Mechanisms of mtDNA segregation and mitochondrial signalling in cells with the pathogenic A3243G mutation

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Suppressed and quantal mtDNA segregation in heteroplasmic cell cultures

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Abstract

At cytokinesis mitochondrial DNA (mtDNA) molecules are assumed to be physically partitioned to daughter cells in a stochastic fashion. In the presence of two mtDNA sequence variants (a situation referred to as heteroplasmy) continuation of this chance distribution process leads to random genetic segregation of the alleles. Here we present results of *in vitro* experiments and computer simulations indicating absence of A3243G mtDNA mitotic segregation. Occasionally, however, discrete heteroplasmy shifts or quantal segregations are observed. To explain the observations a metastable, heteroplasmic mtDNA segregation unit is postulated. Inherently this unit is thus multi-copy in mtDNA and as a rule faithfully replicating its wild type/mutant ratio. The nucleoid, the mitochondrial matrix DNA-protein complex that carries 8 - 10 mtDNA molecules, likely is the physical equivalent of the segregation/segregation suppression unit. The discrete heteroplasmy shifts observed may result from rearrangements leading to nucleoids of variable heteroplasmy, which segregate to fixation at a rate one order of magnitude more rapid than single mtDNAs would do. Reorganization of nucleoids may thus underlie A3243G mtDNA segregation in heteroplasmic cybrid cultures

Introduction

When two neutral mtDNA alleles are present in the founding cell of a clone, the process of random mitotic segregation leads to genetic drift and ultimately to genetic fixation of mtDNA alleles in descendant cells. Two homoplasmic cell populations will eventually emerge with the proportion of their sizes, reflecting the mutation load in the founding cell (1). The lower the mtDNA copy number, the more rapid cells will emerge that have homoplasmic mtDNA, as illustrated in figure 1. mtDNA genetic drift is not only driven by mitosis, but also by mtDNA turnover (2;3).

In case of heteroplasmy with a deleterious mutation, cells drifting towards homoplasmy for the mutant allele can therefore acquire merely on stochastic grounds high levels of the deleterious mtDNA. Up to a point at which the critical threshold heteroplasmy is reached (~80% for the A3243G cell used here), cells experience too little compensation from the neutral variants leading, under selective conditions, to their elimination from the population.

Mice carrying neutral mtDNA haplotypes in a heteroplasmic fashion have been instrumental in establishing the germ line mitochondrial genetic bottleneck/random segregation hypothesis (4). Recently this hypothesis has been challenged by the fact that the dramatic reduction of the mtDNA copy number needed to create a genetic bottle neck that leads by random segregation to great variance in heteroplasmy (and even homoplasmy) in offspring, does not appear to occur in the mouse germ line (5). Studies with these neutral heteroplasmic mice have also shown that post-natally, tissue-specific non-random or directional segregation occurs (6), which appears to be under nuclear genetic control (7) and points to the existence of segregation controlling genes. Also a multitude of clinical studies with pathogenic mtDNA mutations indicate that directional segregation occurs in human post-embryonic life (8;9). It is not understood how diversity in mtDNA mutation load among different tissues, and hence how a wide spectrum of mtDNA disease phenotypes can occur with one specific mutation. Obviously, segregation is important in this respect, but mechanisms are elusive.

In absence of heteroplasmic mouse models for pathogenic mtDNA point mutations, transmitochondrial cybrids provide valuable sources for experimental mitotic segregation analysis. They are created by fusion of enucleated cells carrying two different mtDNA sequence variants with a nucleated cell that has no mtDNA (ρ^0 cells). Mostly by analysis of bulk DNA of passages of cybrid clones carrying the A3243G pathogenic mutation, two patterns have been found:

