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Metabolic and Environmental Conditions Determine Nuclear Genomic Instability in Budding Yeast Lacking Mitochondrial DNA

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ABSTRACT Mitochondrial dysfunctions are an internal cause of nuclear genome instability. Because mitochondria are key regulators of cellular metabolism, we have investigated a potential link between external growth conditions and nuclear chromosome instability in cells with mitochondrial defects. Using Saccharomyces cerevisiae, we found that cells lacking mitochondrial DNA (rho0 cells) have a unique feature, with nuclear chromosome instability that occurs in nondividing cells and strongly fluctuates depending on the cellular environment. Calorie restriction, lower growth temperatures, growth at alkaline pH, antioxidants (NAC, Tiron), or presence of nearby wild-type cells all efficiently stabilize nuclear genomes of rho0 cells, whereas high glucose and ethanol boost instability. In contrast, other respiratory mutants that still possess mitochondrial DNA (RHO+) keep fairly constant instability rates under the same growth conditions, like wild-type or other RHO+ controls. Our data identify mitochondrial defects as an important driver of nuclear genome instability influenced by environmental factors.

KEYWORDS mitochondrial DNA nuclear genome instability metabolism membrane potential calorie restriction

Nuclear genome instability is a threat to normal cellular function, for example, in cancer or aging (Hanahan and Weinberg 2011; Maslov and Vijg 2009). Genomic instability can stem from either internal defects (i.e., cell-cycle errors) or external factors, when genotoxic compounds are present in the environment of the cell (oxidants, chemicals, high-energy rays, and others) (Hanawalt 1998). Among internal sources of instability, dysfunctions of mitochondria also lead to nuclear genome instability (Karthikeyan and Resnick 2009). Genomic instability can stem from either internal or other compartment, the nucleus, via an ISC-dependent pathway...

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Rasmussen et al. 2003). In a more recent study, a severe nuclear chromosome instability (CIN) phenotype was found in budding yeast cells completely devoid of mitochondrial DNA (Veatch et al. 2009). Mutations in genes encoding components of the respiratory chain, but still in possession of their mitochondrial DNA (RHO+), only led to a mild increase in nuclear CIN. Additional important conclusions were drawn from this study. First, in rho0 cells, nuclear DNA damage stems from DNA breaks and mitotic recombination (rather than single nucleotide substitutions). Second, the decrease in mitochondrial membrane potential (and not the mere loss of respiratory capacity) is the main parameter influencing nuclear CIN in rho0 cells. Third, the loss of mtDNA is a common event after replicative aging. Finally, a defect in iron–sulfur cluster (ISC) metabolism, which originates in mitochondria and is partially exported to the cytoplasm (Lill and Muhlenhoff 2008), might ultimately lead to nuclear DNA instability in these cells. Veatch et al. suggested that decreased levels of cytoplasmic ISCs, because of low mitochondrial membrane potential in rho0 cells, impair the proper folding and maturation of a number of nuclear proteins required to maintain genomic stability (such as DNA Pol d and Rad3). This could provide a link between dysfunction in an organelle, the mitochondrion, and its consequences in another compartment, the nucleus, via an ISC-dependent pathway...
(Veatch et al. 2009). A cytoplasmic ISC cofactor, Mms19, was recently characterized and implicated in the maturation of enzymes involved in nuclear genome maintenance (Gari et al. 2012; Stehling et al. 2012; van Wietmarschen et al. 2012), providing a possible link between ISC defect and nuclear genome instability.

Mitochondria contain multiple copies of a DNA molecule (mitochondrial or mtDNA) carrying genes coding for components of the respiratory chain (complex III and IV), for the mitochondrial translation machinery, as well as for components of the FO subunit of mitochondrial ATP synthase. Other proteins required for mitochondrial function (estimated between 500 and 1000) are encoded by the nuclear genome and are imported in mitochondria (Contamine and Picard 2000; Sickmann et al. 2003). Alterations of mtDNA can span from single nucleotide mutations (mt+) to partial deletions (rho−) or full loss of all mtDNA molecules (rho0). Several conditions can promote the loss of mtDNA. Those include replicative aging (Veatch et al. 2009), nuclear mutations (e.g., yeast Frataxin homolog) (Wilson and Roof 1997), defects in mitochondrial fusion process (Rapaport et al. 1998), and the presence of chemicals, including some antitumor drugs (Singh et al. 1992). Ethanol can also lead to mtDNA loss, as shown in mouse liver, as well as in heart, brain, and muscle cells (Demeilliers et al. 2002; Mansouri et al. 2001) and in wine-making flour yeast (Ibeas and Jimenez 1997). Cells lacking mtDNA maintain "proto-mitochondria," with abnormal shape (Holmuhamedov et al. 2003) and limited metabolism, but are still essential for cell survival. Maintenance of a mitochondrial membrane potential (ΔΨ) is essential, because it is involved in protein import into mitochondria and required for mitochondrial biogenesis (Baker and Schatz 1991). In rho0 cells or in cells under anaerobic conditions, glycolytic ATP is imported into mitochondria and hydrolysis of ATP by the F1-ATPase activity is essential to generate a mitochondrial membrane potential and to maintain viability (Giraud and Velours 1997; Lefebvre-Legendre et al. 2003). This low and atypical mitochondrial membrane potential distinguishes rho0 cells from other respiratory mutants with an intact ATP synthase.

Because mitochondria are central organelles for regulation of cellular metabolism, we asked whether cells with dysfunctional mitochondria could still deal with metabolic and environmental variations and, in particular, their capacity to maintain nuclear genome stability when growth conditions are modified. Here, we show that cells lacking mitochondrial DNA (rho0), but not other types of respiration-defective cells, display highly conditional nuclear genome instability, ranging from low/wild-type levels to very high instability, depending on growth parameters and cellular environment. Implications of this finding, comparisons with the current model, and additional hypotheses are presented.

