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Mitochondrial genomic variation associated with higher mitochondrial copy number: the Cache County Study on Memory Health and Aging

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From The 10th Annual Biotechnology and Bioinformatics Symposium (BIOT 2013) Provo, UT, USA. 5-6 December 2013

Abstract

Background: The mitochondria are essential organelles and are the location of cellular respiration, which is responsible for the majority of ATP production. Each cell contains multiple mitochondria, and each mitochondrion contains multiple copies of its own circular genome. The ratio of mitochondrial genomes to nuclear genomes is referred to as mitochondrial copy number. Decreases in mitochondrial copy number are known to occur in many tissues as people age, and in certain diseases. The regulation of mitochondrial copy number by nuclear genes has been studied extensively. While mitochondrial variation has been associated with longevity and some of the diseases known to have reduced mitochondrial copy number, the role that the mitochondrial genome itself has in regulating mitochondrial copy number remains poorly understood.

Results: We analyzed the complete mitochondrial genomes from 1007 individuals randomly selected from the Cache County Study on Memory Health and Aging utilizing the inferred evolutionary history of the mitochondrial haplotypes present in our dataset to identify sequence variation and mitochondrial haplotypes associated with changes in mitochondrial copy number. Three variants belonging to mitochondrial haplogroups U5A1 and T2 were significantly associated with higher mitochondrial copy number in our dataset.

Conclusions: We identified three variants associated with higher mitochondrial copy number and suggest several hypotheses for how these variants influence mitochondrial copy number by interacting with known regulators of mitochondrial copy number. Our results are the first to report sequence variation in the mitochondrial genome that causes changes in mitochondrial copy number. The identification of these variants that increase mtDNA copy number has important implications in understanding the pathological processes that underlie these phenotypes.

Background

Mitochondria are the location of the citric acid or Krebs Cycle, which produces the majority of ATP for cellular work. Each cell has multiple mitochondria and each mitochondrion contains one or more copies of its own circular genome (mtDNA), which is 16569 bases in length and encodes 37 genes. Mitochondria are necessary for survival and malfunctioning mitochondria are

the cause of a variety of diseases [1-11]. Mitochondrial diseases tend to affect the CNS or muscle tissue because of the high energy needs of these tissues [12]. Mitochondrial diseases have been well studied and can be the result of genetic variation in the mitochondrial and/or nuclear genomes. Pathogenic nuclear mutations are inherited in a typical Mendelian pattern and can present with a dominant, recessive, or X-linked dominant or recessive inheritance pattern. Examples of mitochondrial diseases caused by mutations in the nuclear genome

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include Friedrich's ataxia [13], Wilson's disease [14], and Barth syndrome [15].

In contrast, mitochondrial diseases caused by variation in the mtDNA are not as straightforward. Mitochondria are maternally inherited, so mitochondrial disease caused by these variants will display maternal inheritance. However, in most cases both normal and pathogenic mtDNA are inherited together and the mix can vary from predominantly wild type to predominantly pathogenic. Depending on the severity of the mutation, proportion of wild type versus affected mitochondria, and the specific tissue, there may or may not be a disease phenotype. Over the course of life the proportion of diseased mitochondria can change, possibly reaching a critical threshold at which the disease phenotype is expressed. Alternatively, a constant proportion of diseased mitochondria might contribute to disease only when present in combination with one or more additional factors (e.g. stresses of various kinds, and/or aging). In addition to inherited mtDNA variation, mtDNA is prone to somatic mutations [16], and if affected mtDNA are propagated they can eventually reach a threshold at which mitochondrial function is insufficient to support normal cellular functions and disease appears. Some examples of disorders caused by mtDNA mutations are Kearns-Sayre syndrome [8], diabetes mellitus and deafness [7], Leber's hereditary optic neuropathy [9], Leigh Syndrome [11], and Myoclonic Epilepsy with Ragged Red Fibers (a.k.a. MERRF syndrome) [10].

Additionally, mitochondria have a role in aging. The free-radical theory of aging, or mitochondrial free radical theory of aging, hypothesizes that aging occurs as damage from reactive oxygen species (ROS) accumulates. ROS are produced in the electron transport chain [17] and readily oxidize DNA and RNA, amino acids, and fatty acids [18-20]. Damage from ROS can accumulate with time resulting in cellular dysfunction, and death [21].

MtDNA copy number, or the cellular ratio of mitochondrial genomes to nuclear genomes, decreases with age in some, but not all, tissues [22-25] and mtDNA copy number variation has been associated with numerous phenotypes [26-38]. MtDNA copy number is tissue dependent [39] and varies with age and the energy needs of the cell [24,25,40].

Several nuclear genes regulate mtDNA copy number. First, there is substantial evidence that mitochondrial transcription factor A (*TFAM*) regulates mtDNA copy number [41-44]. The *Mec1/Rad53* (yeast) pathway has been implicated in controlling mtDNA copy number, and mtDNA levels can be controlled by any of several genes responsible for regulating the pathway [45]. *p53* deficient cells or mutated *p53* leads to decreased levels of mtDNA [46]. Two common nuclear SNPs in signal transducer and activator of transcription 3 (*STAT3*) were significantly associated with mtDNA levels in leukocytes

[47]. Both the Ras pathway and *p66Shc* likely have roles in regulating mtDNA copy number [48]. *MnSOD* prevents decreases in mtDNA levels by preventing a decrease in mtDNA replication proteins [49]. And finally, overexpression of *Twinkle* increases mtDNA copy number [50].

The direct role for the mitochondrial genome regulating levels of mtDNA has not been studied extensively. Here we conduct a genetic association study of full mitochondrial genome data and mtDNA copy number in individuals from the Cache County Study on Memory Health and Aging. Our results identify association between mitochondrial haplogroups U5A1 and T2 and increased mtDNA copy numbers.

Methods

Ethics statement

As described in [51], all study procedures were approved by the Institutional Review Boards of Brigham Young University, Utah State University, Duke University, and Johns Hopkins University. Written consent was obtained for each individual. To verify a subject's capacity to consent, subjects attempted the Modified Mini-Mental State Exam (3MS). If there was an indication of poor cognitive ability as determined by poor performance on the entire test (scoring below a designated total of 60 points), poor performance on temporal or spatial orientation, or clear difficulty in understanding the nature of the interview, the visit was discontinued and informed consent was obtained from a responsible caregiver- often the next-of-kin. We re-consented subjects/caregivers at each study visit and procedure.

Sample acquisition and sequencing

Samples for this study were selected from the Cache County Study on Memory Health and Aging [52]. This study was initiated in 1994 to investigate associations of genetic and environmental factors with cognitive function. In 1994, the 5,092 individuals enrolled in the study from Cache County, Utah, represented 90% of all Cache County, Utah, residents who were 65 or older. The cohort was followed for 12 years and data (medical histories, demographics, and a multistage dementia assessment) were collected in four triennial waves. The Utah population is similar to other U.S. populations of northern European ancestry characterized by very little inbreeding. The founding group of Utah's population was unrelated and migrated from various locations in Europe [53-55].

The Utah Population database (UPDB) has complete pedigree information going back 14 generations to the original Utah Founders. Using this information we identified individuals from the Cache County Study with the same maternal line of inheritance (matrilineage). We randomly selected one individual from each matrilineage, selecting individuals from the largest matrilineages first to maximize

our ability to infer mitochondrial genomic information. Given our resources, we were able to sequence a representative sample from 274 of the 3151 matrilineages that exist in the Cache County Study samples. The sequenced mitochondrial genomes represent many different major mitochondrial haplogroups (Table 1). 287 samples were sent to Family Tree DNA (<http://www.familytreedna.com>) for Sanger sequencing of the mitochondrial genomes. Two samples failed quality control at Family Tree DNA. Based on maternal inheritance of the mtDNA we inferred that individuals who share matrilineal relationships have the same mtDNA. Using this we inferred the status of full mitochondrial genome sequence for 722 additional individuals for a total of 1007 individuals, not accounting for de novo mutation. The extensive pedigree data in the UPDB allows identification of shared maternal lineages for very distant relationships. As this was a population-based study it is one generation in depth, but there are extended familial relationships, even very distant cousins. Ridge et al [51] contains additional details about the sequencing and inference of the mtDNA status in this dataset.

Measurement of mtDNA copy number

Relative quantitation of the ratio of the copy number of the mitochondrial genome to the copy number of the nuclear single copy gene beta-globin, as compared to that ratio in a reference DNA sample, was determined by monochrome multiplex quantitative polymerase chain reaction (QPCR). Buccal sample cell lysates were diluted in water (containing yeast total RNA as carrier, at 2.5 ng per microliter) to a final total cellular DNA concentration of approximately 1 ng per 10 microliters. QPCR was carried out in 25 microliter reactions, containing 10

microliters of the diluted buccal lysate and 15 microliters of QPCR reagent mix with primers.

The QPCR reagent mix, without primers, was exactly as described by Cawthon [56]. The primers for mtDNA amplification were mt3257u, 5'-GCAGAGCCCGG-TAATCGCA-3', and mt3272d, 5'-TAAGAAGAGGAAT TGAACCTCTGACTGTAA-3'. The mt3272d primer has previously been shown to be specific to mtDNA and unable to amplify any nuclear-embedded mtDNA-like sequences (numts) from rho 0 cell line DNA [57] (Rho 0 cell lines are mtDNA-free). The primers for the beta-globin gene were hbbugc2, 5'-CGGCGGCGGGCGGGCG CGGGCTGGGCGGCTTCATCCACGTTACCTTG-3', and hbgdgc2, 5'-GCCC GGCCCGCCGCGCCCGTCCC GC GAGGAGAAGTCTGCCGTT-3'. Both beta-globin primers contained 5' GC-clamp (non-templated) sequences that confer a high melting temperature on their amplicon. Each of the four primers was present at a final concentration of 900 nM.

