Reactive oxygen species and the segregation of mtDNA sequence variants

To the Editor:

Mutations in mitochondrial DNA (mtDNA) are an important cause of a group of human diseases collectively referred to as mitochondrial encephalomyopathies. Most pathogenic mtDNA mutations coexist with wild-type genomes, and an oxidative phosphorylation defect is not manifest until the proportion of mutant genomes exceeds a particular cellular threshold. The pattern of segregation of mtDNA mutations is thus a key determinant of the onset and severity of disease. The mechanisms regulating tissue-specific mtDNA segregation, however, remain largely unknown.

We have been investigating mechanisms for tissue-specific mtDNA segregation in a heteroplasmic mouse model that shows segregation of two polymorphic mtDNA haplotypes, BALB/c and NZB, whose sequences differ by 101 nucleotides[1–3]. Although transmission of these variants through the germline is stochastic[1], some tissues show age-dependent selective segregation for either mtDNA haplotype[2], a behavior that maps to three quantitative trait loci (*Smdq-1*, *Smdq-2* and *Smdq-3*)[3].

A report from Moreno-Loshuertos *et al.*[4] claims that a SNP in the mitochondrial tRNA arginine gene (*mt-Tr*) provides a unifying genetic explanation for a variety of complex phenotypes associated with common mtDNA sequence variants, including the selective mtDNA segregation that we have documented in our heteroplasmic mouse model. The authors report that the number of adenines in the DHU loop of *mt-Tr* varies from eight to ten among different mtDNA haplotypes and that a higher number correlates with reduced respiratory chain capacity per unit mtDNA in cybrid cell lines, a phenotype that is masked from conventional analysis because of increased reactive oxygen species (ROS) production. Elevated ROS are further proposed to stimulate an increase in mtDNA copy number, and it is inferred that this is the mechanism for the diverse phenotypes associated with mtDNA sequence variants.

We have strong evidence refuting the mechanism proposed by Moreno-Loshuertos *et al.*[4]. Our genetic investigations in mice have demonstrated clearly that nuclear-encoded genes can alter the rate of mtDNA selection in a tissue- and age-specific manner, independent of the poly-A tract in *mt-Tr*. In particular, the *Smdq-1* locus on chromosome 5 has a strong dominant effect on the rate of NZB mtDNA selection in the liver, and the *Smdq-3* locus on chromosome 6 acts additively to select BALB/c mtDNA[3]. Further, most independent hepatocyte clones, established from heteroplasmic mice, reverse their mtDNA haplotype preference in culture, selecting instead the BALB/c haplotype[5]. Direct, *in vivo* measurements show no differences in the rate of replication of the NZB and BALB/c mtDNAs, arguing against selective replication *per se* of a particular mtDNA haplotype[5]. Finally, we have observed that the selective segregation for the BALB/c mtDNA haplotype is completely abolished in hematopoietic tissues on a CAST/Ei nuclear background[6]. The frequency of this phenotype in an F_2 cross is approximately 6%, suggesting that two independent recessive loci regulate this phenotype.

How, then, might one account for the data presented by Moreno-Loshuertos *et al.*[4]? A crucial assumption in their experiments is that they were comparing different mtDNA haplotypes on a uniform nuclear background. The L929 mouse cell line used in their study is, however, highly aneuploid. The ease with which it has been possible to isolate nuclear suppressors of mtDNA mutations in both mouse[7] and human[8] cybrid cells simply by plating them in galactose-containing medium illustrates the extent of nuclear heterogeneity in these cells. We suggest that the authors have not excluded the possibility that differences in nuclear genetic background underlie all of the phenomena they report.

Functional data directly linking the presence of the length variant in the poly-A tract in *mt-Tr* with oxidative phosphorylation dysfunction are completely lacking. The authors propose that low quantities of mutant respiratory chain subunits might escape the quality control system and assemble into functional complexes. However, there are no translation studies to support the idea of amino acid misincorporation in the mitochondrial translation products. There is also no reason to suspect that the AAA ATPase quality control system in the mitochondrial inner membrane[9] could not detect small amounts of defective subunits. Finally, structural subunits containing pathogenic mutations generally fail to incorporate into functional respiratory chain complexes[10]. Consistent with previous reports[5], oxygen consumption and respiratory chain coupling was similar in all of their cybrid lines. We fail to see how this represents an 'apparent' or 'masked' respiratory chain deficiency; there is, in fact, no energetic deficiency.

We see no reason to conclude that the adenine track length of *mt-Tr* initiates selection for a particular mtDNA haplotype through 'masked' functional differences in oxidative phosphorylation function. Although *mt-Tr* could interact with nuclear-encoded genes, the other 100 differences between NZB and BALB/c or CBA mtDNAs are also candidates for such behavior. The identification of nuclear genes whose products interact with mtDNA sequence variants, and the elucidation of their mode of action, will ultimately increase our understanding of mtDNA segregation and its role in complex genetic disorders.

