5 s and extension at 72°C for 15 s for 40 cycles. Samples were quantified by generating a standard curve of a synthesized target from the control cell lines. Each sample was run in triplicates and normalized to beta-actin expression.

Immunoblot and ELISA assays Immunoblot assay

The cell lysis buffer used for preparing total cell extracts was a modified RIPA buffer (50 mM Tris pH 7.4, 100 mM NaCl, 10mM EDTA, 1mM PMSF, 1% IGEPAL) supplemented with complete protease inhibitor cocktail (Sigma, St. Louis, MO) added just before use. Cells were washed twice with PBS and lysed for 30 min at 4°C. The homogenate was centrifuged for 30 minutes at $16,000 \times g$ and the supernatants were saved for immunoblot. Equal quantities of proteins, as determined by Bradford protein assay, were separated by electrophoresis in a 4-20% SDS-polyacrylamide gel (BioRad, Hercules, CA). Anti-syntaxin 16 (rabbit antiserum, Synaptic System) and anti-beta-actin (mouse monoclonal, Sigma) were used as primary antibodies with the suitable HRP-conjugated secondary antibodies. Bound antibodies on nitrocellulose (Amersham Biosciences) membranes were detected using the chemiluminescent substrate ECL (Amersham Biosciences). Densitometry analysis was performed using Quantity One software (Bio-Rad).

Measurement of proteasome activity

Quantitative *in vitro* analysis of 20S proteasome activity was performed by measuring the hydrolysis of the fluorogenic peptidyl substrate Suc-Leu-Leu-Val-Tyr-AMC (Calbiochem, La Jolla CA). Testing was assayed in 143B mutant cybrids with 7.5 kb mtDNA deletions and 143B fusion controls as well as in patient-derived lymphoblast cell lines harbouring a single 4.9 kb mtDNA deletion and lymphoblasts from age and sex matched healthy subjects. Low concentrations of sodium dodecyl sulfate (SDS) were added in order to induce conformational changes allowing access to the central cavity where the active sites residues reside. Briefly, approximately 3 million of cells were lysated and incubated for 1hr at 37°C in 500mM HEPS pH 7.6, 10 mM EDTA, 3% SDS and 200uM of the substrate suc-Leu-Leu-Val-Tyr-AMC. The substrate, cleaved by the chymotrypticlike activity of the proteasome, released free AMC (7-amino-4-methylcoumarin) which was detected using a fluorimeter (Ex380nm; Em460 nm). A standard curve was generated using AMC. Results are presented as fluorescence over time (nM/ mg protein/ 60 min).

Amino Acid Analysis (AAA)

Lymphoblast cell lines from patients and controls were used. The samples were acidified with sulfosalicylic acid (SSA, 3% final concentration) to precipitate any intact protein prior to analysis and centrifuged at 14000 rpm for 15 min at 4 °C. AAA in the supernatants was performed using Beckman 6300 amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA, U.S.A) which utilize a lithium citrate buffer system [43]. Amino acids separation by ion-exchange chromatography was followed by a “post-column” ninhydrin reaction detection system. The standard injection volume was 50.0ul. Aminoethylcysteine (AECys) was used as internal standards to correct for any variation in the operating conditions of the analyser over time. A data file comprised of a series of absorbance values vs. time points was generated during each chromatographic run. The values corresponding to the mutant samples were compared to the control ones; fold changes and p values between the two groups are presented in Table 6.

Statistical analysis

Data are expressed as mean and standard deviation (SD). Comparisons between groups were performed by two-tailed Unpaired Student's *t* test. P values of <0.05 were considered statistically significant. Data were analyzed using GraphPad-Prism software (Prism 4.0, GraphPad Software, Inc.).

Results

Several transcriptional consequences of deletions are significantly shared

Complementary RNA (cRNA) was prepared from 54 samples, labeled and microarrayed on 54 chips. Cell types included differentiated muscle, myoblasts, fibroblasts, lymphoblasts, NT2 cybrids, and 143B osteosarcoma cybrids (Table 1). We also compared the results with those obtained with 143B osteosarcoma rho-zero cells, which lack mtDNA completely, as the transcriptional consequences were similar to those observed in 143B cybrids bearing mtDNA deletions.