The thermal profile for QPCR began with 95 degrees C for 15 minutes to activate the hot-start polymerase and fully denature the DNA; followed by 35 cycles of: 94 degrees for 15 sec, 62 degrees for 20 sec, 72 degrees for 15 sec with signal acquisition (to read the mtDNA amplification signal), 84 degrees for 10 sec, and 88 degrees for 15 sec (to read the beta-globin signal). In this monochrome multiplex QPCR (MMQPCR) strategy, first described by Cawthon [56], the higher copy number target (in this case mtDNA) has its amplification signal collected over a cycle range in which the lower copy number target's (in this case the beta-globin genes) amplification signal is still at baseline, and the lower copy number target's amplification signal is collected in later cycles, at a temperature that is sufficiently high to completely melt the amplicon of the higher copy number target, driving its signal to baseline so that the signal from the high melting amplicon can be cleanly read. All QPCR runs were done on Bio-Rad MyiQ real-time machines, using the manufacturer's accompanying software. The Standard Curve method for relative quantitation was used, with 36 ng of a reference DNA sample as the high end, and four additional standard concentrations obtained via 3-fold serial dilutions from the high end. Each subject's buccal lysate was assayed in triplicate. The average of the three measurements for each sample was used in this study (Additional File 1). DNA is not available from other tissue for the majority of these samples.

Sequence and statistical analyses

We used ClustalW [58] to align the mitochondrial genomes and inferred a haplotype network using TCS [59] and the 285 sequenced mitochondrial genomes. In a haplotype network, segments of branches correspond to a single sequence feature (single nucleotide variant, indel,

Table 1 Distribution of major mtDNA haplogroups/ clusters.

Major Haplogroup	Number	Ethnicities[94,95]
H	424	European
U	147	European
T	121	European
J	99	European
K	95	European
V	34	European
I	21	European
W	20	European
HV	18	European
X	8	European
C	5	Asian
L	4	African
Missing ¹	11	

Here we report the number of individuals belonging to each of the major haplogroups represented in our dataset along with case-control status. This Table first appeared in [51]. ¹Missing major haplogroup

etc.), and nodes in the network correspond to haplotypes. Branches, comprised of one or more segments, connect observed nodes, while clades are comprised of one or more observed nodes, and are defined by a branch.

Genotype-phenotype associations were evaluated using an evolution-based method known as TreeScanning [60,61] that makes use of haplotype networks. Haplotype networks provide a framework from which to select evolutionarily related haplotypes to pool together for comparison. Additional details about the application of TreeScanning to this dataset can be found in Ridge et al [51]. The null hypothesis of TreeScanning is that the phenotype does not differ in distribution across the genotypes derived from allelic classes defined by the branches of the haplotype network. Each branch partitions the haplotypes into bi-allelic pools from which genotypes are constructed and treated as a separate test. Because we have multiple tests that are correlated we obtained multiple-test corrected p-values by a permutation analog of the sequential step-down Bonferroni [62] with 10,000 permutations. If significant branches are found in the first round of TreeScanning, a second round of TreeScanning is performed that can detect phenotypic heterogeneity within the allelic classes of the significant branch. This is accomplished by creating a three-allele system and using conditional permutations that hold one of the alleles constant while subdividing the other class into two alleles [60]. Significant branches define clades.

For these analyses we tested for association with mitochondrial copy number after adjusting for gender, age, and familial relationships. Familial adjustment scores, which quantify the variance in mtDNA copy number that is due to familial relationships between individuals in the dataset, were computed using the method developed by Kerber (modified for a continuous trait) [63]. For each individual we summed the products of the mtDNA copy number and the pairwise kinship coefficient (a pairwise measure of relatedness) with each of the other individuals in the sample. This sum is then divided by the total number of samples in the dataset. Finally, we divide by the mtDNA copy number of the individual, yielding a value, which represents the relationship between mtDNA copy number and relatedness to other individuals in the dataset. We calculated familial adjustment scores for each individual in the dataset using the following equation:

$$\text{familial adjustment score} = \frac{\sum_{j=1}^N \text{copy number } j * f(\text{individual}, j)}{N * \text{individual copy number}}$$

Where N is the number of individuals in the cohort and $f(\text{individual}, j)$ is the kinship coefficient between the individual for whom we are calculating a familial

adjustment score (labeled as 'individual' in the formula) and individual j (representing each of the other individuals in the dataset one at a time). Inclusion of this score as a covariate in our analyses removes variance in mtDNA copy number that is due to relatedness between individuals, making it possible to test for association independently of pedigree relationships in the data. This adjustment addresses both maternal and paternal relationships in the data, thus correcting for possible nuclear genomic confounds as well. Each analysis was performed with 10,000 permutations. Only tests with at least two relevant genotypic classes, each containing five or more individuals, were tested. Significance was inferred if the multiple-test-corrected p-value was less than 0.05.

Bioinformatic analyses of variants

In order to determine the functional impact of variants of interest we applied *in silico* functional prediction algorithms, analyzed pathways, examined protein sequence conservation, and identified conserved domains. We obtained protein sequences from NCBI using blast [64], aligned and analyzed them using the CLCViewer (<http://clcbio.com/>), identified conserved domains using the NCBI conserved domain database [65], identified pathways using Ingenuity (<http://Ingenuity.com/>), and obtained functional predictions from polyphen-2 [66] and SIFT [67,68] webservers. In each case we used default settings.

Results

Haplotype network and mtDNA variation

We sequenced 285 complete mitochondrial genomes from individuals in the Cache County Study on Memory Health and Aging and imputed 722 additional full mitochondrial genomes using maternal lineages for a full dataset of 1007 full mitochondrial genomes. We built our network using the 285 genotyped individuals (Additional Files 2, 3). Our network contained 249 different haplotypes and the majority of haplotypes (152 of 249) were observed in three or fewer individuals with the two most frequently observed haplotypes observed in 39 and 32 individuals, respectively. Our network contained one unresolved loop and the ambiguity was factored into subsequent analyses.

We identified 899 single nucleotide variants (SNVs), 26 insertions, and 20 deletions in our dataset. The most frequently observed SNVs occurred in 281 genomes (m.263A>G, m.8860A>G, and m.15326A>G), and three more SNVs were observed in 280 genomes (m.750A>G, m.1438A>G, and m.4769A>G). Compared to the reference sequence (NC_012920), each person had an average of 25.3 variants (52 variants were the most identified in a single individual and 2 variants the fewest, each extreme observed in one person).

The distribution of major mitochondrial haplogroups within our dataset is reported in Table 1 (major mitochondrial haplogroups/clusters) and Additional File 4 (major mitochondrial haplogroups and sub-haplogroups). Our dataset contained individuals from 102 major mitochondrial haplogroups/clusters (or sub-haplogroups) in our dataset. As expected, the majority (987 of 1007) of individuals in our dataset belonged to European-based major mitochondrial haplogroups. We identified three different branches, corresponding to two different clades, significantly associated with mtDNA copy number.

Branches 124 and 121 are associated with mtDNA copy number

First, branches 124 and 121, p-values of 8.0×10^{-4} and 0.0043 (multi-test corrected p-values), respectively (Table 2, Figure 1), were associated with higher mtDNA copy number. The clade defined by branch 121 is wholly contained within branch 124 (Figure 2); therefore, these two branches are highly correlated and represent the same effect. Branch 124 is defined by a single variant (Table 3), *m.9667A>G*. This is a missense variant, *p.Asn154Ser*, located in cytochrome C oxidase 3 (*COXIII*). Branch 121 is defined by two variants (Table 3), *m.12582A>G* and *m.12879T>C*, both synonymous variants in NADH dehydrogenase 5 (*ND5*).

Since these two branches correspond to a single effect and branch 121 is wholly contained within branch 124, we consider only the clade defined by branch 124 from this point forward. This clade contains 14 individuals for whom we have mtDNA copy number measurements. Pairwise kinship coefficients are reported for these individuals in Additional File 5. Individuals in this clade have a mtDNA copy number nearly 50% higher (3.81 compared to 2.69, p-value 8.0×10^{-4}) than individuals in the rest of the dataset.

All of the individuals in the clade defined by branch 124 belong to major mitochondrial haplogroup U5A1, and have one of four different haplotypes (represented by nodes in Figure 2). Nine other individuals (five different haplotypes) in the dataset also belong to U5A1. These individuals are located in adjacent clades to the one defined by branch 124 and have significantly lower

mitochondrial copy numbers than the other U5A1 individuals (p-value 0.0082). The contrast of all U5A1 individuals against the rest of the dataset was nominally significant (p-value 0.0019). While no d-loop variants define branch 124, *m.16399A>G*, a d-loop variant, is only found in the U5A1 individuals in our dataset and in general appears to be found in all U5A1 individuals [69].

Branch 50 is associated with mtDNA copy number

Branch 50 is the third branch significantly associated higher mtDNA copy number (p-value 0.015, multi-test corrected p-value, Table 2 Figure 1). This represents a statistically separate effect as we controlled for the effect of branch 124 in our analyses (just as we controlled for branch 50 in our analyses of branch 124). Eight sequence features define branch 50: seven single nucleotide variants and one nine base pair deletion (Table 3). Six of the eight features are intergenic or synonymous, but the other two are both missense variants. *m.5277T>C* (*p.Phe270Leu*) is a missense variant in NADH dehydrogenase 2 (*ND2*) and *m.6489C>A* (*p.Leu196Ile*) is a missense variant in cytochrome C oxidase 1 (*COXI*).

In the clade defined by branch 50 there are 12 individuals with mtDNA copy number measurements. Pairwise kinship coefficients are reported for these individuals in Additional File 6. The average mtDNA copy number for individuals in this clade is 3.64 and is significantly higher than the average for the rest of the dataset (2.69, p-value 0.015). Individuals in this clade belong to major mitochondrial haplogroup T2 and all have the exact same haplotype. There were no other T2 individuals in the rest of our dataset; however, there were T2A, T2B, T2C, and T2E individuals. The contrast between T2 and all T2 sub-haplogroups (T2A, T2B, T2C, and T2E) and the rest of the data was nominally significant, p-value 0.019, and the contrast of T2B individuals alone against the rest of the dataset was nominally significant, p-value 0.0062.