MATERIALS AND METHODS

Strain construction
For CIN assay (CINA), a LEU2 wild-type gene was integrated at the ura3 locus (ura3::LEU2, pUL9) (Cross 1997) close to CEN5 in an S288C strain carrying a URA3-CAN1 selection cassette on the distal arm of chromosome V (RDKY3615) (Chen and Kolodner 1999). Integration of the marker was verified by southern blot and genetic analyses. The resulting strain was L1520: MATa, ura3-52::LEU2, leu2Δ1, trp1Δ63, his3Δ200, lys2Δ8gl, hom3-10, ade2Δ1, ade8, hxt13::URA3-CAN1. The mating partner carrying a can1 mutant allele marked with kanMX4 is L1571: MATalpha his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 can1::kanMX4 (Euroscarf, BY4741). Mating of L1520 with L1571 and pulling of a diploid zygote led to L1577, the wild-type CINA strain (for schematic view, see Figure 1A). To generate a CINA strain with a given nuclear mutation, the mutation of choice was introduced separately in two haploid CINA parents by genetic crosses. One CINA parent is L1847, a MATalpha version of L1520. The nuclear mutant was also crossed to the second CINA parent carrying an untagged can1 mutation (L1607: MATAlpha leu2Δ0 lys2Δ0 met15Δ0 trp1::63 ura3Δ0 can1). The correct segregants are crossed and lead to a diploid CINA strain with the homoyzogous deletion for the nuclear gene and appropriate heterozygous selection markers (CAN1/can1 URA3/hxt13/HXT13 ura3::LEU2/ura3) for chromosome instability assay. All CINA strains used in this study are in the S288C strain background and the mutants marked with KanMX4 are from the Euroscarf collection of deletion strains (Supporting Information, Table S1).

To generate rho0 strains, cells were grown to saturation in liquid YPD medium plus ethidium bromide (10 µg/ml) and plated on YPD plates for individual colonies. Clones were checked for growth defects on a nonfermentable carbon source (YPD 2% glycerol) and absence of mtDNA was confirmed by DAPI staining. To further confirm the absence of mtDNA, two distant mitochondrial DNA loci, COX3 and oli1, were checked by PCR (COX3-dir:GAT ATG AAT ATG GTA TAT TTA GC; COX3-rev: GTT ACA GTA GCA CCA GAA GAT AAG; OLI1-dir:ATG CAA TTA GTA TTA GCA GGT AAA T; OLI1-rev: CCG AAT AAT AAG AAT GAA ACC ATT A). RAD5 locus was used as a positive control for a nuclear gene. For assays on newly born rho0 cells, exponentially growing RHO+ cells were treated with ethidium bromide (30 µg/ml) for 6 hr in YEPD, washed, resuspended in fresh medium for 15 hr, and single unbudded cells were micro-manipulated on plates for colony growth. To obtain spontaneous rho0 mutants, untreated wild-type cells were plated to single colonies on YEP plates containing 4% glycerol and 0.1% glucose. This enhances the growth differences between RHO+ and rho− cells. Very slow-growing clones were detected under the microscope, recovered by micromanipulation, and further checked by DAPI stain and PCR reaction for absence of mitochondrial DNA. The RHO+ status of respiratory chain mutants (e.g., cyt1) was checked by complementation with a rho0 strain. Unless stated, all strains were grown at 30°C on YEPD plates (2% glucose).

CINA

For clonal assays, single cells from CINA strains were micromanipulated on solid medium at regular intervals of 0.5 cm, giving rise to nonsyntical colonies (approximately 50 colonies/plate). When grown to a visible size (approximately 104 cells), a representative colony was picked and measured both for cell number and cell size (CASY-1 TTC Cell Analyzer; Schärfe, Germany), and a dilution was plated on YEPD to measure viability. Individual colonies were diluted to allow plating of the chosen number of cells (1–5 × 105 cells, depending on rate of nuclear instability) and allowed to grow for 3 d at 30°C on selective SD minus arginine plates containing canavanine 60 mg/L (L-canavanine sulfate; Sigma) and 5-fluororotic-acid 0.5 g/L (Toronto Research Chemicals) to select for cells with rearranged genomes. CanFoA plates: Yeast minimal SD-Arg medium/L: 2 g yeast nitrogen base without amino acids; 5 g ammonium sulfate; 20 g agar; 50 mg tyrosine; 30 mg isoleucine; 30 mg methionine; 50 mg phenylalanine; 100 mg tryptophan; 25 mg histidine; 50 mg leucine; 50 mg lysine; 50 mg tryptophan, 25 mg uracil, 25 mg adenine. Plating adjustment of the cell numbers were performed depending on the genomic instability of
individual strains. Using this protocol, six assays can easily be performed on a single (10-cm) Petri dish. Rates of instability were calculated by fluctuation analysis of the median by the maximum likelihood method and represented as the median with 95% confidence interval using FALCOR online webtool (Hall et al. 2009). Rates are expressed per 10⁷ viable cells. Data sets were also analyzed using Lea-Coulson estimator and P values were calculated using the Mann-Whitney nonparametric test. Statistical significances are indicated in graphs by the following: ns, not significant, P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; and ****P ≤ 0.0001. At least 24 independent clones were analyzed for each point. Chromosome breakage can be distinguished from full chromosome loss by replica plating of CanFoA-resistant clones on minus leucine plates (Figure 1B) (~LEU). CanFoA-resistant Leu⁺ clones were the result of chromosomal breaks between the URA3-CAN1 and LEU2 markers, whereas CanFoA-r leu⁻ clones resulted from full chromosome loss of the homolog carrying the selective markers (Figure 1). Low-frequency meiosis could lead to false-positive results for CIN (CanFoA-resistant). Unlike the genuine diploid clones, these are haploids and can be measured using mating type tests. Mates can stem from meiotic recombination or from the loss of chromosome III, the chromosome carrying the mating type locus. For a wild-type strain, positive mates ranged from 1% to 5% of total CIN clones, making them negligible in the global percentage of chromosome instability values. The rho0 cells do not sporulate; therefore, they did not suffer from this small bias.