To identify the most consistent consequences of mtDNA deletions across cell types, we looked for transcripts that were significantly different at the $p \leq 0.10$ criterion in each of the seven experiments of mutants and controls. Thus, the combined probability for observing the same gene altered in the same direction across N experiments is: $\{(0.10)(0.5)\}^N$. For example, the nominal p-value for a single transcript to be shared among 4 experiments at the $p \geq 0.10$ criterion in the same direction is $(0.05)^4$, i.e. $p \leq 0.0000063$, $p \leq 6.3 \times 10^{-6}$ [44].

mtDNA deletions cause transcriptional inhibition

Several transcripts were non-randomly shared among experiments (Table 2). In addition, mtDNA deletions caused about twice as many shared downregulated than upregulated transcripts. For example, 38 transcripts were shared and downregulated vs 17 shared and upregulated among 5 experiments, 230 down vs 109 up among 4 experiments, and 983 down vs 684 up among 3 experiments. Thus, it is clear from the most consistently altered transcripts that in cells harboring mitochondrial deletions there are more nuclear gene inhibitions than activations by a ratio of approximately 2:1, when similar categories are compared.

Pathway analysis shows inhibition of ubiquitin-proteasome and mitochondrial transcripts

The Onto-Express bioprocess program objectively reveals the predominance of particular biochemical pathways in microarray data [42]. Using lists encompassing the broadest and most consistent consequences of mtDNA deletions (i.e. genes significantly altered in the same

direction across 4 experiments), we analyzed the pathways emphasized by the bioprocess function of Onto-Express (Table 3).

Onto-Express identified the following pathways as significantly inhibited: ubiquitin-proteasome (corrected p value <0.0007), mitochondrial ATP synthesis (p<0.006), and initiation of protein synthesis (p<0.007). By contrast, intra-Golgi transport, cell cycle arrest, and negative regulation of cell proliferation were significantly activated.

Negative transcriptional consequences of mtDNA deletions: proteasomal, mitochondrial, synthetic, and muscle-specific genes

Of the transcripts shared and downregulated in cells harboring mtDNA deletions in ≥ 3 experiments, four prominent clusters were evident by inspection, three of which had been previously identified by the Onto-Express analysis. First, many members of the ubiquitin-proteasomal complex were downregulated, including the proteasome (PSMB6, PSME3, PSMB4, PSMF1, PSMB2), and the ubiquitin ligase (UBE2E3, UBE2E1, UBE2L6, UBE2I, UBE3B, USP7, USP10, USP22, USP15, USP33, UBR2, CAND2) systems. Thus, mtDNA deletions cause a strong and significant inhibition of many transcripts involved in ubiquitin proteasome activity.

Second, and also consistent with the Onto-express results, many mitochondrially targeted transcripts were inhibited, including those that encode subunits of Complex I, NADH dehydrogenase (NDUFA5, NDUFA6, NDUFAF1, NDUFB3, NDUF3S, NDUFV2), Complex II (SDHC, SDHB), genes involved in the assembly of Complex IV (SURF1, SURF2), and Complex V (ATP5G2, ATP5H, ATP5J2), TCA cycle genes (DLD, SLC25A1, ACO2, IDH3G), mitochondrial ribosomal genes (IARS2, MRPL28, MRPS18B, MRPL23), and a mitochondrial antioxidant gene (SOD2). Thus, mtDNA deletions result in inhibition of nuclear-encoded, mitochondrial-targeted proteins.

mtDNA deletions activate SNARE genes, stress genes, MYC and amino acid degradation transcripts

We inspected the lists of genes shared and activated in ≥ 3 experiments, searching for functional clusters. In agreement with the Onto-Express analysis, several SNARE genes involved in intra-Golgi and vesicular transport and were induced in mutants, including syntaxin 16, (STX16, TGOLN2, ARHGEF, GOLGA8A, RAFTLIN, VAPB, ATP6V0E2L, VPS13A), suggesting an increase in vesicular activity. As discussed further below, these

Multiple stress-related genes of different categories were also induced in 3 or more experiments, including genes involved in the unfolded protein stress response (PERK, HSPA4, DNAJC3, and DNAJC4). Stress genes related to energy depletion were also induced in mutants (PRKAA, AMPK, ATG12, GATM), as were hypoxia-responsive genes (UGCG, ADM, STC2). ADM and STC2 are each targets of Hif1alpha, which was significantly induced in two experiments. Stress genes related to nonsense-mediated decay (SMG1, DICER1) were also induced. These results suggest increased stress in general in cells with mitochondrial deletions, including unfolded protein stress, energy depletion stress, and hypoxic stress. The stress category was not flagged by Onto-Express, probably because these sets of genes belong to separate stress pathways.