stable heteroplasmy and heteroplasmy drift to either wild-type or mutant. The number of stable A3243G clones exceeded the number of drifting clones by a factor of ~3. We counted 42 stable clones against 15 drifting clones in three studies (10-12) (for review see Enriquez (13)). Of significance, stable heteroplasmy as measured on bulk DNA of cells in passages of cultured cybrid clone can be the result of random mitotic mtDNA segregation or from non-segregation. Only single cell analyses can reveal the cellular heteroplasmy distribution of a given cell population, but such studies are rare. In one such study, it was found that the distribution of single cell mutation loads after 30 weeks of culture of A3243G cybrid clones was too narrow to fit the random segregation model (14). It led to the concept of a multi-copy mtDNA segregation unit, the nucleoid, that is heteroplasmic itself and that replicates its wild type/mutant content faithfully. To explain changes in heteroplasmy e.g. in drifting cybrid clones it was proposed that the faithfully replicating nucleoid may occasionally reorganize its wild type/mutant ratio, possibly under genetic control (14; 15). Experimental evidence for reorganizing heteroplasmic nucleoids lying at the base of segregation is, however, very limited. Here we report experiments that are in strong support of the 'metastable' nucleoid model.

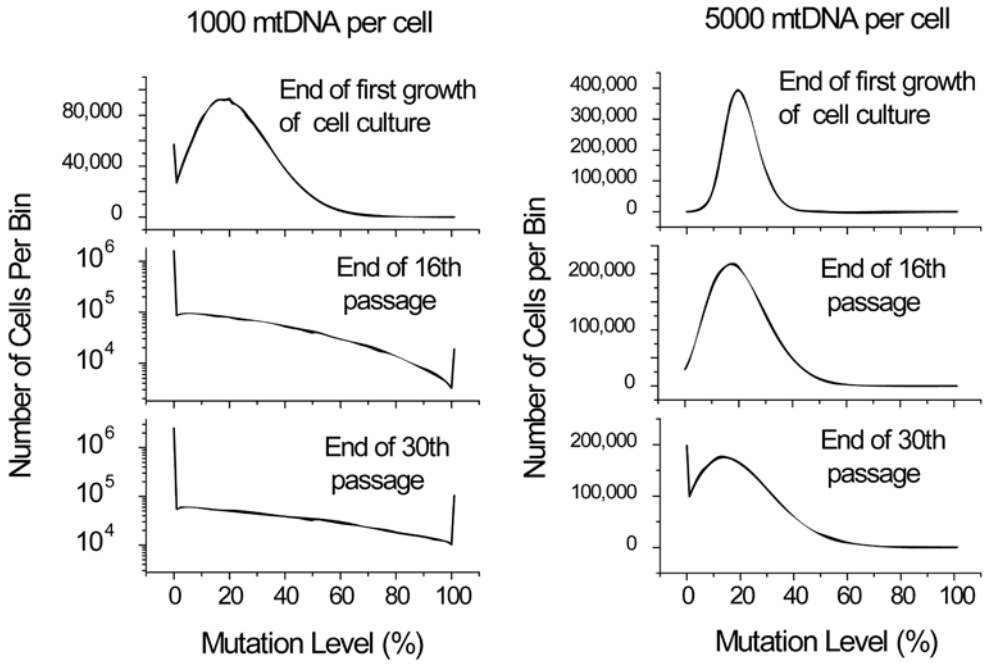


Figure 1: The rate of mitochondrial segregation as a function of the mtDNA copy number

The graphs represent computer-simulated mutation load distributions of the cells in the first outgrowth (passage 1), passage 16 and 30 of a founding cell with 20% mutation load with 1000 (left) and 5000 (right) mtDNA molecules . When reaching 0 and 100% mutation, the cells are genetically fixed on the wild-type and mutant mtDNA respectively. Such cells appear more rapid in the clone with the lower mtDNA copy number. The average mutation level as measured on bulkDNA cells remains steady at the initial value since in this simulation there is no advantage or disadvantage to the mutation. The contributions of mtDNA turnover to genetic drift in this situation is minor (mtDNA half life ~10 days (2)), because mtDNA replication is mostly driven by mitosis (~1 day).