G, branch 124",1,0,1,0,0pc,0pc,0pc,0pc>Bioinformatic Analyses of m.9667A>G, branch 124

m.9667A>G is the defining sequence change between the U5A1 individuals in our dataset who had significantly

Table 2 Demographic information for significant contrasts.

	Individuals/Missing	p-value ¹		p-value ²		Age	Male/Female	Mean copy #
		Nominal	Corrected	Nominal	Corrected			
Whole network	1007/193	N/A	N/A	N/A	N/A	75.6	442/565	2.69
Branch 124	17/3	0	6.0×10^{-4}	0	8.0×10^{-4}	75.2	9/8	3.81
Branch 121	10/1	0	0.002	1.0×10^{-4}	0.0043	76.3	4/6	4.01
Branch 50	15/3	2.0×10^{-4}	0.017	2.0×10^{-4}	0.015	78.4	7/8	3.64

Here we report demographic information for each of the significant contrasts and for all the individuals in the dataset. The clade represented by Branch 121 is wholly contained within Branch 124, so these two contrasts represent a single effect. Branches 124 and 50 represent separate effects. Missing refers to the number of individuals for whom we have no mtDNA copy number measurement.¹p-values were calculated controlling only for the other significant branches ²p-values were calculated using age, gender, mtDNA copy number family risk score, and the other significant effects as covariates

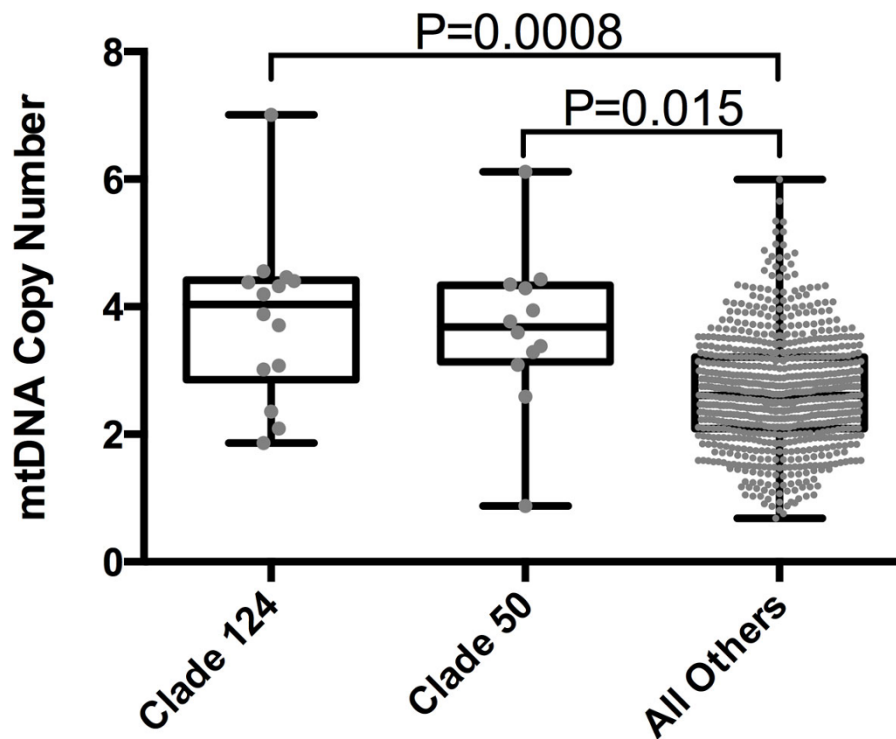


Figure 1 Box plot comparing mitochondrial copy number between different clades. The grey dots represent the mitochondrial copy number for each member of the representative groups. The top and bottom of the boxes correspond to the 75th and 25th percentiles, respectively, and the line through the box is the median mitochondrial copy number for the group. The whiskers correspond to the maximum and minimum mitochondrial copy numbers for the group. Three different groups are represented here: the clades defined by branches 124 and 50, and a group containing all other individuals in the dataset. The y-axis is the mitochondrial copy number. The reported p-values are corrected.

higher mtDNA copy number levels from the other U5A1 individuals in our dataset whose copy number measurements were not statistically different from the rest of the dataset. *m.9667A>G* causes an amino acid substitution, asparagine to serine, at position 154 of *COXIII*, which is located in an 11 residue stretch between transmembrane domains. Since this is a missense mutation, we sought to determine if it changes or inhibits *COXIII* and/or the cytochrome c oxidase complex. We compared *COXIII* sequences in organisms from humans through yeast by aligning a 41-residue stretch of *COXIII*. In Figure 3, position 154 of *COXIII* (the position of the amino acid substitution corresponding to *m.9667A>G*) is in position 21 of the alignment. As seen in Figure 3, two different amino acids appear in this position: asparagine and glycine. Asparagine and glycine are both uncharged amino acids; however, asparagine is polar, whereas glycine is nonpolar. *M.9667A>G* results in serine replacing asparagine. Serine is polar and similar in size to asparagine (asparagine 132.1 g/mol, glycine 75.1 g/mol, and serine 105.1 g/mol).

We further analyzed the effect of this substitution on *COXIII* by using in silico algorithms that predict the effect of amino acid substitutions on protein function

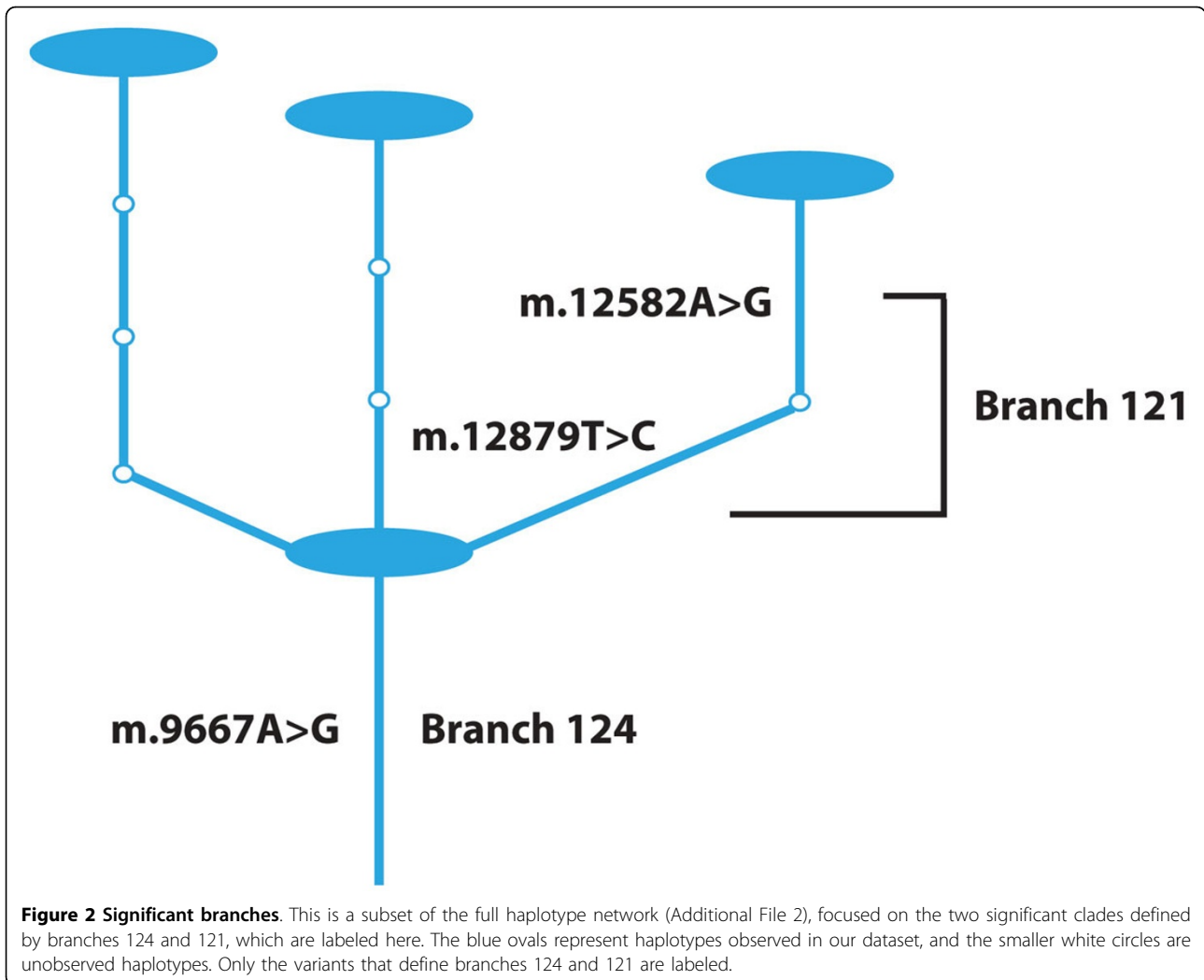
using a variety of criteria such as conservation, amino acid biochemical properties, known domains/structures of the protein, etc. Polyphen-2 predicted the substitution to be benign and SIFT predicted a pathogenic mutation, but noted that its prediction was of very low confidence.

Lastly, we looked at possible interactions of *COXIII* with known regulators (listed in the Introduction) of mtDNA copy number to identify mechanisms *m.9667A>G* could cause the increased copy number. We found common regulators of both *COXIII* and the mtDNA copy number regulators, and we found ways that these regulators could affect *COXIII* expression; however, we identified no pathways by which *COXIII* could regulate mtDNA copy number by known mechanisms (Figure 4).

C and m.6489C>A, branch

50", 1,0,1,0,0pc,0pc,0pc,0pc>Bioinformatic Analyses of m.5277T>C and m.6489C>A, branch 50

It is more difficult to say which variants are causing the increase in mtDNA copy number for the clade-defined by branch 50 since this branch consists of eight different sequence features. We chose to focus our analyses on two of the features: *m.5277T>C* and *m.6489C>A* since these



two variants are missense variants and the six others features are either synonymous or intergenic changes.