**Figure 1** CINA, a sensitive and clonal assay to monitor chromosomal instability in yeast. (A) Genome instability is measured by positive selection of marker loss (URA3 and CAN1 genes) on the distal arm of chromosome V in diploid strains heterozygous for CAN1 and URA3. Combined loss of URA3 and CAN1 genes leads to colonies resistant to the drugs 5-FoA and canavanine (Can FoA R). In CINA, this can be attributable either to chromosome break or to full chromosome loss. (B) Starting from single cells, clones are grown on solid medium and chromosome instability events detected on CanFoA plates. Replica on ~LEU allows distinguishing between chromosome break (CanFoA-resistant, LEU⁺) and chromosome loss (CanFoA-resistant, leu⁻). (C) CIN rates in untreated wild-type (WT) cells, in S-phase cyclin mutant clb5Δ, in WT cells grown in the presence of 50 mM hydroxyurea (+HU) and in S-phase checkpoint mutant mrc1Δ. (D) CIN rates in WT cells grown to colonies in the presence of zeocin. Note the differences in Y scale. All strains were grown on YEPD plates at 30°C. Details of the assay and statistical analysis are described in Materials and Methods.
RESULTS

CINA, a sensitive assay to quantify chromosome instability in yeast

To test nuclear genome instability in a large number of samples, we designed a quantitative and highly sensitive assay called CINA (Figure 1A) with the following features: as in the original GCR assay, chromosome instability is measured by positive selection of two marker losses (URA3 and CAN1, linked in the distal part of chromosome V) (Chen and Kolodner 1999). The assay starts from single cells, CINA measures de novo instability events rather than accidental amplification of preexisting rearrangements present at t = 0 in assays in which large inclusions are required.

When grown on YEPD plates, wild-type cells display CIN rates of approximately 50 marker losses per 10 million cells (5 × 10⁻⁷ CanFoA-resistant/cell) (Figure 1C), i.e. approximately 10,000-fold higher than in the GCR assay; approximately two-thirds of events are attributable to chromosome breaks (CanFoA/66% LEU⁺) and one-third are attributable to chromosome loss (CanFoA/32% leu) (Figure S1). To validate the assay, we used drugs and mutations that affect chromosome stability in yeast. Genomic destabilization can be induced by drugs and mutations that affect chromosome stability in yeast. Genomic destabilization can be induced by etoposide, etidium bromide, or inactivation of the mrc1 gene, which encodes a ribonucleotide reductase (Rnr1p). These treatments increase CIN rates to approximately 50 marker losses per 10 million cells (5 × 10⁻⁷ CanFoA-resistant/cell) (Figure 1C).

Rho0 cells are genetically unstable when grown under standard rich growth conditions

To precisely quantify the rates of nuclear genome instability in cells lacking mitochondrial DNA (rho0 cells), we isolated rho0 clones starting from the diploid CINA parental strain (L1577; S288c background) (Table S1); nuclear chromosome instability was then analyzed many generations after the mtDNA loss event (“established” rho0 clones). Rho0 cells were first obtained after ethidium bromide treatment (Slonimski et al. 1968). When plated, these cells (all rho0) showed a dimorphic colony growth phenotype, with slow-growing and fast-growing clones (L1690) (Figure S2). A similar observation was made with newborn rho0 cells, also in the S288c genetic background (Veatch et al. 2009). Subcloning of slow clones gave rise to a mixture of slow-growing and fast-growing colonies [rho0 (s), L1993], whereas subcloning of fast clones only yielded fast-growing ones [rho0 (f), L1994] (Figure S2A). The frequency (approximately 1 in 10 cells) of conversion from slow-growing to fast-growing clones suggests an efficient suppression of the slow colony growth phenotype after mtDNA loss. No reverse switching from fast-growing to slow-growing clones was observed.

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established rho0 cells display a high rate of nuclear genome instability (mostly chromosome breaks) with two modes: very high CIN in slow-growing rho0 and a more moderate yet highly significant increase in faster growing rho0 cells. Thus, nuclear chromosome breaks occur at high rates when rho0 cells are grown to colonies under optimum, rich growth conditions.

**Nuclear DNA damage in rho0 cells occurs mostly in nondividing cells**

We first explored when in the life cycle of rho0 cells that nuclear genome instability arises. Instability stemming from DNA replication/repair defects (internal sources) is expected to occur in dividing cells, whereas the presence of genotoxic compounds in the environment (external sources) can trigger instability in both dividing and nondividing cells. We thus compared CIN rates in cycling compared with nondividing (postmitotic (PM) cells). When grown on solid media, nonsolitary yeast clones (intercolony distance <3 cm) behave like exponentially growing cells for the first 42 hr (Meunier and Choder 1999). Log phase (42 hr after plating on YPD) and PM cells (4-day-old clones) therefore were compared for CIN rates, both in wild-type and in rho0 strains. Wild-type CIN rates decreased moderately but significantly (P = 0.0001) between cycling (124 × 10⁻⁷) and PM (51 × 10⁻⁷) conditions, as expected (Figure 3, A and B). On the contrary, genome instability in rho0 strains remained surprisingly low in cycling cells and was increased in 4-day-old PM colonies. The effect was very striking in slow-growing rho0 cells [rho0 (s) cycling = 194 × 10⁻⁷ vs. PM = 1792 × 10⁻⁷] (Figure 3A), but was also significant in fast-growing rho0 cells [rho0 (f) cycling = 104 × 10⁻⁷; PM = 257 × 10⁻⁷; P = 0.0003] (Figure 3B). Importantly, in cycling populations, CIN in rho0 cells did not differ from wild-type [not at all for rho0 (f), less than two-fold for rho0 (s)] (Figure 3, A and B).

Thus, nuclear instability in rho0 cells occurs only in nondividing cells, after an exponential phase on rich medium, as if genotoxic compound(s) had appeared in the cell environment. Because the environment was not experimentally modified, these “compounds”/genotoxic effects are likely the result of the colony’s metabolic activity and/or of the modification of the physiology of the cell. Therefore, we next analyzed the effect of changing environments/metabolic conditions on nuclear CIN in rho0 cells in comparison to RHO+ strains.

**Environmental conditions can efficiently stabilize the nuclear genomes in rho0 cells: effects of low temperature and calorie restriction**

The high nuclear genome instability described occurred in standard growth conditions, i.e., in rich medium with high glucose (2%) and optimum temperature (30°). Because mitochondria are key players of cell metabolism, we asked whether modifications of metabolic or environmental conditions could influence the capacity of mitochondria-defective cells to maintain nuclear genome stability. We chose parameters based on their known effects on cellular fitness or longevity (e.g., calorie restriction) or on global metabolism (growth t²).