Material and methods

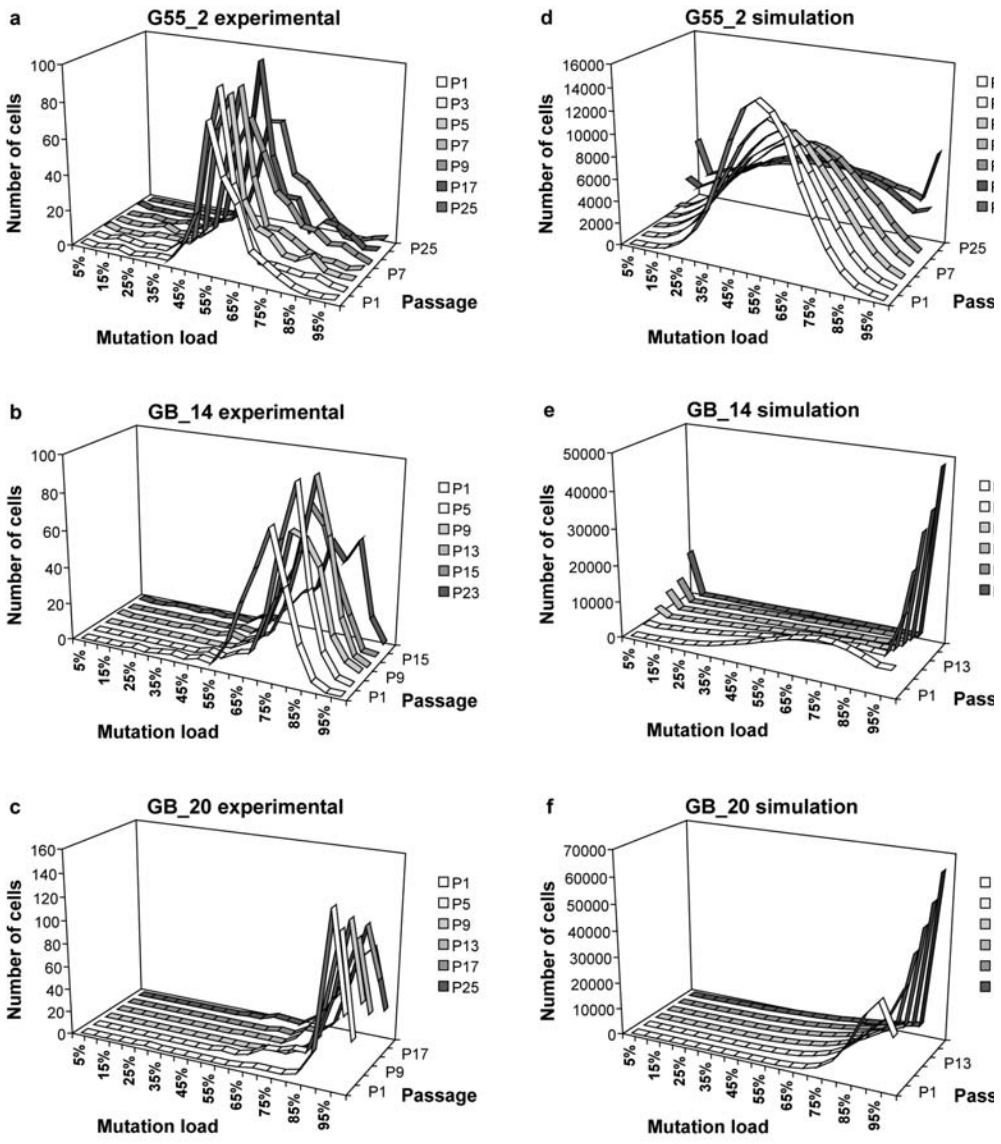
A3243G transmitochondrial 143B cybrid were produced by fusing skin fibroblasts from two maternally inherited diabetes and deafness, MIDD, patients (coded V and GB) and a mitochondrial myopathy encephalopathy lactic acidosis and stroke-like episodes, MELAS, patient (G) with 143 B ρ^0 cells as described (16). Cybrid cloning was by limiting dilution or single cell flow sorting. Cybrid cells were grown on Dulbecco's Modified Eagle's medium containing 4.5 mg/ml glucose and 110 µg/ml pyruvate (DMEM) supplemented with 50 µg/ml uridine and 10% fetal bovine serum. After the first outgrowth to near-confluence (~a million cells) in 9 cm dishes, they were cultured with a 10% split of cells twice a week.
The A3243G mutation load of single cells was determined using a PCR/RFLP method (referred to as PCR/RFMT) as well as by in situ genotyping with Padlock/Rolling Circle Amplification (17) in combination with image analysis as described by Jahangir Tafrechi *et al.* (18). Average cellular mtDNA copy numbers were determined on bulk DNA with the aid of ΔCt method using the SybrGreen Master mix (Applied Biosystems, USA) for real-time PCR, assuming two ß-globin genes per cell. Primers for ß-globin and mtDNA as well as PCR conditions were as described (19).
Array-Comparative Genome Hybridization is described in Knijnenburg *et al.* (20).

