Cell Cycle 13:13, 2056–2063; July 1, 2014; © 2014 Landes Bioscience

Mitochondrial genome regulates mitotic fidelity by maintaining centrosomal homeostasis

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Keywords: mitochondrial genome, centrosome amplification, osteosarcoma, rho0, cybrid

Centrosomes direct spindle morphogenesis to assemble a bipolar mitotic apparatus to enable error-free chromosome segregation and preclude chromosomal instability (CIN). Amplified centrosomes, a hallmark of cancer cells, set the stage for CIN, which underlies malignant transformation and evolution of aggressive phenotypes. Several studies report CIN and a tumorigenic and/or aggressive transformation in mitochondrial DNA (mtDNA)-depleted cells. Although several nuclear-encoded proteins are implicated in centrosome duplication and spindle organization, the involvement of mtDNA encoded proteins in centrosome amplification (CA) remains elusive. Here we show that disruption of mitochondrial function by depletion of mtDNA induces robust CA and mitotic aberrations in osteosarcoma cells. We found that overexpression of Aurora A, Polo-like kinase 4 (PLK4), and Cyclin E was associated with emergence of amplified centrosomes. Supernumerary centrosomes in rho0 (mtDNA-depleted) cells resulted in multipolar mitoses bearing "real" centrosomes with paired centrioles at the multiple poles. This abnormal phenotype was recapitulated by inhibition of respiratory complex I in parental cells, suggesting a role for electron transport chain (ETC) in maintaining numeral centrosomal homeostasis. Furthermore, rho0 cells displayed a decreased proliferative capacity owing to a G_2/M arrest. Downregulation of nuclear-encoded p53 in rho0 cells underscores the importance of mitochondrial and nuclear genome crosstalk and may perhaps underlie the observed mitotic aberrations. By contrast, repletion of wild-type mtDNA in rho0 cells (cybrid) demonstrated a much lesser extent of CA and spindle multipolarity, suggesting partial restoration of centrosomal homeostasis. Our study provides compelling evidence to implicate the role of mitochondria in regulation of centrosome duplication, spindle architecture, and spindle pole integrity.

Introduction

Recent discoveries of mtDNA mutations in a variety of human diseases and cancers have generated an upsurge of interest in mitochondrial gateways to human pathologies.[1-9] Genomic aberrations of mtDNA, which include point mutations, deletions, and rearrangements, have been reported in numerous cancers of the head and neck, breast, hepatic, colon, thyroid, gastric, lung, colorectal, pancreatic, and esophageal tissues.[3-7,9,10] While several studies have reported CIN and a tumorigenic and/or aggressive transformation in mtDNA-depleted or mtDNA-mutated cells,[1-7] the cellular mechanisms underlying these transformative properties remain unexplored. CIN is the most prevalent form of genetic instability in human cancers that facilitates tumor evolution and is associated with poor prognosis in solid tumors.[11,12] Amplified centrosomes occupy center stage in the cascade of events coupled with loss of fidelity in chromosome segregation that results in CIN.[13-17] Defects in pathways that regulate centrosome duplication can lead to disruption of numeral centrosomal homeostasis. Supernumerary centrosomes increase the propensity of CIN and ensuing aneuploidy, via either formation of multiple spindle poles or clustering of multiple centrosomes at a single pole.[13,14,18,19] A recent review by Moore et al.[20] implicated a "centrosomal role" of mitochondrial proteins based on some previous studies showing association of mitochondrial proteins with centrosomal components.[21]

Here we postulated that CIN associated with mtDNA alterations in cancers could be attributable to centrosome amplification (CA). In order to study the role of the mitochondrial genome on centrosomal homeostasis and mitotic spindle architecture, we employed osteosarcoma parental, rho0, and cybrid cells. While rho0 cells are depleted of their mtDNA, parental cells are wild-type (WT), and cybrid are rho0 cells wherein the mitochondrial genome has been reintroduced. The role of the mitochondrial