The MYC gene was also induced in mutants. MYC, which has recently been shown to serve as a transcription factor for mitochondrial biogenesis, responds to mitochondrial functional depletion [40,44-47].

Several transcripts encoding enzymes that degrade amino acids were also induced, including GCAT, METAP2, GLS, AASS, LOX. This suggests an increase in amino acid scavenging.

Decreased proteasome activity in mutant cells

Because the ubiquitin-proteasome pathway was significantly downregulated by the Onto-Express analysis, and was also obvious by inspection of the inhibited gene lists, we first confirmed the downregulation at the RNA level by measuring the proteasomal catalytic 26S subunit through quantitative rt-PCR (Figure 1). We then carried out a functional analysis of proteasome activity in 143B osteosarcoma cybrids harboring a 7.5 kb deletion and in lymphoblasts from patients carrying a 5 kb mtDNA deletion (Figure 2). In agreement with microarray results, we observed decreased proteasomal activity in both mutant cell types.

Amino acid concentration is deficient in mutant lymphoblasts

Proteasome activity is both important for normal degradation of proteins and for providing a substantial fraction of amino acids required for protein synthesis [48]. We compared amino acid concentration in two sets of lymphoblast cells harboring 5 kb and 7.5 kb mtDNA deletions and in control lymphoblasts, and found that 15 out of 20 standard amino acids tested were significantly decreased while one (aspartate) was only mildly increased. In addition, five nonstandard amino acids were also less in mutant lymphoblasts than in controls. All decreased acids are shown in Table 6.

Induction of the autophagy 12 transcript is a specific consequence of mtDNA deletions—The ATG12 transcript encodes the first and most important product of the autophagy pathway [49,50], and was induced in the microarray data from fibroblasts, lymphoblasts, and myoblasts from KSS patients, and in NT2 neural cells bearing deletions. As depletion of steady-state concentrations of amino acids is known to induce autophagy [51, 52], the induction of ATG12 could relate to the significant decreases in several amino acids that we observed in the mutant cells. To determine whether the induction of ATG12 was a specific consequence of mtDNA deletions, we quantified ATG12 transcript levels in 143B osteosarcoma cybrid fusion controls (i.e. cell lines that had gone through the process of cybridization but with normal mtDNA), in osteosarcoma cybrids harboring deletions, and in osteosarcoma cell lines lacking the mitochondrial genome (Figure 3). The ATG12 transcript levels were highly correlated with the presence of deletions, were significantly higher in cells bearing deletions, and highest in cells lacking mtDNA.

Increase in syntaxin 16 protein—Both inspection of the microarray data and the Onto-Express analysis suggested an increase in vesicular activity in the mutant cells. STX16 is a t-SNARE (SNAP receptors) molecule involved in synaptic vesicle docking and fusion, found in the Golgi and trans-Golgi network and essential for autophagy [53-55]. Immunoblot analysis confirmed an increased expression of STX16 in 143B cybrids and 143B rho-zero cells compared to 143B fusion controls (unpaired t test, $p=0.0270$ and $p=0.0036$ respectively, Figure 4).

Increase in oxidized proteins in mutant cells—Since deletions erase not only tRNAs but also mitochondrially-encoded subunits of the respiratory chain, it would not be unexpected if misfolding of electron transport chain complexes caused increased oxidative damage to proteins. Thus we measured oxidatively modified proteins using the Oxyblot method. There were significantly more oxidized proteins in cells bearing mtDNA deletions than fusion controls, i.e. controls that had been through the cybridization process with normal mtDNA.