Computer simulations were started with a single cell containing M mutant mtDNA molecules and W wild-type mtDNA. The mtDNAs in the simulation were not organized into any structure. The simulated cells were divided at random intervals, with a mean division period of D = 1 day. At cell division, the mtDNA molecules were individually randomly distributed to the two daughter cells. The mtDNA molecules were also randomly destroyed with a half-life of $T_{½}$ =10 days (2). mtDNA replication was modeled by copying individual mtDNA molecules at a set rate R = ln(2) Nt [$(1/T_{½})$ + (1/D)], where Nt was the copy number of mtDNA in the simulated cells. The mtDNA molecules to be copied were chosen at random, with no preference to either wild-type or mutant (3). After the simulated cell population has reached 1 million cells we began simulating cell culture passages by selecting 100,000 cells (1/10th of the population) at random from the total cell population, with this sampling taken every 3½ days.

Results and discussion

In a first series of experiments we generated, by PCR-FMT (21), mutation load histograms of individual cells in multiple passages of 3 sub-cloned A3243G mtDNA 143B transmitochondrial cybrid founding cells cultured under non-selective conditions and performed computer simulations of random mitotic segregation specified to these cells in terms of heteroplasmy and mtDNA copy number. The experimental and computer simulation results are presented in Figure 2.
Cells in the first outgrowth of the founding cell (~20 generations; passage 1) of clone G55_2 proved to possess mutation loads of ~50-55 % with a distribution that is substantially narrower than the simulated one. Cells in passage 1 of clones GB_14 and GB_20 had mutation loads of 70-75 % and 90-95 % respectively, also at a much more narrow distribution than predicted. All clones maintained a narrow distribution in subsequent passages, whereas according to the simulation, homoplasmic cells should have been very abundant at passages > 17 for G55_2 and passage ≥ 5 for GB_14 and GB_20. As cellular mtDNA copy number values for the computer simulations, 500 was used for G55_2, which is actually above the experimentally determined copy number of 284 (SD=117, n=3). For G55_2 and for GB_14 and GB_20 1500 was used, which is representative of the mtDNA copy number in 24 GB sub-clones analyzed: 1469 (SD=544, n=24). Under a random segregation regime, the clones theoretically would need to have very much higher mtDNA copy numbers to explain the experimental histograms.

Figure 2: Experimental and computer simulation single cell mutation load histograms of multiple passages of A3243G mtDNA heteroplasmic 143B transmitochondrial stable cybrid clones G55_2 (a,d), GB_14 (b,e), GB_20 (c,f)



(a-c) Cybrids cells were cloned, expanded to a million cells (~20 generations, passage 1) and continuously cultured with a 10% split twice a week. At the indicated passages, single cells were sorted in 96-well optical plates and processed for single cell mutation load measurements by PCR-RFMT (~250 cells per histogram).
(d-f) For computer simulated histograms (10.000 cells per histogram) the algorithm for random segregation used as heteroplasmy value for the founding cell, the average mutation load found in passage 1: 56% for clone G55_2, 70% for GB_14 and 92% for GB_20. As values for cellular mtDNA copy number, 500 was used for G55_2 and for GB_14 and GB_20 1500 was used.