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Submitted: 03/17/2014; Revised: 04/18/2014; Accepted: 04/29/2014; Published Online: 05/05/2014
http://dx.doi.org/10.4161/cc.29061

genome in CIN and tumorigenesis, as evidenced by detrimental changes in nuclear genome status in osteosarcoma rho0 cells, has been previously demonstrated.[7] Here we report for the first time that mtDNA depletion in parental cells leads to robust CA and consequent disruption of mitotic spindle organization. Our data show that rho0 cells exhibit a higher degree and extent of reactive oxygen species (ROS)-independent DNA damage, downregulation of p53, CA, spindle multipolarity, and mitotic arrest as compared with parent cells. Reintroduction of mtDNA largely reversed these phenotypes in cybrid cells. Intriguingly, the aberrant phenotype of rho0 cells was recapitulated by inhibition of Complex I in parent cells, strongly suggesting the role of ETC in maintaining numeral centrosomal homeostasis that evades multipolarity. Taken together, our study suggests a role for mitochondrial genome in regulating centrosomal homeostasis and spindle organization.

Results

mtDNA depletion increases centrosome amplification, spindle multipolarity, spindle disorganization, and mitotic index

Several studies have shown the tumorigenic transformative capacity of mtDNA mutations or depletions in a wide variety of cancer cells.[3-7] In order to differentiate and further characterize the effect of mtDNA depletion on mitosis, we first examined the phenotype of the parent, rho0, and cybrid cells in various phases of the cell cycle, employing immunofluorescence confocal microscopy (**Fig. 1A**). We microscopically visualized a varying number of amplified centrosomes at each spindle pole in all cell lines, as is common in cancer cells (**Fig. 1A**). Although γ-tubulin is preeminently found in centrosomes, it is also distributed in the pericentriolar material (PCM) and can be dispersed during centrosomal splitting.[22] Thus, to confirm the presence of "real centrioles" in these γ-tubulin-positive spots, we immunostained all 3 cell types to visualize the centriolar marker protein, centrin-2 (**Fig. 1B**). Numerous green centrin dots were seen in rho0 mitotic cells, thereby validating that extra centrosomes contained "real" centrioles and not fragments of PCM. Upon quantitating the extent of CA in all 3 cell lines, we found that compared with parent cells (10%), rho0 cells exhibited a 5-fold increase in CA (**Fig. 2A**). Restoration of numeral centrosomal homeostasis in cybrid cells was significant, since only 14% of cybrid mitotic cells showed amplified centrosomes (**Fig. 2A**). Given the pronounced enhancement in the degree of CA in these mtDNA-depleted cells, we investigated any quantitative difference in the associated spindle multipolarity. Indeed, **Figure 2B** shows that mtDNA depletion resulted in a 2-fold decrease in number of normal bipolar cells, from 90% in parent 143 B cells vs. 44% in rho0. In addition, rho0 cells had a 4-fold increase in the number of tripolar cells and a remarkable 8-fold increase in multipolar cells (**Fig. 2B**). Intriguingly, mitotic cybrid cells largely reversed the phenotypes observed in rho0 cells, and 88% of mitotic cybrid cells showed bipolar spindle phenotypes (**Fig. 2B**). Abnormalities in spindle architecture (e.g., spindle elongation, pole fragmentation) also correlated with mtDNA depletion, as 73% of rho0 mitotic cells exhibited these characteristics, vs. only 19% and 28% of parent and cybrid cells, respectively (**Fig. 2A**). Furthermore, we found that rho0 cells harbored a 13% mitotic population (**Fig. 1A**), while parent and cybrid lines had a mitotic index of