Discussion

Single mtDNA deletions cause KSS, CPEO, or PS, whose affected tissues show impaired electron transport activity, ATP production, and mitochondrial protein synthesis [26,56,57]. Muscle biopsies from patients with KSS or CPEO show ragged-red fibers and cytochrome

oxidase-negative fibers [58,59]. Clinical signs vary widely in different syndromes but include muscle weakness, heart block, retinopathy, deafness, diabetes, short stature and dementia [2, 60]. We have used microarray in an attempt to link the primary cause of KSS/CPEO/PS with cellular, morphological, and clinical consequences. We examined the common transcriptomal consequences of mtDNA deletions in 6 cell types. Because deletions erase essential parts of the mitochondrial protein synthetic machinery (i.e. tRNAs) [58,61] as well as essential components of the respiratory chain complexes that produce energy and other metabolites for the cell (Figure 5), we expected a strong 'nuclear response' to the 'mitochondrial stimulus'.

Single mtDNA deletions produce inhibition of multiple mitochondrial transcripts.

Since the mitochondrial and nuclear genome exist symbiotically, and co-encode several multisubunit complexes, it was interesting to see that mtDNA deletions had a strong negative effect on nuclear-encoded mitochondrially targeted genes. This was especially evident on Complex I transcripts, but also on Complex II and Complex IV assembly genes, on Complex V, on several TCA cycle genes, and on components of the mitochondrial ribosome. These data suggest that the nucleus senses the irreversible depletion of mtDNA-encoded mitochondrial subunits and tRNAs, and responds by down-regulating the interacting subunits that would normally form a functional complex. The downregulation of nuclear-encoded mitochondrial ribosomal subunits, OXPHOS, and TCA cycle transcripts thus appears to reinforce the mitochondrial defect initiated by the deletions, and add to the mitochondrial metabolic defect in the patients.

The depletion of mitochondrial function has recently been shown to induce MYC overexpression, and we also observed a substantial upregulation of MYC in cells bearing mtDNA deletions [40,44-47,62]

Deletions inhibit the ubiquitin and proteasome system

Deletions of the mitochondrial genome erase not only subunits of the respiratory chain that must co-operate with nuclear-encoded subunits [63], but also tRNAs necessary for mitochondrial protein synthesis [57].

Deletion of specific mtRNAs not only decreases mitochondrial translation rates [64,65] but is also expected to result in increased mitochondrial protein unfolding and damage.

Although it has been known for some time that mildly damaged mitochondrial proteins are degraded by the proteasome, it hasn't been appreciated until recently that heavily damaged mitochondrial proteins inhibit the proteasome [66-70]. Thus, the very strong inhibition of many subunits of the ubiquitin-proteasome system is likely a consequence of misfolded and damaged mitochondrial proteins specified by the mtDNA deletions. Also, recent studies have demonstrated that mtDNA deletions increase ROS production, which would seem to compound the misfolding/damage problem [31]. To test these ideas, we measured oxidative damage to proteins by the Oxyblot method, and observed a >300% increase in oxidized proteins. Thus, mtDNA deletions result not only in increased protein oxidative damage but also decreased proteasome activity.

The downregulation of the proteasome microarray signal appears to be a unique consequence of mtDNA deletions, as it was not observed in a larger microarray study of other mitochondrial diseases, including Friedreich's ataxia, Leber's Hereditary Optic Neuropathy, Myoclonic Epilepsy with Ragged Red Fibers, and Neuropathy, Ataxia, and Retinitis Pigmentosa [44]. Thus, it seems likely that inhibition of the proteasomal transcripts is a direct consequence of mtDNA deletions.

Decreased amino acid concentration in mutant cells

One potential consequence of decreased ubiquitin protease activity is decreased salvage of amino acids, and recently the proteasome has been shown to be an important provider of amino acids for protein synthesis, specifically under conditions of minimal nutrient status [48].

Consistent with this prediction, we observed a significant and substantial decrease in steady-state amino acid concentration in mutant cells, and significant increases in transcripts that encode several amino acid-degrading enzymes, possibly as a response to amino acid depletion.

Induction of autophagy transcript in mutant cells

Deletions erase essential tRNA genes responsible for decoding mitochondrial RNA transcripts, which should result in irreparable protein damage. Since proteasomal activity is inhibited in cells with deletions, this raises the question of how the cell may deal with mitochondria carrying irreparable protein damage. ATG12, which is the first and most important gene in the mammalian autophagy cascade [49,50], was upregulated in response to deletions, as shown by microarray across cell lines (Table 5), and the degree of upregulation was correlated with deletion status in the osteosarcoma cell line (Figure 3). It has recently been shown that autophagy is induced in response to impaired ubiquitin proteasome system activity [71,72]. Also, it is known that low amino acid concentration triggers autophagy via inhibition of mTOR, thus providing a possible pathogenic mechanism for KSS: deletions> misfolding> proteasome inhibition> AA depletion> mTOR inhibition> autophagy, inhibition of protein synthesis and of cell growth.