Yet we considered whether at moderate copy numbers, the stable heteroplasmies observed can still be explained by random segregation of mtDNAs. Theoretically negative selection for cells drifting towards mutant and wild-type mtDNA provides an explanation, but this is biologically highly improbable. Alternatively, it can be envisaged, that a delicate balance between negative selection of cells that drift to mutant and replicative advantage for mutant mtDNA creates a situation in which a population gets enriched for cells that have a heteroplasmy just under the threshold level. However, we observed stable heteroplasmy at 3 different levels, including one above the threshold level.

In view of the existence of the nucleoid with its multi-copy mtDNA nature, absence of segregation can best be explained with the nucleoid being a multi-copy segregation unit that is discretely heteroplasmic and faithfully replicating its wild type/mutant ratio as proposed by Lehtinen (14) and illustrated in Figure 3a.

In our cybrid repository we identified a A3243G cybrid sub-clone (V_50) that by gel-based PCR-RFLP mutation load measurement on bulk DNA showed to be drifting from ~ 98% mutant to wild type over a period of a year under non-selective culture conditions. (Figure 4, left panel). It provided a unique occasion to test the idea of a reorganizing nucleoid underlying segregation by analyzing its heteroplasmy evolution at the single cell level. Individual cells of the first passage analyzed were mostly 95-100% wild type. At passage 6 the great majority of the individual cells was shifted to 90-95% heteroplasmy, following which a second discrete shift to ~50-55% heteroplasmy occurred as evidenced by the significant subpopulations at 50-55% heteroplasmy in passages 42 48 and 62. Finally, a third shift to homoplasmy wild type occurred (Figure 4, right panel).

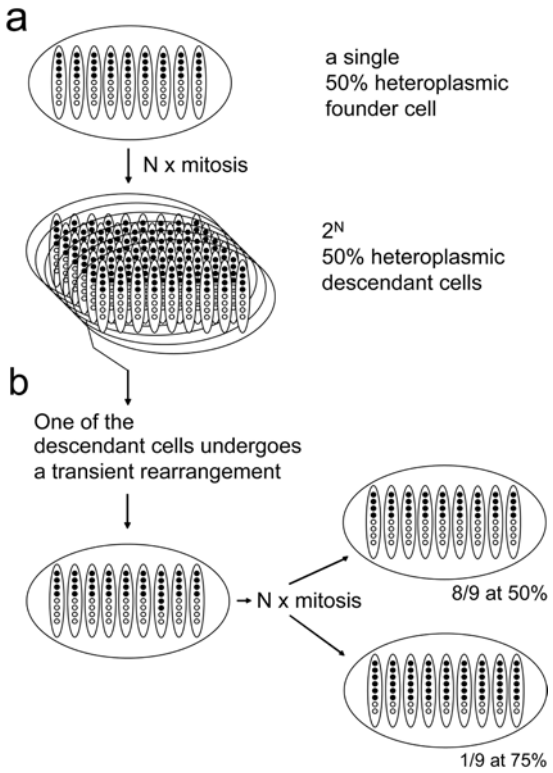


Figure 3: Model for the mitotic consequences of a genetically stable heteroplasmic segregation unit and of its rearrangement.

(a) Schematic representation of the mitotic consequences of random mtDNA segregation and a stable heteroplasmic mtDNA segregation unit. The hypothetical founding cell is 50% heteroplasmic with 72 mtDNA arranged in 9 segregation units each with 8 mtDNAs, 4 of which are variant. Upon their faithful replication and partitioning to daughter cells for N mitosis, all 2N descendant cells acquire the same 50% heteroplasmy level.

(b) Schematic representation of the mitotic consequences of a transient loss of faithful replication in one segregation unit of a cell from (a) which acquired 6 instead of 4 variant mtDNA molecules. Upon random mitotic segregation of the units, 1/9th of the cells will become fixed at 75% heteroplasmy and 8/9th at 50%. Reassembly of mtDNAs in nucleoids following a complete transient loss of nucleoid structure will result in descendant cells with heteroplasmies from 0% to 100% in discrete steps of 12.5% with binomially distributed frequencies.