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COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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Enriquez *et al.* reply:

We have read with interest the letter from Battersby and Shoubridge, but we are unable to find any fundamental contradiction between their previous results[1–3] and ours[4]. We all agree on a basic principle: selective segregation of two mtDNA haplotypes is a consequence of sequence differences between them.

In their experiments, Battersby and Shoubridge tested whether the sequence differences between two selectively segregating mtDNA haplotypes (NZB and BALB/c) induce functional differences in oxidative phosphorylation (OXPHOS). They analyzed respiration and found no differences[3]. Consequently, to explain the selective segregation of these mtDNA haplotypes, they hypothesized that the mtDNA sequence differences between NZB and BALB/c are recognized by protein factors (encoded by genes in the nucleus) involved in replication and/or maintenance of the mtDNA.

The discrepancy between Battersby and Shoubridge's view and ours is that we demonstrated that different mtDNA haplotypes, including NZB and BALB/c, promote functional differences in OXPHOS.

Battersby and Shoubridge claim that they have strong evidence to refute our conclusions and the mechanisms that we propose to explain why they failed to see these OXPHOS differences. Their arguments are based on (i) their demonstration that nuclear-encoded genes can alter the rate of mtDNA selection to the point where it can be abolished, (ii) the reversion of the mtDNA haplotype preference when the hepatocytes are established in culture and (iii) the absence of differences in the rate of replication among NZB and BALB/c mtDNAs.

Battersby and Shoubridge mapped three quantitative trait loci (*Smdq-1, Smdq-2* and *Smdq-3*), but the nuclear genes influencing segregation and their function remain to be identified. We never concluded that nuclear genes would not be involved in the selective segregation of a particular mtDNA haplotype, and we do not see any contradiction between their findings and our results. Quite the opposite: our model allows speculation about the nature of some nuclear-encoded genes that would be able to modulate the phenotype: (i) these genes could interact with the function of the $tRNA^{Arg}$, (ii) they could participate in ROS handling and in the signaling cascade that ROS may trigger and (iii) they could participate in the quality control system of the mitochondrial translated products.

We are aware that the L929 mouse cell line used in our study is aneuploid, as is the case for almost every immortalized animal cell line. We are also aware that this is a relevant issue in any study performed with cybrid cells, an approach that the Shoubridge group and many others have employed repeatedly and successfully for more than 15 years. Because of this, in our report we have avoided the use of individual cybrid clones. To randomize for any particular nuclear contribution that could be accidentally selected, in our report we used several clones or a nonclonal culture representative of each haplotype. Moreover, we used five sources of mtDNA that ended up being only four different mtDNA haplotypes. As stated in our article, cells with identical mtDNA showed an identical phenotype, and the two types of OXPHOS phenotypes segregated with a particular mtDNA polymorphism, ten adenines in *mt-Tr*. Therefore, we have excluded the possibility that differences in nuclear genetic background underlie the phenomena that we report.

Battersby and Shoubridge comment how 'easy' it is to isolate nuclear suppressors for mtDNA mutations. We disagree. Only two reports in 15 years of exhaustive use of cybrid models have documented putative, yet undetermined, nuclear suppressors for mtDNA mutations. A critical issue that is omitted in their argument is that such suppressor clones appear at frequencies in the range of 10^{-6}. This is not trivial; it means that one would have to analyze 1 million individual clones to get one suppressor. Those clones could be obtained only by submitting several million cybrid cells to strong positive selection[5,6].

We agree that future work should provide functional data that directly link the presence of the polymorphic A tract in *mt-Tr* with OXPHOS performance (the term 'dysfunction' used by Battersby and Shoubridge in their letter is not appropriate, since all the variants are present in healthy animals). Accordingly, in our report, we proposed a hypothetical scenario to address these future investigations. A similar situation also applies in the case of the three *Smdq* quantitative trait loci described by Battersby *et al.*[1], for which functional data directly linking the genes to the phenotype are also missing.

Finally, we identified a polymorphic A tract in *mt-Tr* as the mtDNA sequence variation responsible for the phenotype because, among other genetic arguments, the NIH3T3 and NZB mtDNA haplotypes induced the same specific OXPHOS phenotype, and the only shared difference between these two haplotypes and the CBA, BALB/c or C57BL6/J mtDNA haplotypes is that they harbor ten adenines in the polymorphic A tract in *mt-Tr*. There is no room to consider any of the other 100 differences in the NZB mtDNA haplotype, since they are not present in the NIH3T3 mtDNA and are not associated with the phenotype.

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COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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