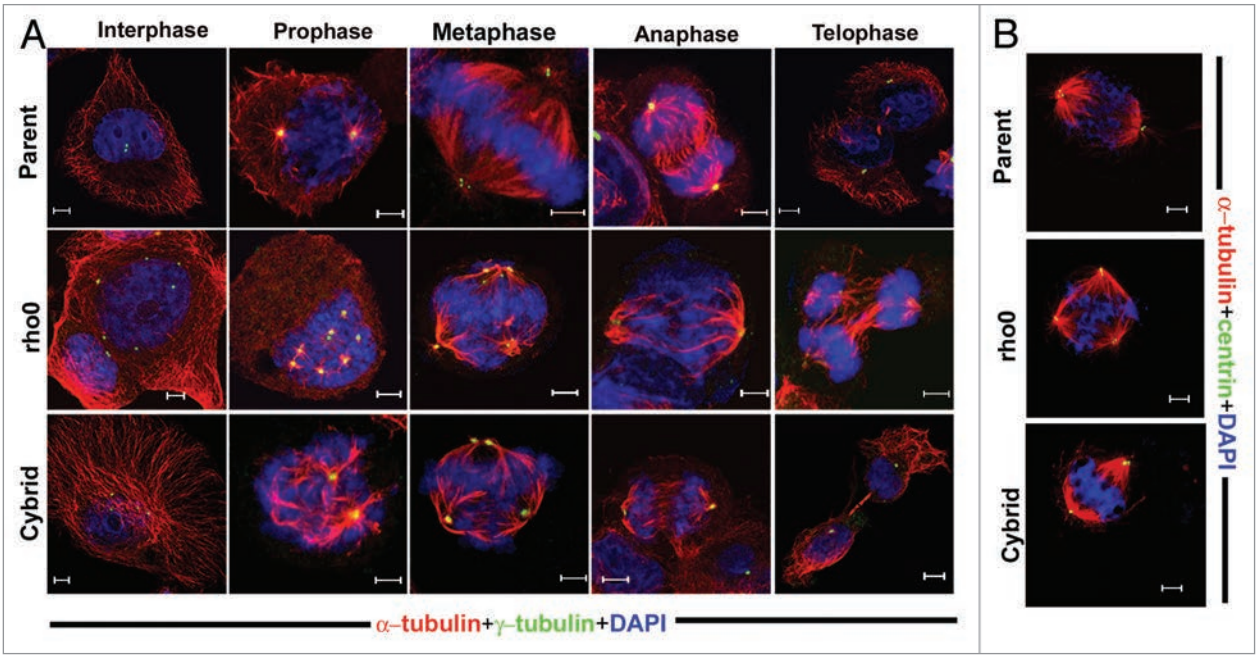


Figure 1. Cell cycle phenotype of parental, rho0, and cybrid cells. (**A**) Parental, rho0, and cybrid cells were stained with α-tubulin (red; γ-tubulin, green; DAPI, blue) to visualize microtubules, centrosomes, and DNA, respectively. Confocal microscopic images above represent cells throughout the sequential stages of the cell cycle. Bar represents 5 μm. (**B**) Confocal micrograph images showing real centriole amplification in rho0 cells. Cells were stained with α-tubulin (red; centrin-2, green; DAPI, blue) to visualize microtubules, centrioles, and DNA, respectively. Bar represents 5 μm.

6.4% and 7.3%, respectively. This 2-fold increase in the proportion of mitotic cells in mtDNA-depleted rho0 cells indicated a delayed passage through mitosis, a phenotype which suggests the presence of defects in kinetochore–microtubule attachments.

To further substantiate aberrant spindle assembly and chromosome missegregation, we coimmunostained with anti-γ-tubulin and α-tubulin antibodies along with CREST, a human autoimmune antibody used to indicate the location of sister kinetochores (**Fig. 2C**). Immunostaining using the CREST antibody to label kinetochores showed that in metaphase cells (**Fig. 2C**, upper panel), kinetochores were dispersed all over the region between multiple spindle poles perhaps due to widespread merotelic attachments, where a single kinetochore attaches to more than one centrosome perhaps from more than one spindle pole. In support of this notion, we found an increased number of bipolar anaphase cells with lagging chromosomes (**Fig. 2C**, lower panel) indicative of pronounced chromosomal missegregation in rho0 cells.