Autophagy requires the action of SNARE and vesicular proteins

Consistent with the hypothesis of an induction of autophagy in mutant cells, we observed the induction of several SNARE/vesicular transcripts, syntaxin 16 (STX16, TGOLN2, ARHGEF, GOLGA8A, VAPB, ATP6V0E2L, VPS13A). Induction of SNARE proteins is essential for the process of autophagy [53,73-75]. Thus it is possible that the subsarcolemmal proliferation of mitochondria observed in KSS/CPEO and revealed by the Gomori trichrome stain in ragged-red fibers is in fact the result of increased autophagic activity.

Amino acid deprivation induces autophagy

Amino acid deprivation is known to induce autophagy via inhibition of the mTOR pathway [51,52]. Amino acid deprivation is also known to signal GCN2 and stimulate eIF2- α phosphorylation [76-78], resulting in increased expression of unfolded protein response (UPR) genes and endoplasmic reticulum (ER) stress-related genes, many of which are induced in mutant cells. Both the inhibition of mTOR and the phosphorylation of eIF2 α should result in the inhibition of protein synthesis transcripts that we observe in the microarray data.

A pathogenetic mechanism for KSS

mtDNA deletions remove tRNAs and functional components of the respiratory chain. The resulting mitochondrial protein damage has multiple consequences. Perhaps as a result of oxidative protein damage, one consequence is inhibition of the ubiquitin proteasome system, which may cause amino acid depletion through decreased amino acid salvage. A second generally accepted consequence is the reduction of ATP synthesis [26,79]; and ATP depletion is known to activate the AMPK response [80] as a protective mechanism. Both amino acid depletion and AMPK activation can result in inhibition of mTOR [51,81] which in turn inhibits translation, protein synthesis, and cellular growth, and induces autophagy (Figure 6).

Implications of the proposed mechanism for KSS patients

If this mechanism is correct, it suggests potential interventions for patients harboring mtDNA deletions. If the ubiquitin proteasomal defect is a primary consequence of mtDNA deletions, as we suspect, and causes the decrease in cellular amino acid concentration, which in turn signals mTOR and causes decreased protein synthesis transcripts, then supplementation of free amino acids might benefit the patients, because amino acids in general (and leucine in particular) are known to potently stimulate mTOR and inhibit autophagy [82-85].

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List of Abbreviations

CPEO, Chronic Progressive External Ophthalmoplegia; FBS, fetal bovine serum; FC, fusion control cell lines; KSS, Kearns Sayre Syndrome; OE, Onto-Express; mtDNA, mitochondrial DNA; OPG, Osteoprotegerin; PS, Pearson's Syndrome; PEG, Poly Ethylene Glycol; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SSA, sulfosalicylic acid; SD, standard deviation; UPR, unfolded protein response.

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Figure 1.

Quantitative rt-PCR validation of microarray data.

Fold changes of 4 selected genes obtained by microarray analysis and by quantitative rt-PCR are shown. QRT-PCR results were normalized to beta-actin expression. Significant differences from the control groups: * unpaired t test, $p < 0.05$, ** unpaired t test, $p < 0.005$.

Figure 2.

Measurement of proteasome activity in 143B cybrids and lymphoblasts.

In vitro assessment of 20S proteasome activity was done by measuring the hydrolysis of the fluorogenic peptidyl substrate Suc-Leu-Leu-Val-Tyr-AMC by the SDS-activated proteasome. (A) Average activity in three 143B fusion controls and two osteosarcoma cybrids 3.6 and 16.3 with 70% of 7,5 kb mtDNA deletions. (B) Average activity in lymphoblast cell lines from three control subjects, and two patient cell lines, (848) with 57% of 4,9 kb mtDNA deletion and (63358) with 27% of 7,4 kb mtDNA deletion. C, control cells; M, mutant cells. Error bars represent SEM. * Unpaired t test, $p < 0.02$.

Figure 3.