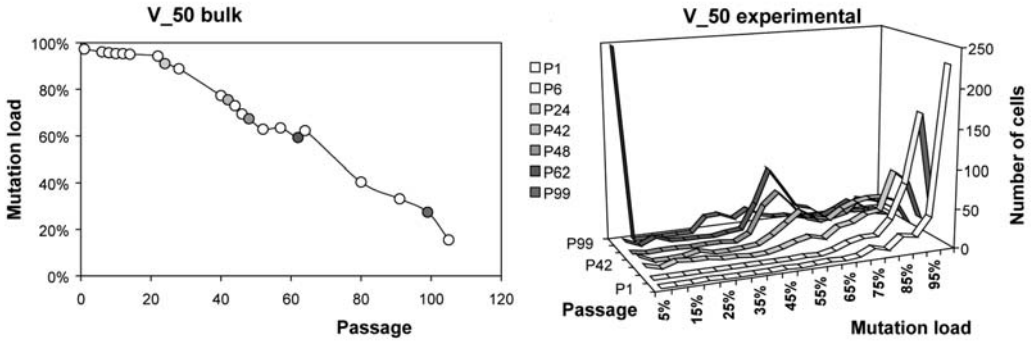


Figure 4: Bulk and single cell mutation loads of the shifting clone V_50
Heteroplasmy evolution in V_50 by bulk DNA mutation load analysis as a function of passage number showed apparent drift for this clone. Grey symbols correspond to the passages analyzed at the single cell level. Single cell analysis of selected passages of V_50 (~350 cells per histogram) revealed discrete shifts in cellular mutation load.

Results described thus far were obtained with a PCR-RFLP mutation load assay based on melting curve analysis (PCR-RFMT). We confirmed qualitatively and quantitatively the G55_2 results as well as the discretely shifted 50-55 % heteroplasmy peak in V_50 with a different method based on direct *in situ* hybridization and ligation of A3243G padlock probes and target-primed rolling circle amplification (padlock/RCA) in combination with image analysis for quantitation purposes (17;22) as shown in Figure 5.

We explained the heteroplasmy shifts of V_50 in terms of the metastable nucleoid, but not after having considered random segregation in combination with selection against the higher mutant cells either and/or replicative advantage for wild type. These combinations intuitively explain the overall shift from near 100% mutant to wild type. However, these combinations do predict gradual appearance of homoplasmic wild type cells only and no heteroplasmic peak such as the one seen at 50-55%, making it very unlikely that random mitotic segregation of mtDNAs is operational in these cells.

For the emergence in V_50 of the 90-95% heteroplasmy subpopulation, we inferred that in the founding cell at least one faithfully replicating 90-95% heteroplasmic segregation unit (nucleoid) was present amongst homoplasmic mutant nucleoids and that it segregated randomly so that in relative low frequency stable heteroplasmic cells emerged with uniform, 90-95 % heteroplasmic, faithfully replicating nucleoids. Such a cell then clonally expanded due to growth advantage as witnessed by the significant sub-population in passage 6 at 90-95% heteroplasmy.

With chromosome instability and presence of multiple karyotypically different subclones being a hallmark of cancer cell lines, we reasoned that genetic instability of the 143B nucleus generated, by chance, nuclear determined growth advantage to one or more such 90-95% heteroplasmy cells. Chromosome analysis by Combined Binary Ratio FISH (23) showed the cybrids to possess aberrant karyotypes (not shown). Although this is indicative for genomic instability it is no formal proof. Array Comparative Genomic Hybridization (array-CGH), however, proved the nuclear genomic instability of the 143B nuclear genome (Fig. 6). Thus a scenario is plausible in which the nuclear genomic constitution of a cell, that hosted uniform 90-95% heteroplasmic mtDNA nucleoids, provided it with growth advantage, effectively leading to genetic hitchhiking of the heteroplasmic nucleoid.