First, *m.5277T>C* results in a phenylalanine to leucine change in *ND2*. Position 270 of *ND2* is column 21 in Figure 5. At this position, primates have phenylalanine and other species before have leucine. *p.Phe270Leu* changes the human sequence back to the historical residue. Polyphen-2 and SIFT predict that this substitution is benign and tolerated, respectively.

Next, *m.6489C>A* causes a leucine to isoleucine change at position 196 of *COXI*. This region of *COXI* is highly conserved. Position 196 is leucine in every species we examined from humans to yeast except nematodes that have valine at this position (Figure 6). Polyphen-2 predicts that this substitution is probably damaging, and SIFT also predicts that this substitution affects function, but it is a low confidence prediction. Lastly, we identified pathways in which *COXI* malfunction could cause an increase in mtDNA copy number. First we analyzed

pathways for all nuclear genes known to modify mtDNA copy number and found no obvious pathways for genes other than *p53* and *TFAM*. We identified several pathways in which *COXI* malfunction could change mtDNA copy number, the majority of which function through intermediate genes activated by reactive oxygen species (Figure 7).

Discussion

Using 1007 full mitochondrial genome sequences we have identified sequence variation in mtDNA that affects mtDNA copy number. Two different clades were significantly associated with higher mtDNA copy number. Each of these clades represents statistically separate effects. The first was defined by branch 124 and consisted of individuals with haplogroup U5A1, and is defined by *m.9667A>G* (*p.Asn154Ser*). This variant has also been reported in D2A1, D4M1, and J1B2A haplogroups [69]; however, no individuals in our dataset

Table 3 Defining variants for the three significant contrasts.

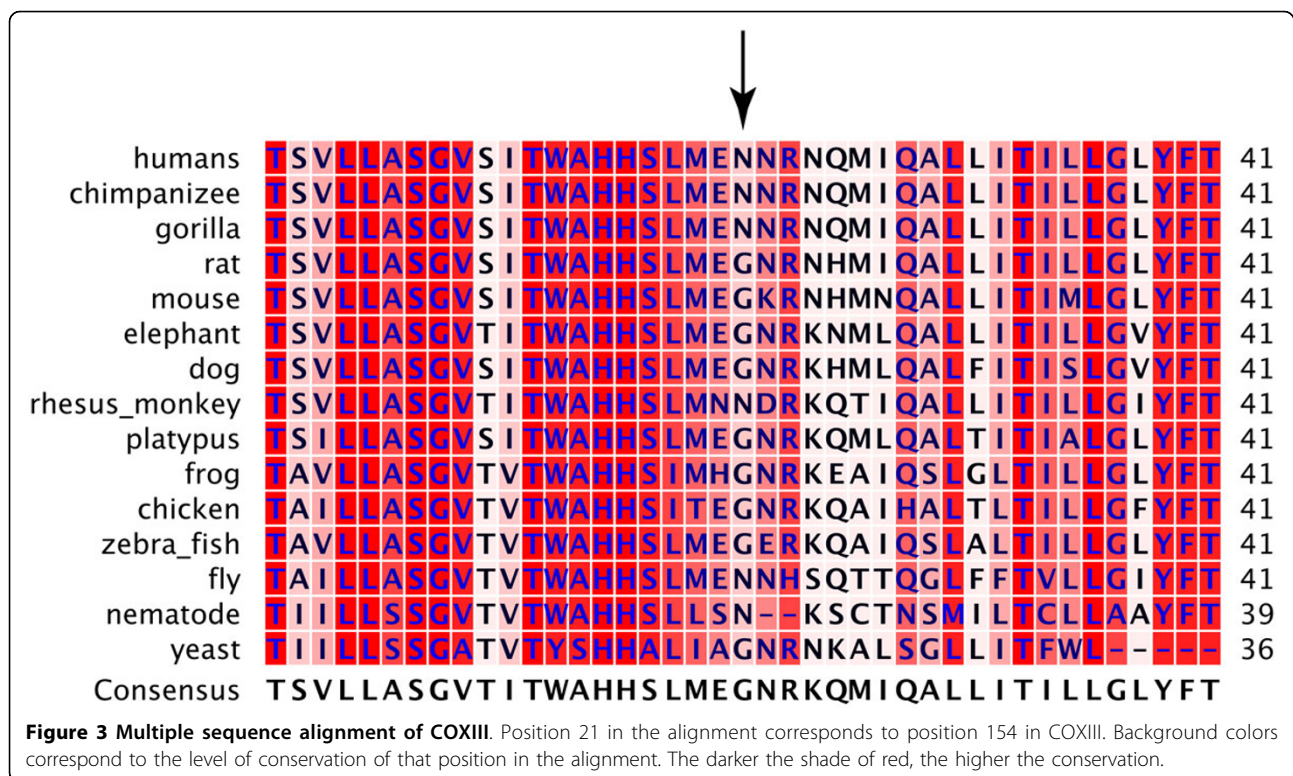
Branch	Nucleotide Change	Amino Acid Change	Gene
Branch 124	m.9667A>G	p.Asn154Ser	Cytochrome C Oxidase 3
Branch 121	m.12582A>G	p.Leu82Leu	NADH Dehydrogenase 5
	m.12879T>C	p.Gly181Gly	NADH Dehydrogenase 5
Branch 50	m.5277T>C	p.Phe270Leu	NADH Dehydrogenase 2
	m.5426T>C	p.His319His	NADH Dehydrogenase 2
	m.6489C>A	p.Leu196Ile	Cytochrome C Oxidase 1
	m.8270C>T	N/A	Intergenic
	m.del8281-8289	N/A	Intergenic
	m.14458C>T	p.Ala72Ala	NADH Dehydrogenase 6
	m.15028C>A	p.Leu94Leu	Cytochrome B
	m.15043G>A	p.Gly99Gly	Cytochrome B

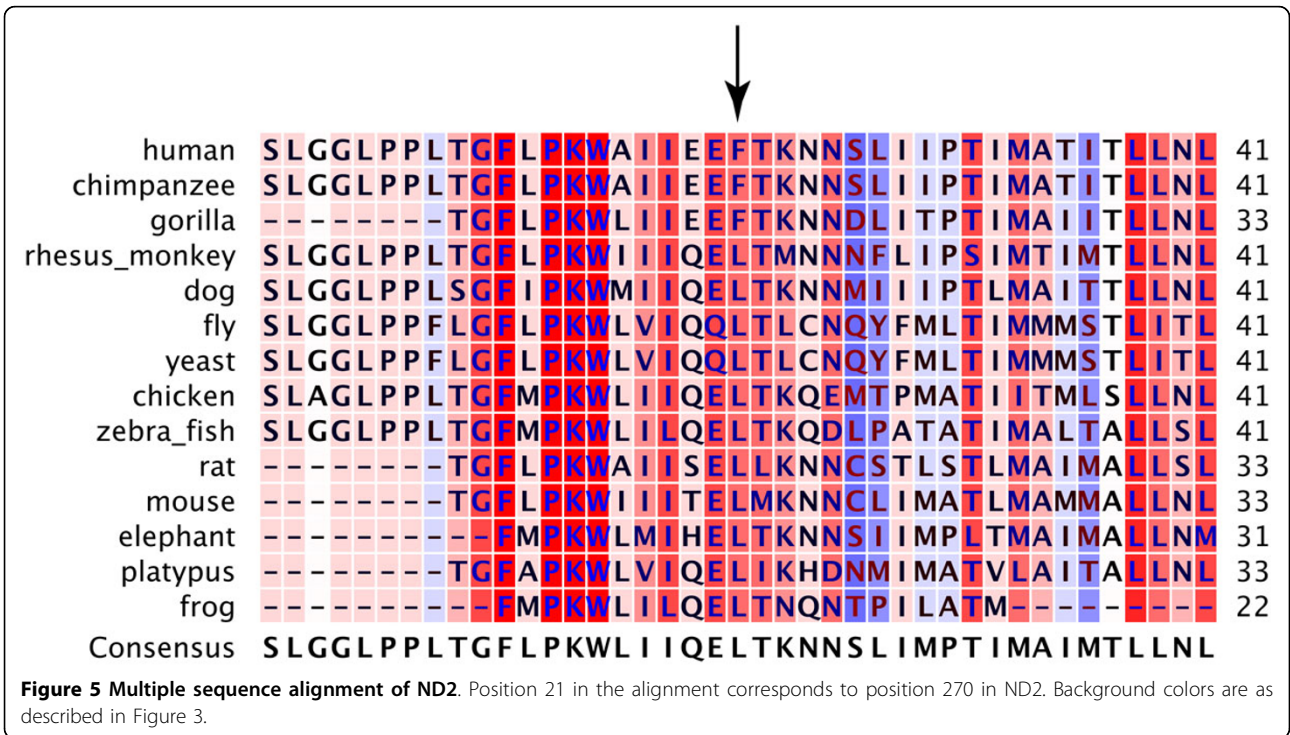
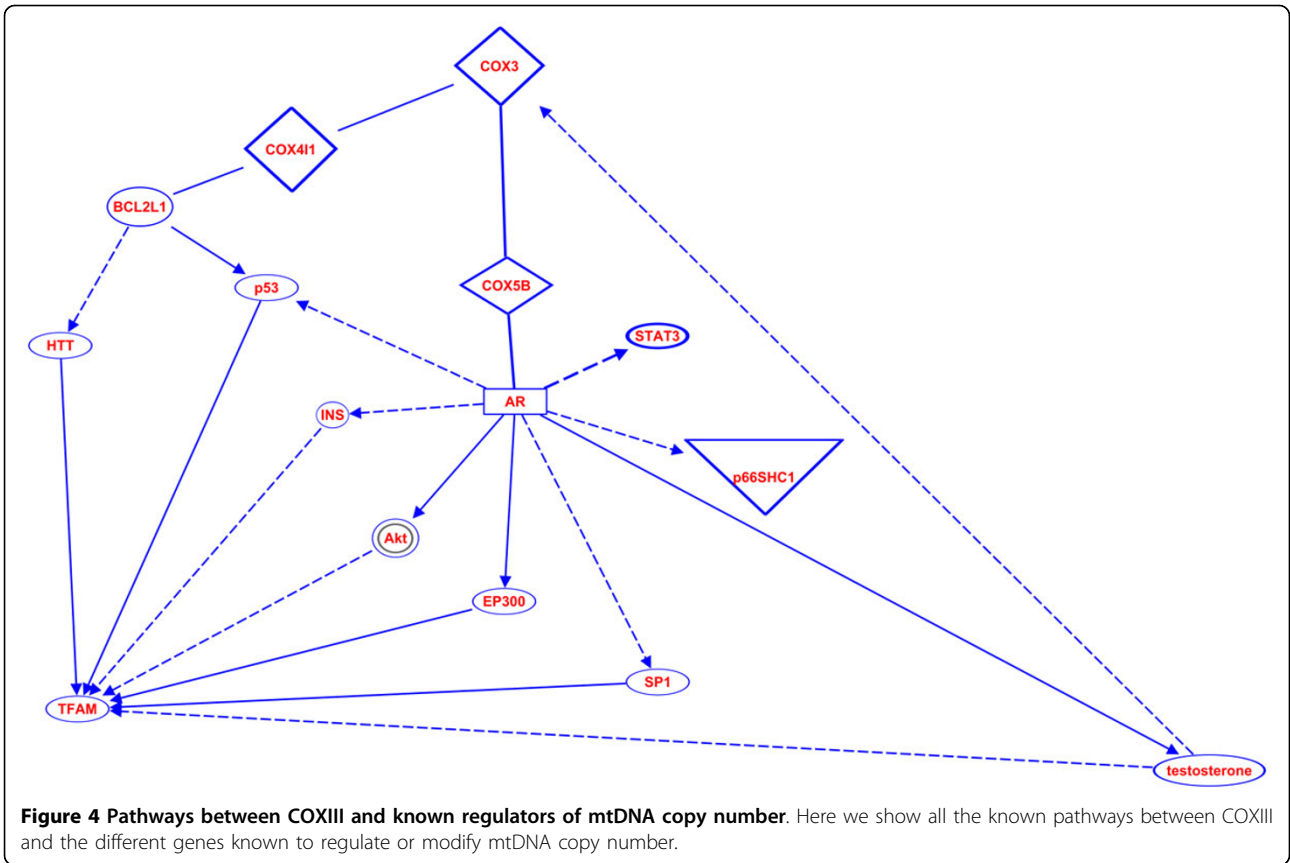
There were three significant contrasts in our dataset, two of which, 124 and 121, which represent a single effect. One or more sequence features define each of the branches, and each is listed here with the resulting protein change, and the gene the feature is located in.