ATG12 RNA overexpression in mutant 143B cybrids

Quantitative rt-PCR analysis of ATG12 RNA expression in 143B osteosarcoma cybrids. QRT-PCR results were normalized to 18sRNA expression. (A) QRT-PCR results of three separate experiments each done in duplicate. Black bars represent fusion control cell lines (FC), white bars mutant cell lines (M) and striped bars mtDNA depleted rho-zero 143B cell lines, cell line mutation status is described in Table 7. (B) Average relative ATG12 RNA expression of fusion controls, mutant cybrids and 143B rho-zero cells at left, summed. Error bars represent SEM.

* Unpaired t test, $p < 0.05$. ** Unpaired t test, $p < 0.01$.

Figure 4.

Syntaxin 16 protein overexpression in mutant 143B cybrids.

Relative expression of syntaxin 16 (STX16) in 143B mutant cybrids (M) compared to 143B fusion control cybrids (FC). (A) Representative immunoblot for STX16 out of three separate experiments. From left, the samples were: FC (fusion controls)-HGA13 & HPC7; M-Mutants: 14.6, 16.3. and 51-18. The blots were stripped and reprobed against beta-actin. (B) Average relative density (syntaxin 16 over beta-actin) in fusion controls, mutant cybrids and 143B rho-zero cells. Error bars represent SEM. * Unpaired t test, $p < 0.05$. ** Unpaired t test, $p < 0.005$.

Figure 5.

Deletion sites within mtDNA.

Deletions of mtDNA may occur within a certain region (shown in light blue). The deleted genes code for respiratory chain subunits of Complex I (ND3, ND4, ND4L, ND5 and ND6), Complex III (Cyt b), Complex IV (COII and COIII), and Complex V (ATPase 6 and 8). The deletions may erase also transfer RNA genes (indicated in red) such as S, D, K, G, R, H, S, L, E, T, and P.

Figure 6.

A mechanistic hypothesis for the consequences of mtDNA deletions.

Expected consequences of mtDNA deletions. Deletions must perforce erase essential tRNAs and components of multisubunit complexes of the OXPHOS chain, and result in increased protein oxidative damage. Increased protein oxidative damage has been demonstrated to inhibit ubiquitin-proteasomal activity, which is expected to decrease the rate of salvage of amino acids, and result in decreased amino acid concentration in cells, which has previously been demonstrated to decrease the activity of mTOR, which then represses protein synthesis and activates autophagy. Deletions were shown to result in decreased expression of nuclear-encoded OXPHOS complex (of both the electron transport chain and ATP synthase), which must also inhibit ATP synthesis (which has been demonstrated at the biochemical level by others), and stimulate the activity of AMP kinase, which then should inhibit mTOR, which should result in the inhibition of protein synthesis and the activation of autophagy [52].

Table 1

Summary of basic microarray statistics.

Cell types	Chips number	Mutants vs Controls	# Unique altered genes
Differentiated muscles	11	7/4	499
Myoblasts	4	2/2	1417
Fibroblasts	7	4/3	1309
Lymphoblasts	5	2/3	1988
+NT2 cybrids	6	4/2	2444
143B cybrids	13	8/5	2077
143B rho-zero	8	2/6	2413
Tot	54	29/25	3661

Table 2

Transcriptional results shared among experiments

Transcripts shared among experiments	≥ 3	≥ 4	≥ 5	≥ 6
<i>Down-regulated</i>	983	230	38	8
<i>Up-regulated</i>	684	109	17	0
<i>Individual P value</i>	0.00012	6.3×10^{-6}	3×10^{-7}	1.6×10^{-8}

Table 3
Onto-Express analysis of pathways affected by mtDNA deletions

<i>Function Name</i>	<i>Unique altered genes</i>	<i>Total unique genes</i>	<i>P-Value</i>
<i>Downregulated bioprocesses</i>			
ubiquitin cycle	11	116	3.46E-04
ubiquitin-dependent protein catabolism	8	74	7.17E-04
transcription from RNA polymerase II promoter	11	159	0.005158
ATP synthesis coupled proton transport	4	28	0.005166
regulation of transcription	6	50	0.005441
regulation of translational initiation	3	23	0.006789
vesicle-mediated transport	4	39	0.007359
<i>Upregulated bioprocesses</i>			
intra-Golgi transport	3	17	4.33E-04
intracellular signaling cascade	9	227	0.003112
regulation of cell growth	4	60	0.003343
cell surface receptor linked signal transduction	6	120	0.003429
regulation of transcription from RNA polymerase II promoter	6	151	0.007731
cell cycle arrest	3	50	0.007767
negative regulation of cell proliferation	5	117	0.009219