Calorie restriction is known to increase life span and organosomal fitness (Anderson and Weintraub 2010; Fontana 2009; Longo and Fontana 2010). Strikingly, we found that calorie restriction also has very beneficial effects on the stability of nuclear genomes in rho0 cells. CIN reduction is already very strong in slow rho0 cells grown under moderate calorie restriction (MCR; 0.5% glucose; CIN = 121 × 10⁻⁷/cells) or at lower glucose concentration (CIN = 76 × 10⁻⁷/cells in ECR; 0.05% glucose) compared to standard 2% glucose concentrations (CIN = 1792 × 10⁻⁷/cells) (Figure 4A). Nuclear genomes in spontaneously rho0 cells were also stabilized efficiently by growth under MCR conditions (Figure 4B). However, unexpectedly, calorie restriction did not reduce CIN rates in the wild-type RHO+ strain (Figure 4A). Instead, a mild CIN increase was measured in wild-type after growth under MCR (CIN = 129 × 10⁻⁷/cells; P = 0.004) and extreme (CIN = 102 × 10⁻⁷/cells; P = 0.040) compared to standard 2% glucose concentration (CIN = 51 × 10⁻⁷/cells) (Figure 4A).

Another global means of tuning metabolism is modifying the growth temperature. We therefore repeated CINA on rho0 cells grown on YEPD (2% glucose) at 25°, which is the low edge for optimal growth temperature in S. cerevisiae (Watson 1987). Remarkably, this moderate temperature decline reduced nuclear genome instability with great efficiency, again in rho0 cells only but not in wild-type cells (Figure 4C). Nuclear genomes were stabilized at 25° both in slow-growing (CIN 30° = 1281 × 10⁻⁷ vs. CIN 25° = 74 × 10⁻⁷) and fast-growing (CIN 30° = 338 × 10⁻⁷ vs. CIN 25° = 66 × 10⁻⁷) rho0 cells to wild-type levels (CIN 30° = 57 × 10⁻⁷ vs. CIN 25° = 56 × 10⁻⁷). Again, rho0 cells showed a highly conditional CIN phenotype in response to temperature, unlike wild-type cells. We conclude that reducing growth conditions from optimum parameters to either lower glucose concentrations or lower growth temperature strongly stabilizes the nuclear genome in cells lacking mitochondrial DNA, both in rho0 (s) and in rho0 (f), but not in RHO+, cells.
Factors with a negative impact on genome stability in rho0 cells

To search for conditions that, on the contrary, could negatively impact rho0 cells, we looked for drugs that differentially affect cell survival between wild-type and rho0 strains. Tested drugs included an inhibitor of DNA synthesis (HU), a DNA alkylating agent [methylmethanesulfonate (MMS)], a DNA double-strand break inducer (bleomycin), and a microtubule poison (benomyl). As control strains, wild-type (checkpoint proficient) and checkpoint mutants for DNA damage/DNA replication mec1 sml1 and spindle checkpoint mutant bub1 were used. Both rho0 (f) (L1994) and rho0 (s) (mip1, L1779) were tested. Figure S5A shows that rho0 cells are not hypersensitive to any of the tested drugs, compared to wild-type. This suggests that rho0 cells have no major defects in sensing, signaling, or repairing these types of damage. Consistently, the Rad53-dependent checkpoint was found to be functional in yeast cells lacking mitochondrial DNA (Crider et al. 2012). In contrast, rho0 cells show striking growth defects when plated on media containing hydrogen peroxide (Figure S5, A and B), as previously reported (Grant et al. 1997), unlike wild-type and bub1 control strains that formed colonies in the same conditions. Interestingly, RHO+ mec1 sml1 were as sensitive as rho0 cells to H2O2 (Figure S5A). Unlike peroxide, rho0 cells were not hypersensitive to the superoxide-generating agent menadione (Figure S5C). Hence, rho0 cells seem particularly sensitive to oxidative stress by peroxide but not to the other drugs tested. Because rho0 cells are sensitive to oxidants, we wondered whether intracellular oxidative stress might be responsible for the elevated CIN observed in unperurbed rho0 cells (Figure 2). If such were the case, then one might expect to reverse the effect by the mere addition of an antioxidant to the medium. Addition of the oxygen scavenger and redox modulator N-acetyl cysteine (NAC) to the medium strikingly lowered the high CIN of rho0 cells, both in slow and fast rho0. Most strikingly, at 20 mM NAC, CIN in rho0 (s) became indistinguishable from CIN in wild-type (P = not significant) (Figure 5A). Addition to the medium of another antioxidant, the electron-scavenger Tiron (Taiwo 2008), also stabilized nuclear genomes in both fast and slow rho0 strains (Figure 5B). Although the stabilizing effect of Tiron on slow rho0 is very significant (P = 0.0001), it was not quite as efficient as with NAC, and it remained significantly higher in rho0 (s) compared to wild-type (Figure 5B). Again, wild-type cells behaved differently from rho0 cells because addition of either NAC (20 mM) or Tiron (1 mM) slightly increased CIN in both instances (two-fold; P = 0.011 and 0.002, respectively). Thus, rho0, but not wild-type cells, undergo far less genomic instability in the presence of an antioxidant. These data suggest the presence of an unidentified oxidation-dependent damage in rho0 cells, leading to higher genomic instability in standard conditions.

To explore this phenomenon further, we deleted the thioredoxin peroxidase gene TSA1, encoding a major ROS detoxifying enzyme with an important function of protecting from nuclear genome instability (Chae et al. 1994; Iraqi et al. 2009; Ragu et al. 2007). Either wild-type or rho0 cells lacking TSA1 were challenged with sublethal H2O2 (1 mM), a concentration that mildly increased CIN (two-fold) in wild-type and left CIN in rho0 (f) cells unchanged (Figure S6A). Control tsal cells showed high CIN (25-fold more than wild-type), as described (Ragu et al. 2007), also moderately increased by H2O2 (two-fold) (Figure S6A). Removing TSA1 from a rho0 (f) had a very strong impact on nuclear CIN. First, the basal level of CIN in tsal rho0 was much higher [80-fold more than wild-type CIN levels, 16-fold more than rho0 (f)] (Figure S6A). Second, the mild H2O2 treatment further increased this instability to extreme proportions (400-fold more than wild-type) (Figure S6A). Despite their sensitivity at high peroxide concentrations (4 to 6 mM), we observed that rho0 cells still possessed efficient CIN protection against peroxides at sublethal doses [1 mM; rho0 (f)± H2O2]; however, when their defense against peroxides was
ethanol as well as hydrogen peroxide (but only in the absence of the protection by thioredoxin enzymes). Finally, the presence of antioxidants can efficiently stabilize the nuclear genome of rho0 cells, showing the importance of an unidentified oxidant-dependent reaction in the process of nuclear DNA damage in rho0 cells.