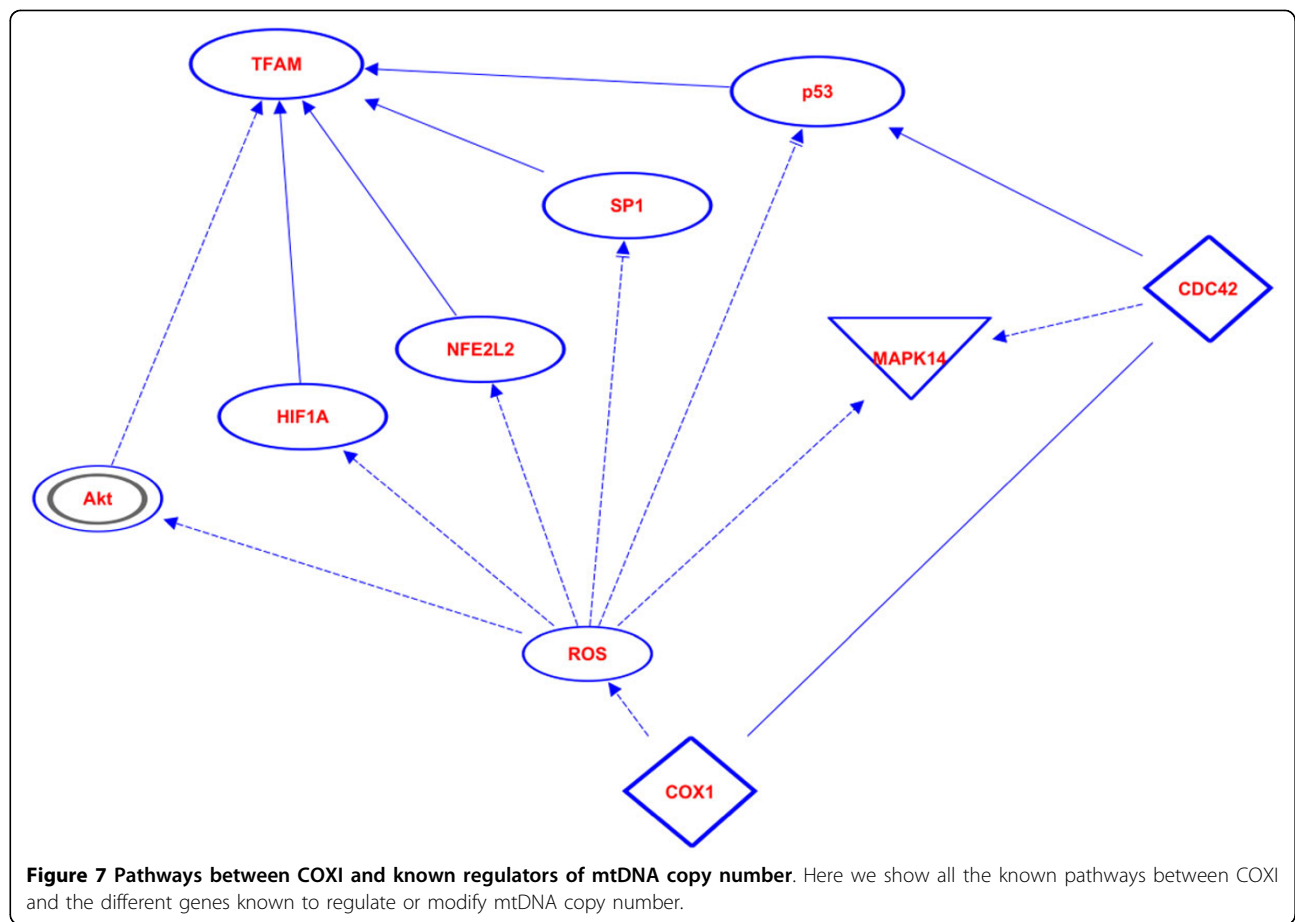
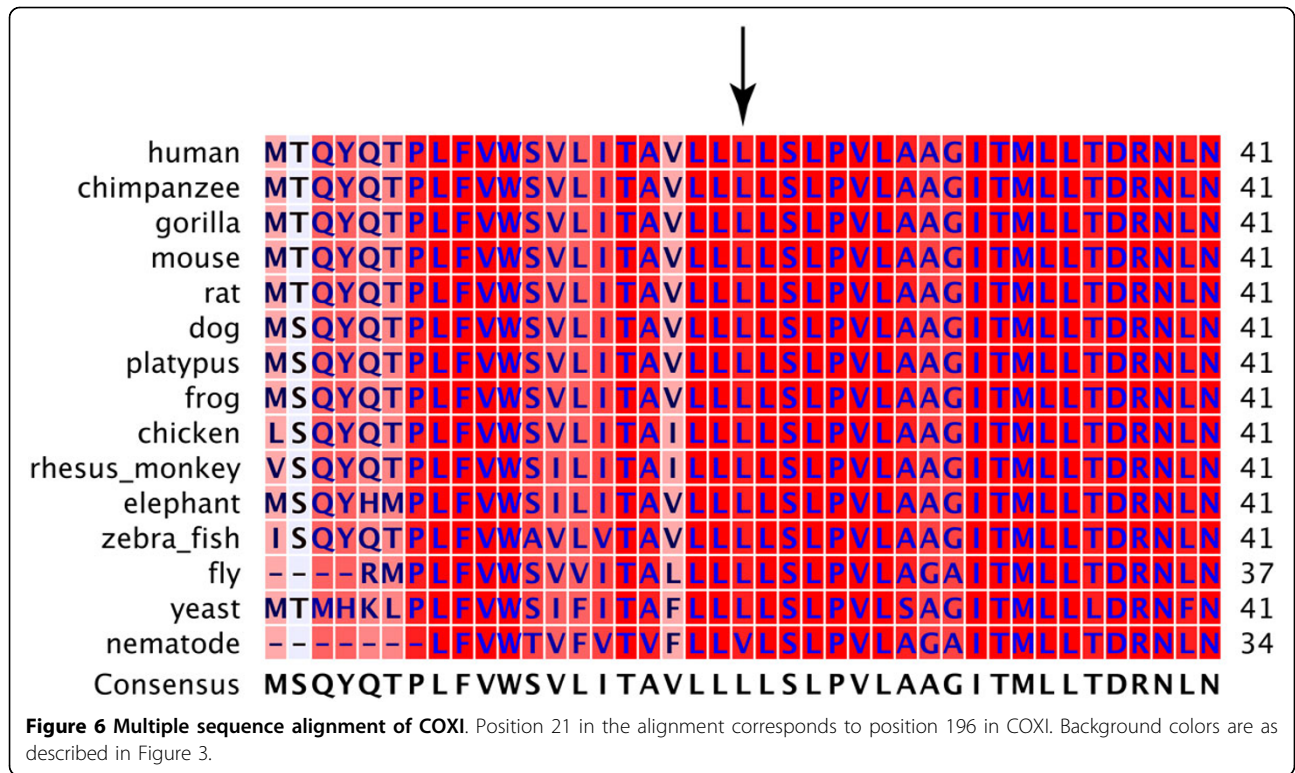
belong to these haplogroups. We analyzed this substitution to determine if it likely causes *COXIII* malfunction, and then to determine whether or not it could cause the observed increase in mtDNA copy number. Our analyses

suggest this substitution does not impact *COXIII* function. This conclusion is based on several lines of evidence; first, this is a high frequency, known substitution [70], second the substitution occurs in an unconserved site (Figure 3), third asparagine and glycine, two very different amino acids, appear historically in this position and a change from asparagine to the more similar serine is likely to be tolerated, and finally this position is in a short stretch of sequence located between transmembrane domains and is not a known position of importance in the heme-copper oxidase subunit III super family, of which it is a part.