Table 4

Downregulated genes

Function	Gene name	Gene	Times Down	Fold
Ubiquitin-proteasome system				
Proteasome	proteasome beta type, 6	PSMB6	4	-1.2
	proteasome activator 3	PSME3	3	-1.6
	proteasome , beta type, 4	PSMB4	3	-1.3
Ubiquitin ligase system	proteasome inhibitor subunit 1	PSMF1	3	-1.4
	proteasome subunit, beta type, 2	PSMB2	4	-1.3
	ubiquitin-conjugating enzyme E2E 3	UBE2E3	6	-1.3
	ubiquitin-conjugating enzyme E2E 1	UBE2E1	3	-1.4
	ubiquitin-conjugating enzyme E2L 6	UBE2L6	4	-1.7
	ubiquitin-conjugating enzyme E2I	UBE2I	4	-1.2
	ubiquitin protein ligase E3B	UBE3B	3	-1.1
	Ubiquitin specific peptidase 7	USP7	4	-1.4
	ubiquitin specific peptidase 22	USP22	4	-1.4
	ubiquitin specific peptidase 15	USP15	3	-1.6
	ubiquitin specific peptidase 33	USP33	4	-1.4
	ubiquitin protein ligase E3 n-recogin 2	UBR2	5	-1.3
	cullin-associated & neddylation-dissociated 2	CAND2	3	-1.5
Mitochondrially-targeted transcripts				
Complex I	NADH dehydrogenase 1 alpha	NDUFA5	5	-1.8
	NADH dehydrogenase 1 alpha	NDUFA6	3	-1.4
	NADH dehydrogenase 1 alpha	NDUFAF1	5	-1.5
	NADH dehydrogenase 1 beta	NDUFB3	3	-1.5
	NADH dehydrogenase Fe-S protein 3	NDUFS3	3	-1.4
	NADH dehydrogenase flavoprotein	NDUFV2	3	-1.3
Complex II	succinate dehydrogenase	SDHC	3	-1.4
	succinate dehydrogenase complex	SDHB	3	-1.3
Complex IV assembly	surfeit 1	SURF1	3	-1.4
	surfeit 2	SURF2	3	-1.5
Complex V	ATP synthase F0 complex, subunit c	ATP5G2	3	-1.4
	ATP synthase, F0 complex, subunit d	ATP5H	4	-1.3
	ATP synthase, F0 complex, subunit f	ATP5J2	3	-1.3
TCA cycle	dihydrolipoamide dehydrogenase	DLD	3	-1.4
	aconitase 2, mitochondrial	ACO2	3	-1.7
	isocitrate dehydrogenase 3 (NAD+) gamma	IDH3G	4	-1.4
Mitochondrial ribosome	isoleucine-tRNA synthetase 2, mitochondrial	IARS2	4	-1.4
	mitochondrial ribosomal protein L28	MRPL28	3	-1.3
	mitochondrial ribosomal protein S18B	MRPS18B	4	-1.3
	mitochondrial ribosomal protein L23	MRPL23	4	-1.4
Mitochondrial antioxidant	superoxide dismutase 2, mitochondrial	SOD2	3	-1.7