**The peroxiredoxin mutant tsa1 behaves differently than rho0 cells**

Another potential explanation for the beneficial effect CIN-stabilizing factors (MCR, low t′, and others) on rho0 cells could be the reduction of an oxidative-dependent damage. CIN in rho0 is very much reduced by antioxidants (Figure 5). To assess the potential in vivo benefits of slower metabolism in cells with a high oxidative load, we used a mutant in one of the main H2O2 detoxification enzymes, TσA1, known to be prone to high CIN (Ragu *et al.* 2007). We might expect to reduce CIN in tσA1 mutants by slowing metabolism, just like in rho0 cells. Under standard growth conditions, CIN in the tσA1 mutant is 30-fold higher [in the same range as in rho0 (s)]; however, unlike in rho0 cells, growth under MCR or at 25°C did not stabilize CIN in tσA1 cells (Figure S6B). Thus, these environmental conditions do not simply counteract a peroxide-dependent nuclear DNA damage. The addition of previously tested compounds, including NAC, ethanol, or peroxide, all led to a moderate CIN increase (less than two-fold). However, extreme calorie restriction (ECR) or growth on a poor carbon source, such as glycerol, reduced CIN quite efficiently, from 1424 × 10⁻⁷ (D) to 391 × 10⁻⁷ (ECR) and 142 × 10⁻⁷ (glycerol). Globally, CIN in tσA1 appears to be fairly invariant, like in other RHO⁺ strains, with the exception of ECR, which brings significant benefit to the stability of that strain. Thus, MCR or low growth temperature is not sufficient to reduce DNA damage in RHO⁺ cells with a high oxidative load.

**Presence of a wild-type cell neighbors stabilizes rho0 clones**

The rho0 cells are not born in an isolated environment but are surrounded by RHO⁺ cells. We therefore wished to investigate the effect of wild-type RHO⁺ cells on nuclear CIN in nearby rho0 cells. Because of multiple and opposite influences of the environment on genomic

![Figure 5](image)

**Figure 5** Nuclear genome stabilization in rho0 cells grown in the presence of antioxidants. (A) Wild-type (WT; L1577) and slow rho0 cells (L1690) grown at 30°C on YEPD medium in the presence of 20 mM of N-acetyl-cysteine (NAC) and tested in CIN assay. Addition of NAC strongly decreases genome instability of rho0 cells, close to WT levels. (B) The same effect on CIN is obtained by addition of electron scavenger Tiron at 1 mM on fast rho0 (f) and slow rho0 (s) cells. Addition of NAC or Tiron slowed but could be used for CINA (Figure S7B). Those clones grown on YEPD medium in the presence of 20 mM of N-acetyl-cysteine (NAC) and tested in CIN assay. Addition of NAC strongly decreases genome instability of rho0 cells, close to WT levels. (B) The same effect on CIN is obtained by addition of electron scavenger Tiron at 1 mM on fast rho0 (f) and slow rho0 (s) cells. Addition of NAC or Tiron slowed but could be used for CINA (Figure S7B). Those clones grown on YEPD medium in the presence of 20 mM of N-acetyl-cysteine (NAC) and tested in CIN assay. Addition of NAC strongly decreases genome instability of rho0 cells, close to WT levels.

crippled (by deletion of *TSA1*), rho0 cells became extremely prone to genome instability [tsa1 rho0 (f) ± H2O2] (Figure S6A).

In addition to its sensitivity to hydrogen peroxide, we found that rho0 cells are also hypersensitive to the presence of ethanol. Ethanol, the main fermentation product in yeast, cannot be processed in the absence of a functional respiratory chain; therefore, rho0 cells do not grow with ethanol as a sole carbon source. Both slow-growing and fast-growing rho0 cells showed a marked sensitivity when a mild concentration of ethanol (2%) was added to YEPD (2% glucose) medium (YEPD plus ethanol) (Figure S7A). Growth of rho0 (f) was slowed but could be used for CINA (Figure S7B). Those rho0 colonies grown on glucose plus ethanol medium were not suppressors, because they behaved just like the parental strain after a second passage on ethanol-containing medium (Figure S7B, passage 2). In rho0 (f), ethanol further increased the nuclear genome instability five-fold, from 338 × 10⁻⁷ to 1468 × 10⁻⁷ (Figure 6), i.e., up to the high level observed in highly unstable rho0 (s) strain. Moreover, the ratio between chromosome breaks and loss was not grossly affected by exposure to ethanol (Figure S7C). At the same concentration, ethanol had no gross effect on wild-type growth (Figure S7A), and a moderate but significant (two-fold to three-fold) increase in CIN rate was observed in the same conditions (Figure 6). In summary, exogenous factors with a negative impact on nuclear genome of rho0 cells include...
CIN in rho0 cells, as described, one cannot easily predict the outcome of such an experiment (for instance, glucose exhaustion would create a calorie restriction zone and reduce CIN, but production of ethanol would increase CIN).

At an intercolony distance less than 3 cm (0.5 cm in our experimental settings), colonies are of non-solitary type and can therefore influence each other (Meunier and Choder 1999). To assess whether CIN in rho0 could be influenced by the proximity of wild-type cells, we alternatively micro-manipulated cells of wild-type and rho0 (s) genotypes (Figure 7A, mixed) on rich medium at 30°C (high instability conditions for a rho0 cell) and compared them to plates containing unmixed clones (Figure 7A) [wild-type and rho0 (s)]. Genomes of highly unstable rho0 (s) cells are clearly stabilized when surrounded by wild-type clones [rho0 (s), mixed], still more than wild-type levels but close to the CIN level typical of the more stable rho0 (f) (nonmixed) (Figure 7B). We conclude that global modifications to the environment by wild-type cells lead to a clear decrease in nuclear DNA damage in nearby rho0 clones.