Several mitotic kinases are known to play a role in centriole duplication, specifically Polo-like and Aurora kinases.[23,24] PLK4 localizes to centrioles and regulates centriolar duplication during the cell cycle. PLK4 overexpression has been shown to induce multiple daughter centrioles at a single maternal centriole.[24] Aurora A kinase is involved in centrosome duplication and paired centriole separation.[23] Thus, we next asked whether alterations of PLK4, Cyclin E, and Aurora A levels underlie the robust CA seen in rho0 cells. Indeed immunoblotting confirmed a massive increase in PLK4 levels in rho0 cells. Likewise, Cyclin E and Aurora A blots confirmed their overexpression in rho0 cells compared with parent cells, suggesting a causative role of these proteins in inducing CA (**Fig. 2D**). Our data show that cybrids partially reversed the protein levels of Cyclin E and PLK4, while Aurora A levels did not change compared with that of parent cells. It is likely that cybrids recruit compensatory mechanisms and pathways to adapt to the loss of mitochondrial DNA gene products, and thus repletion of mtDNA in cybrids precludes reversal to "near" parental levels of the said proteins.

mtDNA depletion reduces proliferative capacity

Given the higher frequency of mitotic cells observed in rho0 cells, we were inquisitive to learn whether rho0 cells were undergoing rapid proliferation or were stalled in the cell cycle. To this end, we seeded parent, rho0, and cybrid cells for a 10-d clonogenic assay (**Fig. 3A and B**). The rho0 cells had a ~49% decrease in the number of colonies when compared with parent cells (**Fig. 3B**). Cybrids were partially restored in proliferation, showing ~29% decrease in the number of colonies compared with the parent cell line (**Fig. 3B**). Having identified that rho0 cells had a decreased proliferative capacity, we sought to determine whether these cells were stalled in the cell cycle. Cell cycle profiles of all 3 cell types were analyzed using dual-color flow cytometry. The rho0 cells showed a 10% increase in G_2/M population (**Fig. 3C**) when compared with parent cells. Cell cycle profile of cybrids was quite similar to parent cells, suggesting near-complete restoration in rho0 cells. Thus, these data suggest that the decreased proliferation capacity of rho0 cells compared with parent cells is due to G_2/M cell cycle arrest.