We next reasoned that the heteroplasmy shifts in V_50 from 90-95% to 50-55% and then on to 0-5% can be explained with the nucleoid if it occasionally makes errors in replication of its wild type/mutant ratio or undergoes genetic rearrangements, similar but not identical to imbalanced nuclear chromosome translocations (see Figure 3b). Such events will lead to altered wild type/mutant ratios of the nucleoids. In the example of Figure 3b, such newly emerged 'homologous' nucleoids will segregate at a rate 8-fold faster than single mtDNAs would and thus rapidly seed cells in the population with uniform heteroplasmic nucleoids of altered genetic composition, which then may hitchhike with cells with nuclear determined growth advantage, as outlined above.

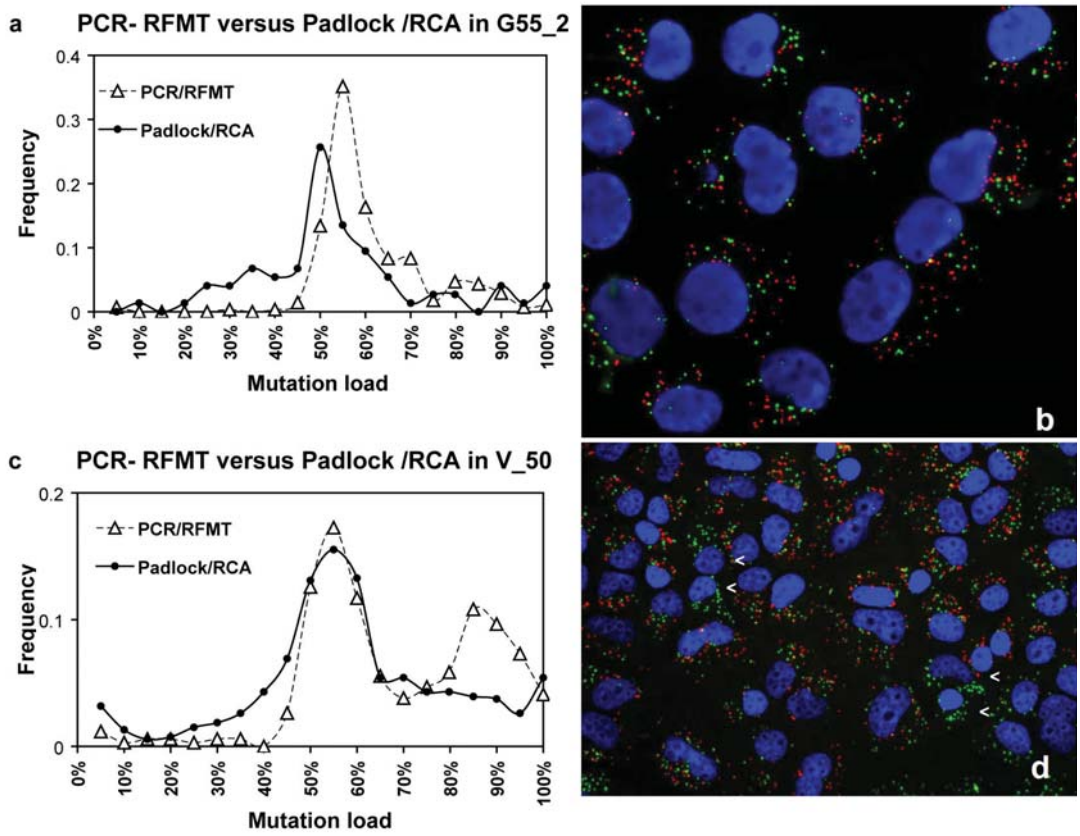


Figure 5:Quantitative and qualitative correlations between single cell A3243G mtDNA PCR-RFMT and Padlock/RCA results.
(a) Relative single cell mutation load histograms generated by PCR/RFMT for G55_2 passage 17 (n=276) and by Padlock/RCA for passage 17 + 4 (n=74). For Padlock/RCA a liquid nitrogen aliquot was re-grown for an additional 4 passages.
(b) Photomicrographs of cells from clone G_55 passage 17 + 4 (400x) genotyped *in situ* by Padlock/RCA. Red dots correspond to mutant and green dots to wild type mtDNA.
(c) Relative single cell mutation load histograms generated by PCR/RFMT for V_50 passage 48 (n = 342) and by Padlock/RCA for passage 48 + 4 (n= 535). The reduction in frequency of the 85-95% range for the Padlock/RCA likely relates to the liquid nitrogen archiving and re-growth. See also Jahangir Tafrechi *et al.* for a similar correlation for GB_20 Passage 17 (21).
(d) Photomicrographs of cells from V_50 passage 62 + 4 (630x). Note the presence in this image of wild type daughter cells (arrow heads) amidst the heteroplasmic cells. Eventually these overtook the culture in passage 99.

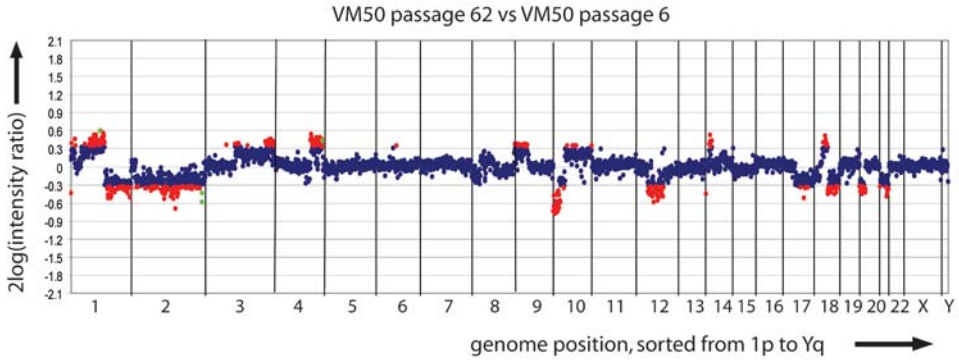


Figure 6: Chromosome instability of the 143B nuclear genome assessed by Array-Comparative Genome Hybridization with ~ 1 Mbp resolution of genomic DNA from V_50 passage 6 as test-DNA and V_50 passage 62 as reference-DNA.