The nuclear genome in newborn rho0 cells is also stabilized by MCR

Previous experiments in this report were performed on established rho0 cells (i.e., many generations after the loss of mtDNA). To assess whether the same observations apply to newborn rho0 cells, we repeated our experiments in cells immediately after mtDNA loss during a period termed "crisis," which is known to be prone to high CIN in rho0 cells (Veatch et al. 2009). In a first step, mtDNA loss was induced in wild-type cells by growth in the presence of ethidium bromide, followed by recovery in either rich YEPD liquid medium or under MCR for 15 hr (t1) (Figure 8A). Thereafter, single cells were micro-manipulated on plates and allowed to grow to colonies for 4 d under the same conditions as during the liquid growth (D or MCR; t2) and then used for CINA. First, we detected no increase in nuclear chromosome instability in the first hours after the loss of mtDNA (t1) compared to the untreated rho0+ control (Figure 8B) (t1, D). When single cells from these cultures were allowed to grow to colonies and were restet for CIN, chromosome instability was readily detected in rho0 cells grown on YEPD (2%) (Figure 8B) (t2, D) to the same extent as in the established slow-growing rho0 (s) (Figure 2) (L19293 and L2232). Second, when recovery and colony growth were performed under a MCR regimen after mtDNA loss, it drastically reduced CIN (eight-fold) (Figure 8B) (t2, MCR), just like in established rho0 (s) under MCR (Figure 4C) (P = 0.20, not significant). Thus, it appears that newborn rho0 cells also undergo conditional instability, like the long-established rho0 clones described.

Unlike in rho0 cells, CIN is constitutive in wild-type, cell-cycle, and respiratory RHO* mutants

Our results clearly indicate that CIN in rho0 strongly depends on the environment of the cell. To test whether other mutants with unstable genomes but still in possession of their mitochondrial DNA behave likewise, we measured CIN in a few selected RHO* cell-cycle mutants grown under various conditions shown to affect rho0 cells (MCR, in the presence of ethanol or antioxidant Tiron). Those included deletion mutants in the SIC1 (p27KIP1 ortholog), WHI5 (yeast Rb), and MRC1 (Claspin) genes. The tested cell-cycle mutants had a general trend of constitutive CIN rates (Figure S8) very different from the environment-modulated genome instability seen in rho0 cells. Wild-type cells showed a very mild yet significant increase in CIN after growth under MCR (P = 0.0019), as well as in the presence of ethanol (P < 0.0001) or Tiron (P = 0.0002) (Figure S8). Thus, the highly variable CIN rates in rho0 strains are a characteristic not shared by RHO* cells.

To see if mutants with mitochondrial dysfunctions but still in possession of their mtDNA also display a high conditional CIN phenotype, like in rho0 cells, we examined the behavior of individual mutants in the respiratory chain as well as in the F1 and FO subunits of ATP synthase. In all instances, the single mutants analyzed showed a moderate increase in nuclear genome instability (two-fold to three-fold increase more than wild-type) and, contrary to rho0 cells, showed no major fluctuation of the CIN rates in changing environments (Table S2). Thus, a simple loss of ATP production from the mitochondria is not sufficient to induce a high CIN phenotype, as previously found for other respiratory mutants (Veatch et al. 2009). Importantly, none of the respiratory mutants still in possession of their mitochondrial DNA had a clear conditional CIN phenotype. Thus, so far, the strong conditional CIN phenotype is a unique feature of rho0 cells.

An increase in pH protects from nuclear CIN in rho0 cells

Disruption of vacuolar biogenesis or alkalinization of the yeast vacuole (that can be obtained by growth on alkaline medium) improves mitochondrial protein import, a sign of increased mitochondrial ΔΨ, through an unknown mechanism (Garipler and Dunn 2013). Because increased membrane potential is known to stabilize the nuclear
Inactivation of the ISC-adaptor MMS19 leads to a moderate CIN phenotype different from rho0 mutants

A defect in the maturation of ISC-dependent DNA metabolism and genome integrity enzymes could be responsible for the high CIN crisis in rho0 cells, as proposed in a previous study (Veatch et al. 2009). A specific branch of the ISC pathway involved in the maturation of these genome integrity enzymes was recently found to depend on the ISC adaptor Mms19 (Gari et al. 2012; Stehling et al. 2012). Taken together, these results predict that deletion of MMS19 might lead to a high chromosome instability rate, similar to CIN in rho0 cells and that deletion of mtDNA in an mms19Δ strain should not increase nuclear CIN much more.

However, we found that deletion of MMS19 (L2360) only leads to a moderate increase in nuclear CIN (rate mms19Δ = 144 × 10⁻⁷ on YEPD, 30°C) (Figure 10), approximately three-fold more than wild-type but significantly lower (P < 0.0001) than in any rho0 strain tested, either fast-growing or slow-growing, and especially much lower than slow rho0 "in crisis" [rho0 (s)]. We then removed mtDNA from an mms19Δ mutant and obtained a fast-growing mms19Δ rho0 strain that showed an increased nuclear CIN compared to mms19 alone [CIN mms19 rho0 (f) = 557 × 10⁻⁷ (L2372); P < 0.0001] (Figure 10). In conclusion, deletion of MMS19 might have only a partial effect on this branch of the ISC pathway, there could be an Mms-19-independent branch of the ISC pathway defective in rho0 cells, or, finally, additional sources of nuclear genome instability might exist in rho0 cells. This could include an ISC-independent but ΔΨ-dependent mechanism (Figure 11).

**DISCUSSION**

Cells with mitochondrial defects are known to be prone to nuclear genome instability. Use of a highly sensitive and quantitative nuclear genome instability assay made it possible to examine the features of cells lacking mitochondrial DNA. Regarding nuclear CIN, we report here that rho0 cells strikingly differ from most RHO+ WT cells in two major aspects. First, regarding timing, CIN in rho0 cells occurs in PM cells. Second, it strongly responds to changing environments (e.g., to glucose concentration or to growth temperature). This highly conditional CIN phenotype in rho0 cells raises the question regarding how nuclear chromosomes can break in nondividing cells in response to nongenotoxic factors.