While it seems likely this variant does not disrupt *COXIII* function, it is still possible that it could alter protein-protein interactions or specific dynamics associated with the electron transport chain and ultimately lead to changes in mtDNA copy number. Our initial analyses of known regulators of mtDNA copy number with *COXIII* (Figure 4) revealed no obvious mechanism for *COXIII* to directly modify mtDNA copy number; however, Pello et al [71] reported that *m.9667A>G* causes respiratory chain assembly deficiencies in patients with Leber's hereditary optic neuropathy. *TFAM* (the main known regulator of mtDNA copy number) concentration and mtDNA copy number are proportional [42]; therefore, upregulators of *TFAM* increase mtDNA copy number. *TFAM* is regulated by *NRF-1* and *NRF-2*, and all three are sensitive to the energy needs of the cell [72,73]. Silencing of *NRF-1* is known to lead to lower levels of *TFAM* and *NRF-1* expression is known to increase in response to signals







meant to increase energy production [73]. We propose the following model for *m.9667A>G* to increase mtDNA copy number. First, *m.9667A>G* can decrease the efficiency of complex assembly and decrease overall energy production of the mitochondria, in response, *NRF-1* expression increases, which in turn increases *TFAM* expression, and *TFAM* expression increases mtDNA copy number.

The second clade significantly associated with higher mtDNA copy number is defined by branch 50. Branch 50 consists of eight sequence features, six of which are synonymous changes or located in intergenic regions. We focused our functional analyses on the two missense variants. The first is *m.5277T>C* (*p.Phe270Leu*) in *ND2*. Besides T2, this variant has also been reported in L1C1A1B individuals [69] and there are no L1C1A1B individuals in our dataset. This variant is in an unconserved region immediately adjacent to a low complexity region, predicted to not affect protein function, and is not novel [74,75]. These data suggest this variant is not functionally deleterious.

In contrast, there is evidence that the second of the two variants that define this clade, *m.6489C>A* (*p.Leu196Ile*), is functionally deleterious and can explain changes in mtDNA copy number. *m.6489C>A* is specific to T2 [69]. This variant in *COXI* occurs in a highly conserved region in both *COXI* and the heme-copper subunit I domain it is in, and is predicted to affect function. *COXI* and *COXII* form the catalytic center of cytochrome c oxidase (COX), or complex IV, in the respiratory complex. *m.6489C>A* is not a novel mutation and has been reported to lead to COX deficiency and a destabilization of complex IV [76]. It does appear, however, that a high threshold of mutant mtDNA is required before a phenotype appears [76,77]. The variant has only been observed in mitochondrial haplogroups T2F1 [69] and in our T2 individuals here.

COXI is involved in several pathways that include known regulators of mtDNA copy number (Figure 7). Mutations in mtDNA in general, and cytochrome c oxidase malfunction specifically, lead to an increase in reactive oxygen species (ROS) [78]. ROS increase expression and/or activate protein kinase B (*Akt*) [79,80], HIF-1 Alpha (*HIF1A*) [81,82], nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2*) [83], *SP1* transcription factor [84], and *p53* [85,86]. *p53* could also be potentially activated by *COXI* binding *CDC42* [87,88]. Of these genes, two suppress *TFAM* (*Akt* and *HIF1A*) [89,90], and three increase *TFAM* expression (*NFE2L2*, *SP1*, and *p53*) [91-93].

Conclusions

As we outlined previously, mtDNA copy number is related to several important human health phenotypes including several age-related disorders. The identification of these variants that increase mtDNA copy number has

important implications in understanding the pathological processes that underlie these phenotypes. We have used bioinformatics analyses to generate hypotheses for the mechanisms by which these variants influence mtDNA copy number, successfully generating several hypotheses. Future work to characterize these mechanisms will provide important insights into the effects of mitochondrial genomic variation on mtDNA copy number and broader human phenotypes.

Additional material

Additional file 1: (docx) Mitochondrial copy number measurements.

List of mitochondrial copy number measurements, age, and gender for all individuals used in our analyses.

Additional file 2: (pdf) Haplotype network. Our haplotype network was constructed using TCS and 285 full mitochondrial genomes. The arrows point to each of the three branches representing the significant contrasts. The blue arrow points to branch 124, the red to branch 121, and the green to branch 50.

Additional file 3: (tiff) Haplotype network. We collapsed our haplotype network (Additional File 2) into nodes corresponding to the major mitochondrial haplogroups present in our network.

Additional file 4: (xlsx) Mitochondrial haplogroups. We have listed all the major mitochondrial haplogroups as well as sub-haplogroups in our dataset. The numbers in parenthesis represent the number of individuals in our dataset that belong to the haplogroup. There were 1007 total individuals in our dataset.

Additional file 5: (docx) Kinship coefficients for the clade defined by branch 124. We have listed the pairwise kinship coefficients for all the individuals in this clade. The IDs (row and column titles) correspond to the same IDs used in Additional File 1.

Additional file 6: (docx) Kinship coefficients for the clade defined by branch 50. We have listed the pairwise kinship coefficients for all the individuals in this clade. The IDs (row and column titles) correspond to the same IDs used in Additional File 1.

List of abbreviations

mtDNA, mitochondrial genome; CNS, central nervous system, AD, Alzheimer's disease; ROS, reactive oxygen species; ETC, electron transport chain; SNV, single nucleotide variant; TFAM, mitochondrial transcription factor A; STAT3, signal transducer and activator of transcription 3; D-loop, displacement loop; COX, cytochrome C oxidase; COXI, cytochrome C oxidase 1; COXIII, cytochrome C oxidase 3; ND2, NADH dehydrogenase 2; ND5, NADH dehydrogenase 5; Akt, activate protein kinase B; HIF1A, HIF-1 Alpha; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; CEPH, Centre d'Etude du Polymorphisme.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PGR, TYM, RMC, and JSKK designed analyses; PGR2, TJM, and RAK performed the analyses; CDC, JTT, MCN, RGM, EO, and RAK collected the data; RMC measured mitochondrial copy number; PGR2 and SJF wrote the paper; all authors contributed to revisions and approved of the final draft.

Acknowledgements

The authors thank the participants and staff of the Dementia Progression Study, the Utah Population Database, and the Cache County Study on Memory Health and Aging for their important contributions to this work. Additionally, the authors acknowledge the assistance of Drs. David Ward and Ned Weinschenker. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declarations

This work was supported by grants from NIH (R01AG11380, R01AG21136, R01AG31272, R01AG042611), the Alzheimer's Association (MNRG-11-205368) and the Utah Science, Technology, and Research initiative (USTAR), and the Brigham Young University Gerontology Program (JSKK).

This article has been published as part of BMC Bioinformatics Volume 15 Supplement 7, 2014: Selected articles from the 10th Annual Biotechnology and Bioinformatics Symposium (BIOT 2013). The full contents of the supplement are available online at <http://www.biomedcentral.com/bmcbioinformatics/supplements/15/S7>