Red, blue and green dots represent respectively, copy number change, no copy number change and natural copy number polymorphisms. Thresholds for gains and losses were set at a ^{2}log change of ± 0.33 (y-axis) and all data were sorted according to chromosome number (x-axis).
These results indicate the changes which did occur during 28 weeks of culturing.

This line of reasoning (reorganization and subsequent segregation of heteroplasmic nucleoids, followed by nuclear determined growth advantage of a cell with altered but uniform heteroplasmic nucleoids) may as such explain the discrete shifts from mutant to wild type seen in V_50; it also predicts discrete shifts in cellular mutation loads from wild type to mutant. In an ongoing second similar long term segregation analysis initiated during this Thesis work with 10 subclones of passage 42 of V_50 this was exactly what was observed. Clone, V3.18 by bulk DNA analysis gradually shifted to mutant and showed upon single cell padlock/RCA analysis a discrete cellular heteroplasmy shift from wild type to mutant (see Chapter 6 for Figure). Furthermore in support of the 'stable' element of the metastable nucleoid model, another subclone with ~ 1800 mtDNAs proved to be stable at ~ 65% heteroplasmy up to passage 81 (see Chapter 6, page 81 for Figure).
Mitochondrial nucleoids are nucleo-protein complexes located in the matrix of the mitochondrial compartment. They physically are associated with multiple mtDNAs. Molecular cytological studies indicated that the average number of mtDNA per nucleoid varies per cell type, but that it is in the range of 2-10, with ~8 for the 143B host cell line used here (24;25). This approximate one order of magnitude difference between mtDNA and nucleoid copy number is compatible with our results. Thus the nucleoid, as a metastable segregation unit with multiple mtDNAs, likely mediates the segregation suppression and quantal segregation observed here. More similar, long term segregation studies will be needed, however, to arrive at the exact quantal number, to document cell type dependence and to answer the question whether or not the metastable nucleoid model holds for neutral and other pathogenic mtDNA mutations.

Acknowledgements

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