**Figure 9**  
Alkaline growth pH stabilizes the nuclear genome of both fast (L2249) and slow (L2232) rho0 cells. Comparison of control (C) YEPD medium (not buffered) and YEPD (pH = 7.6) medium buffered with 50 mM potassium phosphate buffer.
High glucose and fast metabolism induce a genotoxic response in mitochondria-defective rho0 cells

Glucose is essential for growth of respiratory-defective cells, which fully rely on glycolysis for ATP production. If anything, high glucose should protect nuclear genomes in rho0 cells because glycolytic ATP is also required to maintain a mitochondrial membrane potential in rho0 cells (Giraud and Velours 1997; Lefebvre-Legendre et al. 2003), a key parameter to protect nuclear genomes in those cells (Veatch et al. 2009). Instead, we found that high glucose concentrations lead to high nuclear genome instability in rho0 cells. However, our data suggest that high glucose is not by itself genotoxic in rho0 cells. Nuclear chromosomal instability occurs in PM cells, when colonies have grown and glucose is metabolized, replaced by “waste” products (such as ethanol, which cannot be used as a carbon source by mitochondria-defective cells). Secondary metabolites of glucose, rather than glucose itself, are more likely involved in this apparent genotoxicity in glucose-rich medium. Three observations are in agreement with this idea: nuclear genomes of rho0 cells are stable during the exponential growth period, even in the presence of high glucose; addition of ethanol further destabilizes nuclear genomes in rho0 cells; and glucose limitation (even MCR) prevents high nuclear CIN in rho0 cells. Thus, after the growth period, a combination of secondary glucose metabolites and cell physiology (exponential vs. PM cells) is the likely combination that triggers high CIN in rho0 cells. At this point, we do not know whether exponentially growing rho0 cells are truly “protected” from nuclear chromosome damage or whether instability is not present because the CIN-inducing metabolic conditions are not met yet.

Oxidative stress and CIN in rho0: a paradox

The behavior of rho0 cells in response to oxidative stress/antioxidants is somewhat paradoxical. Our data indicate that rho0 cells still possess efficient defenses against oxidants, including peroxides. First, nuclear CIN does not increase in the presence of “low” \( \text{H}_2\text{O}_2 \) (1 mM) treatment (Figure S6) \( \text{rho0} (f) \). Second, the main thioredoxin \( \text{Tsa1} \) still protects rho0 cells, as shown by the strong additive effects of rho0 and \( \text{tsa1} \) mutations (Figure S6). Moreover, levels of endogenous \( \text{H}_2\text{O}_2 \) are fairly normal in rho0 cells, whereas superoxides are very low (Delsite et al. 2003; Rasmussen et al. 2003). The hypersensitivity of rho0 cells to peroxide (approximately 4 mM) is still unexplained and we have no evidence that it may be linked to nuclear CIN. Yet, we found that antioxidant treatment efficiently stabilizes CIN in rho0, but not in wild-type cells. Combined, these data suggest that chromosome instability in rho0 may not be attributable to abnormally high peroxide levels, but rather to some deleterious event that involves an oxidant-dependent reaction. Interpreting experiments using antioxidants is difficult and is more of a hint for further experiments than an explanation by itself (Murphy et al. 2011). Still, genome stabilization by addition of antioxidants to rho0 cells is clear, and this should be taken into account when proposing a mechanism for CIN.

Potential causes for nuclear CIN in PM rho0 cells

Chromosome breakage in unperturbed wild-type cells occurs mostly in the sensitive S-phase of the cell cycle (Libsy et al. 2001), with an open chromatin and active replication forks that represent a potential internal source of DNA breaks and genome instability. However, genome instability in rho0 cells is atypical, because it occurs not in dividing but in PM cells (Figure 3). What mechanism could explain this unusual feature of CIN in resting cells? Production of high-energy radicals by rho0 cells, leading to a direct chemical break of DNA, is one possibility. The Fenton reaction, involving the reaction of hydrogen peroxide with reduced iron \( \text{Fe}^{2+} \), could be a source of such high-energy hydroxyl radicals (Winterbourn 1995). The rho0 cells are hypersensitive to peroxides, and the regulon controlling iron uptake is activated in rho0 cells (Veatch et al. 2009); yet, we found only mild effects of modulating iron concentrations on CIN in rho0 cells (data not shown) and inactivation of the iron regulon does not abrogate high CIN in newborn rho0 cells (Veatch et al. 2009). High-energy radicals could also stem from reaction between metabolitic byproducts, driven by an enzyme; in peroxiredoxin \( \text{tsa} \) mutants, the reaction of ethanol with hydrogen peroxide leads to high-energy radicals (1-ethoxyhydroxyl). This reaction is catalyzed by the peroxidase activity of superoxide dismutase \( \text{Sod1} \), when this cytoplasmic enzyme is mislocalized to the nucleus (Ogusucu et al. 2009). In a similar way, \( \text{Sod2} \), a nuclear-encoded mitochondrial superoxide dismutase, could potentially drive such a reaction; mislocalized \( \text{Sod2} \) (misimported in the mitochondria because of low \( \Delta \Psi \) in rho0 cells) could induce nuclear DNA damage in the presence of \( \text{H}_2\text{O}_2 \) and ethanol. The rho0 cells are hypersensitive to both ethanol and \( \text{H}_2\text{O}_2 \) (Figure S5 and Figure S7), but we found that deletion of \( \text{SOD2} \) in rho0 cells does not prevent high genomic instability (data not shown).