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Published: 28 May 2014

References

- Sequeira A, Martin MV, Rollins B, Moon EA, Bunney WE, Macciardi F, Lupoli S, Smith EN, Kelson J, Magnan CN, et al: **Mitochondrial mutations and polymorphisms in psychiatric disorders.** *Frontiers in genetics* 2012, **3**:103.
- Konradi C, Eaton M, MacDonald ML, Walsh J, Benes FM, Heckers S: **Molecular evidence for mitochondrial dysfunction in bipolar disorder.** *Archives of general psychiatry* 2004, **61**(3):300-308.
- Vilming ST, Dietrichson P, Isachsen MM, Lovvik L, Heiberg A: **Late-onset hereditary myopathy with abnormal mitochondria and progressive dementia.** *Acta neurologica Scandinavica* 1986, **73**(5):502-506.
- Swerdlow RH, Burns JM, Khan SM: **The Alzheimer's disease mitochondrial cascade hypothesis.** *J Alzheimers Dis* 2010, **20** Suppl 2: S265-279.
- Swerdlow RH, Khan SM: **A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease.** *Med Hypotheses* 2004, **63**(1):8-20.
- Swerdlow RH, Khan SM: **The Alzheimer's disease mitochondrial cascade hypothesis: an update.** *Exp Neurol* 2009, **218**(2):308-315.
- Ballinger SW, Shoffner JM, Gebhart S, Koontz DA, Wallace DC: **Mitochondrial diabetes revisited.** *Nature genetics* 1994, **7**(4):458-459.
- Bianchi M, Rizza T, Verrigni D, Martinelli D, Tozzi G, Torraco A, Piemonte F, Dionisi-Vici C, Nobili V, Francalanci P, et al: **Novel large-range mitochondrial DNA deletions and fatal multisystemic disorder with prominent hepatopathy.** *Biochemical and biophysical research communications* 2011, **415**(2):300-304.
- Jun AS, Brown MD, Wallace DC: **A mitochondrial DNA mutation at nucleotide pair 14459 of the NADH dehydrogenase subunit 6 gene associated with maternally inherited Leber hereditary optic neuropathy and dystonia.** *Proceedings of the National Academy of Sciences of the United States of America* 1994, **91**(13):6206-6210.
- Shoffner JM, Lott MT, Lezza AM, Seibel P, Ballinger SW, Wallace DC: **Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation.** *Cell* 1990, **61**(6):931-937.
- Taylor RW, Morris AA, Hutchinson M, Turnbull DM: **Leigh disease associated with a novel mitochondrial DNA ND5 mutation.** *European journal of human genetics : EJHG* 2002, **10**(2):141-144.
- Finsterer J: **Hematological manifestations of primary mitochondrial disorders.** *Acta haematologica* 2007, **118**(2):88-98.
- Koutnikova H, Campuzano V, Foury F, Dolle P, Cazzalini O, Koenig M: **Studies of human, mouse and yeast homologues indicate a mitochondrial function for frataxin.** *Nature genetics* 1997, **16**(4):345-351.
- Gu M, Cooper JM, Butler P, Walker AP, Mistry PK, Dooley JS, Schapira AH: **Oxidative-phosphorylation defects in liver of patients with Wilson's disease.** *Lancet* 2000, **356**(9228):469-474.
- Schlame M, Towbin JA, Heerdt PM, Jehle R, DiMauro S, Blanck TJ: **Deficiency of tetralinoleoyl-cardiolipin in Barth syndrome.** *Annals of neurology* 2002, **51**(5):634-637.
- Larsson NG: **Somatic mitochondrial DNA mutations in mammalian aging.** *Annual review of biochemistry* 2010, **79**:683-706.
- Adam-Vizi V: **Production of reactive oxygen species in brain mitochondria: contribution by electron transport chain and non-electron transport chain sources.** *Antioxid Redox Signal* 2005, **7**(9-10):1140-1149.
- Cooke MS, Evans MD, Dizdaroglu M, Lunec J: **Oxidative DNA damage: mechanisms, mutation, and disease.** *FASEB J* 2003, **17**(10):1195-1214.
- Li Z, Wu J, Deleo CJ: **RNA damage and surveillance under oxidative stress.** *JUBMB Life* 2006, **58**(10):581-588.
- Stadtman ER, Levine RL: **Free radical-mediated oxidation of free amino acids and amino acid residues in proteins.** *Amino Acids* 2003, **25**(3-4):207-218.
- Vendelbo MH, Nair KS: **Mitochondrial longevity pathways.** *Biochim Biophys Acta* 2011, **1813**(4):634-644.
- Hartmann N, Reichwald K, Wittig I, Drose S, Schmeisser S, Luck C, Hahn C, Graf M, Gausmann U, Terzibas E, et al: **Mitochondrial DNA copy number and function decrease with age in the short-lived fish *Nothobranchius furzeri*.** *Aging cell* 2011, **10**(5):824-831.
- Cree LM, Patel SK, Pyle A, Lynn S, Turnbull DM, Chinnery PF, Walker M: **Age-related decline in mitochondrial DNA copy number in isolated human pancreatic islets.** *Diabetologia* 2008, **51**(8):1440-1443.
- Barazzoni R, Short KR, Nair KS: **Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart.** *The Journal of biological chemistry* 2000, **275**(5):3343-3347.
- Miller FJ, Rosenfeldt FL, Zhang C, Linnane AW, Nagley P: **Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age.** *Nucleic acids research* 2003, **31**(11):e61.
- Lee JE, Park H, Ju YS, Kwak M, Kim JI, Oh HY, Seo JS: **Higher mitochondrial DNA copy number is associated with lower prevalence of microalbuminuria.** *Experimental & molecular medicine* 2009, **41**(4):253-258.
- Hirai K, Aliev G, Nunomura A, Fujioka H, Russell RL, Atwood CS, Johnson AB, Kress Y, Vinters HV, Tabaton M, et al: **Mitochondrial abnormalities in Alzheimer's disease.** *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2001, **21**(9):3017-3023.
- Santos RX, Correia SC, Wang X, Perry G, Smith MA, Moreira PI, Zhu X: **Alzheimer's disease: diverse aspects of mitochondrial malfunctioning.** *International journal of clinical and experimental pathology* 2010, **3**(6):570-581.
- Lynch SM, Weinstein SJ, Virtamo J, Lan Q, Liu CS, Cheng WL, Rothman N, Albanes D, Stolzenberg-Solomon RZ: **Mitochondrial DNA copy number and pancreatic cancer in the alpha-tocopherol beta-carotene cancer prevention study.** *Cancer prevention research* 2011, **4**(11):1912-1919.
- Yu M, Zhou Y, Shi Y, Ning L, Yang Y, Wei X, Zhang N, Hao X, Niu R: **Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients.** *JUBMB life* 2007, **59**(7):450-457.
- Thyagarajan B, Wang R, Barcelo H, Koh WP, Yuan JM: **Mitochondrial copy number is associated with colorectal cancer risk.** *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2012.
- Yu M, Wan Y, Zou Q: **Decreased copy number of mitochondrial DNA in Ewing's sarcoma.** *Clinica chimica acta; international journal of clinical chemistry* 2010, **411**(9-10):679-683.
- Lamson DW, Plaza SM: **Mitochondrial factors in the pathogenesis of diabetes: a hypothesis for treatment.** *Alternative medicine review : a journal of clinical therapeutic* 2002, **7**(2):94-111.
- Blokhin A, Vyshkina T, Komoly S, Kalman B: **Variations in mitochondrial DNA copy numbers in MS brains.** *Journal of molecular neuroscience : MN* 2008, **35**(3):283-287.
- Coskun P, Wyrembak J, Schriener SE, Chen HW, Marciniack C, Laferla F, Wallace DC: **A mitochondrial etiology of Alzheimer and Parkinson disease.** *Biochimica et biophysica acta* 2012, **1820**(5):553-564.
- Lee JW, Park KD, Im JA, Kim MY, Lee DC: **Mitochondrial DNA copy number in peripheral blood is associated with cognitive function in apparently healthy elderly women.** *Clinica chimica acta; international journal of clinical chemistry* 2010, **411**(7-8):592-596.