Table 5
Upregulated genes

Function	Gene name	Gene	Up	Fold
Stress response				
Unfolded protein	heat shock 70kDa protein 4	HSPA4	3	1.8
	DnaJ member 3	DNAJC3	4	1.9
	DnaJ member 4	DNAJC4	3	1.2
Energy depletion	AMP kinase, alpha 1	PRKAA	3	2.1
	ATG12 autophagy related 12	ATG12	4	1.7
	glycine amidinotransferase	GATM	3	1.9
Hypoxia	UDP-glucose ceramide glucosyltransferase	UGCG	4	2.1
	adrenomedullin	ADM	3	3.6
	stanniocalcin 2	STC2	4	3.0
Nonsense-mediated decay	PI-3-kinase-related kinase SMG-1	SMG1	4	2.0
	Dicer1, Dcr-1 homolog (Drosophila)	DICER1	3	1.7
DNA damage	glycine amidinotransferase	ATM	3	1.9
Mitochondrial depletion	v-myc myelocytomatosis viral oncogene	MYC	5	1.9
SNARE/ intra-golgi transport				
	syntaxin 16	STX16	5	1.5
	trans-golgi network protein 2	TGOLN2	4	1.6
	rho/rac guanine nucleotide exchange factor	ARHGEF	4	1.3
	golgi autoantigen, golgin subfamily a, 8A	GOLGA8A	3	2.8
	raft-linking protein	RAFTLIN	3	2.8
	VAMP (vesicle-associated membrane protein)	VAPB	3	2.1
	ATPase, H ⁺ transporting V0 subunit E isoform	ATP6V0E2	3	1.6
	vacuolar protein sorting 13A (yeast)	VPS13A	3	1.6
Amino acid degradation				
	glycine C-acetyltransferase (2-amino-3-oxo)	GCAT	3	1.2
	methionyl aminopeptidase 2	METAP2	3	1.8
	glutaminase	GLS	3	1.6
	amino acid oxidase-semialdehyde synthase	AASS	3	2.2
	lysyl oxidase	LOX	4	1.6

Table 6
Altered cytoplasmic amino acids in mutant lymphoblast cell lines

Amino acid	Fold Change	P value
<i>Standard amino acids</i>		
Alanine	-2.5	0.00004
Asparagine	-1.7	0.005
Aspartate	1.4	0.05
Glutamine	-1.3	0.05
Glycine	-1.8	0.002
Histidine	-1.9	0.001
Isoleucine	-1.7	0.003
Leucine	-1.7	0.006
Methionine	-1.9	0.002
Phenylalanine	-1.8	0.0006
Proline	-1.4	0.02
Serine	-3	0.0002
Threonine	-2.7	0.05
Tryptophan	-1.9	0.004
Tyrosine	-2	0.0007
Valine	-1.8	0.004
<i>Nonstandard amino acids</i>		
Citrulline	-6.1	0.001
G-aminobutyric acid	-4.2	0.002
Methylhistidine	-1.9	0.0005
Ornithine	-2.3	0.002
Phosphoethanolamine	-2.2	0.02

Table 7
Cell lines and tissues used in the study, with size and percent of deletion and disease status.

	Line	Size	Disease	%	array	QPCR	UPS
Osteosarcoma	3.7	7.5kb	kss	70	*		*
	16.3	7.5kb	kss	70	*		*
	51-18	5kb	kss	21	*		
	BH.10.59	5kb	kss	50		*	
	14-6	5kb	kss	21		*	
	dH.2.1	7.5kb	kss	50		*	
	143GA	0	Normal	0	*		
	143MK	0	Normal	0	*		
	hga13	0	Control Fused	0		*	
	hpc7	0	Control Fused	0		*	
Lymphoblasts	h1959	0	Control Fused	0		*	
	hba2	0	Control Fused	0		*	
	848	5kb	kss	57	*	*	*
	63358	7.4kb	kss	27	*		*
	C536	0	Normal	0			
	C333	0	Normal	0	*	*	
	C621	0	Normal	0	*		
	AC	5kb	kss	20	*		
	RJ	5kb	kss	72	*		
	3511	NA	kss	NA	*		
Fibroblasts	3246	NA	kss	NA	*		
	C305	0	kss	NA	*		
	C572	0	Normal	0			
	4.9-1(8)	5kb	Normal	0	*		
	4.9-2 (48)	5kb	kss	16	*		
	AW Ctl	0	kss	75	*		
	4317	5kb	Normal	0	*	*	
	8279	5kb	kss	NA	*	*	
	2f0135	0	kss	NA	*	*	
	2f0873	0	Normal	0	*	*	
NT2 cybrids	1348	5 kb	Normal	NA	*	*	
	3515	5kb	kss	NA	*	*	
	3516	5kb	kss	NA	*	*	
	3517	5kb	kss	NA	*	*	
	2378	0	kss	NA	*	*	
	2666	0	normal	0	*	*	
	2794	0	normal	0	*	*	
	2815	0	normal	0	*	*	
Myoblasts							
Muscle							