As previously shown, defects in mitochondrial iron–sulfur cluster biogenesis could be a main cause of CIN in rho0 cells; decreased ISC biogenesis is sufficient to generate nuclear CIN even in RHO+ cells (Veatch et al. 2009). However, unlike in rho0 cells, DNA damage induced by low ISC occurs in cycling cells (Veatch et al. 2009) and, unlike expected, deletion of \( \text{MMS19} \), the ISC cofactor for the maturation of genome stability enzymes, only leads to a mild CIN increase, lower than CIN in rho0 cells (Figure 10). Loss of \( \text{Mms19} \) could only partially affect this branch of ISC biogenesis, or another \( \text{Mms19} \)-independent ISC branch could also be important for genome maintenance. Several DNA repair pathways and DNA synthesis are predicted to be affected in cells with low ISC function (Veatch et al. 2009). However, a repair defect does not account for the initial source of the damage. Because damage in rho0 cells is strongly reduced in the presence antioxidants (Figure 5), oxidative-dependent damage combined with a reduced ISC-dependent DNA repair could be yet another potential source of instability in nondividing cells.
Suppression of CIN in rho0 cells by environmental conditions

How do external factors affect CIN in rho0 cells? Affecting mitochondrial ΔΨ is one possibility. Increasing ΔΨ is sufficient to reduce genomic instability in rho0 cells (Veatch et al. 2009). External factors that can affect mitochondrial ΔΨ are therefore expected to influence CIN in rho0 cells. Recently, it was found that one external condition, increased pH, improves mitochondrial ΔΨ and growth properties in rho0 cells (Garipler and Dunn 2013). Growth at alkaline pH is therefore expected to suppress CIN in rho0 cells. We found this to be the case (Figure 9). Similarly, indirect evidence suggests that MCR might act through the same pathway (Figure S9). Thus, one category of environmental factors likely influences genomic instability in rho0 cells through their effect on vacuolar pH and mitochondrial ΔΨ. Increased ΔΨ should improve ISC biogenesis (hence, improving DNA repair) and increase mitochondrial protein import more generally (Figure 11). We noted that the same external conditions can have opposite effects on wild-type and on rho0 cells, because MCR prevents early alkalinization of the vacuole in wild-type cells, therefore extending lifespan (Hughes and Gottschling 2012). It was also concluded that increased vacuolar pH has an opposite effect on RHO+ and on rho0 cells (Garipler and Dunn 2013). Our data suggest that external

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**Figure 11** Environment-dependent nuclear genome instability in rho0 cells. (A) Wild-type cells grown under standard/rich conditions with normal metabolic activities, including mitochondrial membrane potential ΔΨ, iron–sulfur cluster (ISC) biogenesis, and efficient import of mitochondrial protein from the cytoplasm. Metabolic byproducts can be properly processed by the cell (e.g., ethanol produced from glucose can be oxidized in the mitochondria). CIN = 1X refers to the standard chromosome instability in these cells, which mainly occurs during the cell cycle. (B) In the same wild-type cells grown in different environments (calorie restriction, alkaline pH, lower t, and others), CIN rates remain fairly constant, despite metabolic changes. (C) Cells lacking mitochondrial DNA [rho0 (slow) or newborn rho0 cells] grown under standard/rich conditions display very high rates of chromosome instability in postmitotic cells. Low ΔΨ leads to inefficient mitochondrial (mt) protein import and low ISC biogenesis. Metabolic byproducts like ethanol cannot be processed in the dysfunctional mitochondria. Nuclear genome instability could stem from low ISC activity (Veatch et al. 2009), but deletion of the ISC cofactor MMS19 alone does not lead to high rho0-like instability, suggesting the existence of an additional source of nuclear CIN. An Mms19-independent branch of ISC-dependent proteins is one possibility. High genomic instability in rho0 cells could arise from the combination of increased metabolic-dependent DNA damage (after growth in rich conditions) and defective DNA repair because of low ISC biogenesis. (D) Modified environmental conditions can fully stabilize the nuclear genome of rho0 cells. In this model, external factors could influence nuclear CIN in rho0 by increasing ΔΨ (like growth at alkaline pH or under moderate calorie restriction), which would increase ISC biogenesis (hence, improving DNA repair) and improve mitochondrial protein import more generally, or by decreasing metabolic byproducts (decreased DNA damage?). So far, those extreme variations of nuclear CIN in response to multiple environmental conditions are only observed in rho0 cells.
conditions could also influence genomic instability in rho0 cells by affecting metabolic byproducts (ethanol, oxidants, and others) through an unknown mechanism. This is summarized in a speculative model (Figure 11).

In a yeast colony and beyond

One technical feature of this study is that all chromosome instability assays are performed on colonies (see CINA). Growth within a colony on solid medium is complex and heterogeneous compared to growth of cells in liquid shaken media. A cell at the center or at the margin of a clone experiences increasingly different conditions as the colony grows. This difference will be marked after approximately 10 d, when a differentiation occurs (triggered by ammonia signaling in yeast) and cells at the colony center undergo apoptosis (Palkova and Vachova 2006). In our study, we distinguished between early time points (less than 42 hr), equivalent to an exponentially growing population, when we observed low nuclear CIN and an intermediate stage at 4 d, when most cells are PM, but the differentiation step described has not yet taken place; at this stage, rho0 cells already show high nuclear CIN. This distinction is fairly crude in time and space and deserves more study. What we can state is that nuclear genome instability in rho0 cells is not like in wild-type cells in several aspects, including timing. Yet, even though instability occurs in the PM stage in rho0 cells, these cells are still able to resume proliferation on return to growth, because they are capable of generating CanFosA-resistant clones (in our CINA) or colony sectors (Veatch et al. 2009). As a colony grows, a decrease in nutrients and appearance of metabolites in the medium are likely to influence chromosome stability in rho0 cells. The capacity of wild-type colonies to stabilize the genome of nearby rho0 clones gives weight to this hypothesis (Figure 7); fast-growing wild-type colonies likely generate a calorie restriction zone for rho0 cells (a CIN-stabilizing condition) and could deplete metabolites like ethanol (CIN destabilizing), a reaction that rho0 cells are unable to perform.

If our observations apply to metazoans, then liver would seem like one place where such effects may occur because most liver cells are nondividing and highly exposed to incoming nutrients (glucose, ethanol, or others). Under specific nutrient conditions, this could lead to genotypic changes, including loss of heterozygosity in cells lacking mtDNA. Although cancer cells often display mutations in mitochondrial genes, the depletion of mtDNA from cancer cells reduced mtDNA. Although cancer cells often display mutations in mitochondrial metabolism. Science 337: 243–245.


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