37. Suomalainen A, Isohanni P: **Mitochondrial DNA depletion syndromes—many genes, common mechanisms.** *Neuromuscular disorders : NMD* 2010, **20**(7):429-437.
38. Lebedeva MA, Shadel GS: **Cell cycle- and ribonucleotide reductase-driven changes in mtDNA copy number influence mtDNA inheritance without compromising mitochondrial gene expression.** *Cell cycle* 2007, **6**(16):2048-2057.
39. Clay Montier LL, Deng JJ, Bai Y: **Number matters: control of mammalian mitochondrial DNA copy number.** *Journal of genetics and genomics = Yi chuan xue bao* 2009, **36**(3):125-131.
40. Hassanin A, Ropiquet A, Couloux A, Cruaud C: **Evolution of the mitochondrial genome in mammals living at high altitude: new insights from a study of the tribe Caprini (Bovidae, Antilopinae).** *Journal of molecular evolution* 2009, **68**(4):293-310.
41. Carling PJ, Cree LM, Chinnery PF: **The implications of mitochondrial DNA copy number regulation during embryogenesis.** *Mitochondrion* 2011, **11**(5):686-692.
42. Ekstrand MI, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K, Rustin P, Gustafsson CM, Larsson NG: **Mitochondrial transcription factor A regulates mtDNA copy number in mammals.** *Human molecular genetics* 2004, **13**(9):935-944.
43. Matsushima Y, Goto Y, Kaguni LS: **Mitochondrial Lon protease regulates mitochondrial DNA copy number and transcription by selective degradation of mitochondrial transcription factor A (TFAM).** *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(43):18410-18415.
44. Guo J, Zheng L, Liu W, Wang X, Wang Z, French AJ, Kang D, Chen L, Thibodeau SN: **Frequent truncating mutation of TFAM induces mitochondrial DNA depletion and apoptotic resistance in microsatellite-unstable colorectal cancer.** *Cancer research* 2011, **71**(8):2978-2987.
45. Taylor SD, Zhang H, Eaton JS, Rodeheffer MS, Lebedeva MA, O'Rourke T W, Siede W, Shadel GS: **The conserved Mec1/Rad53 nuclear checkpoint pathway regulates mitochondrial DNA copy number in *Saccharomyces cerevisiae*.** *Molecular biology of the cell* 2005, **16**(6):3010-3018.
46. Kulawiec M, Ayyasamy V, Singh KK: **p53 regulates mtDNA copy number and mitochekpoint pathway.** *Journal of carcinogenesis* 2009, **8**:8.
47. Gianotti TF, Castano G, Gemma C, Burgueno AL, Rosselli MS, Pirola CJ, Sookoian S: **Mitochondrial DNA copy number is modulated by genetic variation in the signal transducer and activator of transcription 3 (STAT3).** *Metabolism: clinical and experimental* 2011, **60**(8):1142-1149.
48. Trinei M, Berniakovich I, Pellicci PG, Giorgio M: **Mitochondrial DNA copy number is regulated by cellular proliferation: a role for Ras and p66 (Shc).** *Biochimica et biophysica acta* 2006, **1757**(5-6):624-630.
49. Tewari S, Santos JM, Kowluru RA: **Damaged mitochondrial DNA replication system and the development of diabetic retinopathy.** *Antioxidants & redox signaling* 2012, **17**(3):492-504.
50. Ylikallio E, Tynymäa H, Tsutsui H, Ide T, Suomalainen A: **High mitochondrial DNA copy number has detrimental effects in mice.** *Human molecular genetics* 2010, **19**(13):2695-2705.
51. Ridge PG, Maxwell T, Corcoran C, Norton M, Tschanz J, O'Brien E, Kerber R, Cawthon R, Munger R, JSK K: **Mitochondrial genomic analysis of late onset Alzheimer's disease reveals protective haplogroups H6A1A/H6A1B: the Cache County Study on Memory in Aging.** *PLoS One* 2012, **7**(9):e45134.
52. Breitner JC, Wyse BW, Anthony JC, Welsh-Bohmer KA, Steffens DC, Norton MC, Tschanz JT, Plassman BL, Meyer MR, Skoog I, et al: **APOE-epsilon4 count predicts age when prevalence of AD increases, then declines: the Cache County Study.** *Neurology* 1999, **53**(2):321-331.
53. Jorde LB: **The genetic structure of the Utah Mormons: migration analysis.** *Hum Biol* 1982, **54**(3):583-597.
54. Jorde LB, Morgan K: **Genetic structure of the Utah Mormons: isonymy analysis.** *Am J Phys Anthropol* 1987, **72**(3):403-412.
55. O'Brien E, Rogers AR, Beesley J, Jorde LB: **Genetic structure of the Utah Mormons: comparison of results based on RFLPs, blood groups, migration matrices, isonymy, and pedigrees.** *Hum Biol* 1994, **66**(5):743-759.
56. Cawthon RM: **Telomere length measurement by a novel monochrome multiplex quantitative PCR method.** *Nucleic acids research* 2009, **37**(3):e21.
57. Parfait B, Rustin P, Munnich A, Rotig A: **Co-amplification of nuclear pseudogenes and assessment of heteroplasmy of mitochondrial DNA mutations.** *Biochemical and biophysical research communications* 1998, **247**(1):57-59.
58. Thompson JD, Gibson TJ, Higgins DG: **Multiple sequence alignment using ClustalW and ClustalX.** *Curr Protoc Bioinformatics* 2002, **Chapter 2**(Unit 2.3).
59. Clement M, Posada D, Crandall KA: **TCS: a computer program to estimate gene genealogies.** *Mol Ecol* 2000, **9**(10):1657-1659.
60. Templeton AR, Maxwell T, Posada D, Stengard JH, Boerwinkle E, Sing CF: **Tree scanning: a method for using haplotype trees in phenotype/genotype association studies.** *Genetics* 2005, **169**(1):441-453.
61. Posada D, Maxwell TJ, Templeton AR: **TreeScan: a bioinformatic application to search for genotype/phenotype associations using haplotype trees.** *Bioinformatics* 2005, **21**(9):2130-2132.
62. Westfall P, Young SS: *Resampling-Based Multiple Testing: Examples and Methods for p-Value Adjustments.* New York City, NY: Wiley-Interscience; 1993.
63. Kerber RA: **Method for calculating risk associated with family history of a disease.** *Genet Epidemiol* 1995, **12**(3):291-301.
64. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *Journal of molecular biology* 1990, **215**(3):403-410.
65. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, et al: **CDD: a Conserved Domain Database for the functional annotation of proteins.** *Nucleic acids research* 2011, **39**(Database):D225-229.
66. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR: **A method and server for predicting damaging missense mutations.** *Nature methods* 2010, **7**(4):248-249.
67. Kumar P, Henikoff S, Ng PC: **Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm.** *Nature protocols* 2009, **4**(7):1073-1081.
68. Kumar P, Henikoff S, Ng PC: **Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm.** *Nature protocols* 2009, **4**(7):1073-1081.
69. van Oven M, Kayser M: **Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation.** *Human mutation* 2009, **30**(2):E386-394.
70. Saxena R, de Bakker PI, Singer K, Mootha V, Burt N, Hirschhorn JN, Gaudet D, Isomaa B, Daly MJ, Groop L, et al: **Comprehensive association testing of common mitochondrial DNA variation in metabolic disease.** *American journal of human genetics* 2006, **79**(1):54-61.
71. Pello R, Martin MA, Carelli V, Nijtmans LG, Achilli A, Pala M, Torroni A, Gomez-Duran A, Ruiz-Pesini E, Martinuzzi A, et al: **Mitochondrial DNA background modulates the assembly kinetics of OXPHOS complexes in a cellular model of mitochondrial disease.** *Human molecular genetics* 2008, **17**(24):4001-4011.
72. Piantadosi CA, Suliman HB: **Mitochondrial transcription factor A induction by redox activation of nuclear respiratory factor 1.** *The Journal of biological chemistry* 2006, **281**(1):324-333.
73. Hock MB, Kralli A: **Transcriptional control of mitochondrial biogenesis and function.** *Annual review of physiology* 2009, **71**:177-203.
74. Finnila S, Lehtonen MS, Majamaa K: **Phylogenetic network for European mtDNA.** *American journal of human genetics* 2001, **68**(6):1475-1484.
75. Herrnstadt C, Elson JL, Fahy E, Preston G, Turnbull DM, Anderson C, Ghosh SS, Olefsky JM, Beal MF, Davis RE, et al: **Reduced-median-network analysis of complete mitochondrial DNA coding-region sequences for the major African, Asian, and European haplogroups.** *American journal of human genetics* 2002, **70**(5):1152-1171.
76. Varlamov DA, Kudin AP, Vielhaber S, Schroder R, Sassen R, Becker A, Kunz D, Haug K, Rebstock J, Heils A, et al: **Metabolic consequences of a novel missense mutation of the mtDNA CO I gene.** *Human molecular genetics* 2002, **11**(16):1797-1805.
77. Kleefstra T, Wortmann SB, Rodenburg RJ, Bongers EM, Hadzsiev K, Noordam C, van den Heuvel LP, Nillesen WM, Hollody K, Gillessen-Kaesbach G, et al: **Mitochondrial dysfunction and organic aciduria in five patients carrying mutations in the Ras-MAPK pathway.** *European journal of human genetics : EJHG* 2011, **19**(2):138-144.
78. Murphy MP: **How mitochondria produce reactive oxygen species.** *The Biochemical journal* 2009, **417**(1):1-13.
79. Zou T, Yang W, Hou Z, Yang J: **Homocysteine enhances cell proliferation in vascular smooth muscle cells: role of p38 MAPK and p47phox.** *Acta biochimica et biophysica Sinica* 2010, **42**(12):908-915.
80. Lu Y, Azad N, Wang L, Iyer AK, Castranova V, Jiang BH, Rojanasakul Y: **Phosphatidylinositol-3-kinase/akt regulates bleomycin-induced fibroblast proliferation and collagen production.** *American journal of respiratory cell and molecular biology* 2010, **42**(4):432-441.

81. Sitkovsky MV, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A, Thiel M: **Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors.** *Annual review of immunology* 2004, **22**:657-682.
82. Gao N, Jiang BH, Leonard SS, Corum L, Zhang Z, Roberts JR, Antonini J, Zheng JZ, Flynn DC, Castranova V, *et al*: **p38 Signaling-mediated hypoxia-inducible factor 1alpha and vascular endothelial growth factor induction by Cr(VI) in DU145 human prostate carcinoma cells.** *The Journal of biological chemistry* 2002, **277**(47):45041-45048.
83. Nathan C, Ding A: **Nonresolving inflammation.** *Cell* 2010, **140**(6):871-882.
84. Carnesecchi S, Carpentier JL, Foti M, Szanto I: **Insulin-induced vascular endothelial growth factor expression is mediated by the NADPH oxidase NOX3.** *Experimental cell research* 2006, **312**(17):3413-3424.
85. Lee JJ, Lee JH, Ko YG, Hong SI, Lee JS: **Prevention of premature senescence requires JNK regulation of Bcl-2 and reactive oxygen species.** *Oncogene* 2010, **29**(4):561-575.
86. Li M, Fang X, Baker DJ, Guo L, Gao X, Wei Z, Han S, van Deursen JM, Zhang P: **The ATM-p53 pathway suppresses aneuploidy-induced tumorigenesis.** *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(32):14188-14193.
87. Bandyopadhyay S, Chiang CY, Srivastava J, Gersten M, White S, Bell R, Kurschner C, Martin CH, Smoot M, Sahasrabudhe S, *et al*: **A human MAP kinase interactome.** *Nature methods* 2010, **7**(10):801-805.
88. Lassus P, Roux P, Zugasti O, Phillips A, Fort P, Hibner U: **Extinction of rac1 and Cdc42Hs signalling defines a novel p53-dependent apoptotic pathway.** *Oncogene* 2000, **19**(20):2377-2385.
89. Liu HY, Yehuda-Shnaidman E, Hong T, Han J, Pi J, Liu Z, Cao W: **Prolonged exposure to insulin suppresses mitochondrial production in primary hepatocytes.** *The Journal of biological chemistry* 2009, **284**(21):14087-14095.
90. Krishnan J, Danzer C, Simka T, Ukropec J, Walter KM, Kumpf S, Mirtschink P, Ukropcova B, Gasperikova D, Pedrazzini T, *et al*: **Dietary obesity-associated Hif1alpha activation in adipocytes restricts fatty acid oxidation and energy expenditure via suppression of the Sirt2-NAD+ system.** *Genes & development* 2012, **26**(3):259-270.
91. McGill JK, Beal MF: **PGC-1alpha, a new therapeutic target in Huntington's disease?** *Cell* 2006, **127**(3):465-468.
92. Zaid A, Li R, Luciakova K, Barath P, Nery S, Nelson BD: **On the role of the general transcription factor Sp1 in the activation and repression of diverse mammalian oxidative phosphorylation genes.** *Journal of bioenergetics and biomembranes* 1999, **31**(2):129-135.
93. Park JY, Wang PY, Matsumoto T, Sung HJ, Ma W, Choi JW, Anderson SA, Leary SC, Balaban RS, Kang JG, *et al*: **p53 improves aerobic exercise capacity and augments skeletal muscle mitochondrial DNA content.** *Circulation research* 2009, **105**(7):705-712, 711 p following 712.
94. Hermstadt C, Elson JL, Fahy E, Preston G, Turnbull DM, Anderson C, Ghosh SS, Olefsky JM, Beal MF, Davis RE, *et al*: **Reduced-median-network analysis of complete mitochondrial DNA coding-region sequences for the major African, Asian, and European haplogroups.** *Am J Hum Genet* 2002, **70**(5):1152-1171.
95. Ruiz-Pesini E, Lott MT, Procaccio V, Poole JC, Brandon MC, Mishmar D, Yi C, Kreuziger J, Baldi P, Wallace DC: **An enhanced MITOMAP with a global mtDNA mutational phylogeny.** *Nucleic acids research* 2007, **35**(Database): D823-828.

doi:10.1186/1471-2105-15-S7-S6

Cite this article as: Ridge *et al*: Mitochondrial genomic variation associated with higher mitochondrial copy number: the Cache County Study on Memory Health and Aging. *BMC Bioinformatics* 2014 **15**(Suppl 7):S6.

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