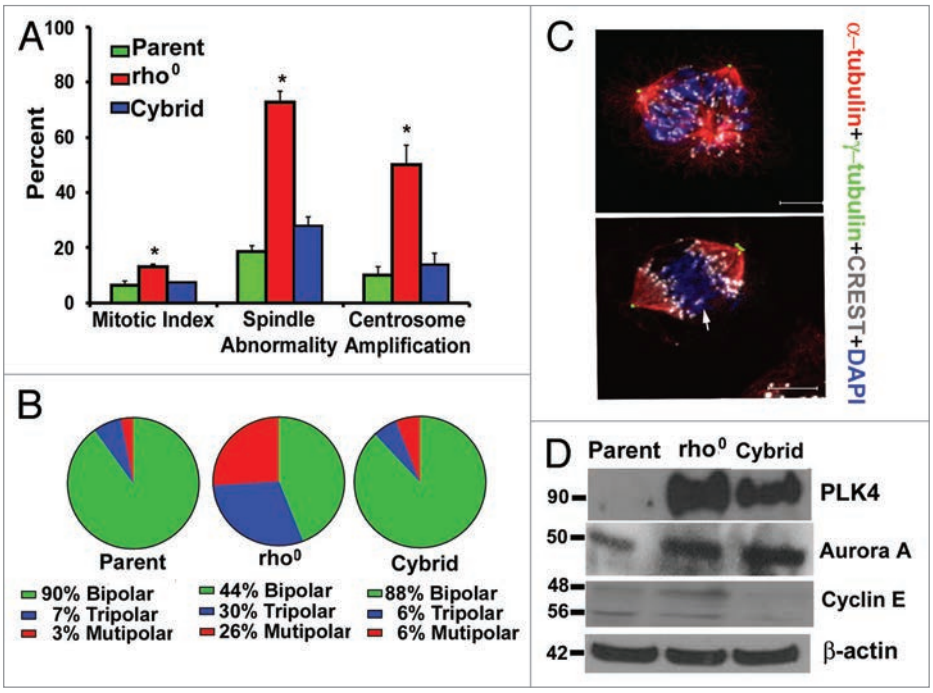


Figure 2. Phenotype quantitations and determination of centrosome amplification (**A**) Bar graph representation of mitotic index, spindle abnormality, and centrosome amplification in parental, rho0, and cybrid cell lines. Five hundred (500) cells were counted. (*$P < 0.05$ compared with parental and cybrid using 2-sample *t* test). (**B**) Quantitative pie chart representation of numeral spindle polarity in parental, rho0, and cybrid cell lines. 500 cells were counted. (**C**) Confocal micrograph images showing kinetochores in rho0 cells. Cells were stained with α-tubulin (red; CREST, white; γ-tubulin, green; DAPI, blue) to visualize microtubules, kinetochores, centrosomes and DNA, respectively. Arrow indicates lagging chromosomes. Bar represents 5 μm (**D**) Western blot analysis of proteins associated with centrosome amplification. Immunoblotting of PLK4, Cyclin E, and Aurora A in parental, rho0, and cybrid cells lines. β-actin was used as a loading control.

DNA damage in mtDNA-depleted cells is largely ROS-independent

We next examined whether DNA damage in rho0 cells was responsible for the G_2/M arrest. γH2AX immunofluoresence staining detected double-stranded DNA breaks in parent, rho0, and cybrid cells (**Fig. 4A**). DNA damage was 2.7-fold higher in rho0 cells (80% γH2AX-positivity) than parent cells (30% γH2AX-positivity) (**Fig. 4B**). Interestingly, while restoration in cybrid cells was seen in many characteristics as described above, DNA damage was evident in 65% of cybrid cells, a significant 2.1-fold increase when compared with parent cells. γH2AX immunoblotting confirmed higher levels of DNA damage in rho0 and cybrid

cells (**Fig. 4C**). Since the mitochondrial genome encodes subunits of each complex within the ETC, it is reasonable to speculate that ROS leakage from the ETC can cause DNA damage. Thus, we next asked if DNA damage in rho0 cells was due to an increase in ROS, mainly hydrogen peroxides (H_2O_2) and superoxides (O_2^-), from a "leaky" and incomplete ETC. We found that 2,7-Dichlorofluorescin (DCFDA), which detects H_2O_2, was not significantly higher in rho0 than parent or cybrid cells (P = 0.27 and P = 0.14, respectively) (**Fig. 4D**). In addition, levels of Dihydroethidium (DHE), which detects O_2^-, were similar among the 3 cell lines (**Fig. 4D**). Furthermore, treatment of rho0 cells with 5 mM of ROS-scavenger tiron failed to significantly reduce DNA damage in rho0 cells (70% positive, P = 0.1725) (**Fig. 4B**). These results suggest that loss of DNA integrity as seen in rho0 cells is ROS-independent and can be ascribed to mechanisms that respond to the depletion of mitochondrial genome and the absence of its gene products.

Telomeric loss in mtDNA depleted cells

Our studies so far confirmed that mitochondrial dysfunction leads to increased DNA damage. Given that DNA damage is significantly linked to loss of telomeric ends,[25] we next performed fluorescence in situ hybridization (FISH) to evaluate telomeric loss.[26] Interestingly, a high percentage of rho0 cells showed significant telomere loss when compared with parent cells. However, telomeric loss in cybrid cells was comparable to parental cell line (**Fig. 4F**). These studies suggested that DNA damage induced due to mitochondrial dysfunction results in loss of telomeric ends.

Depletion of mitochondrial genome downregulates p53

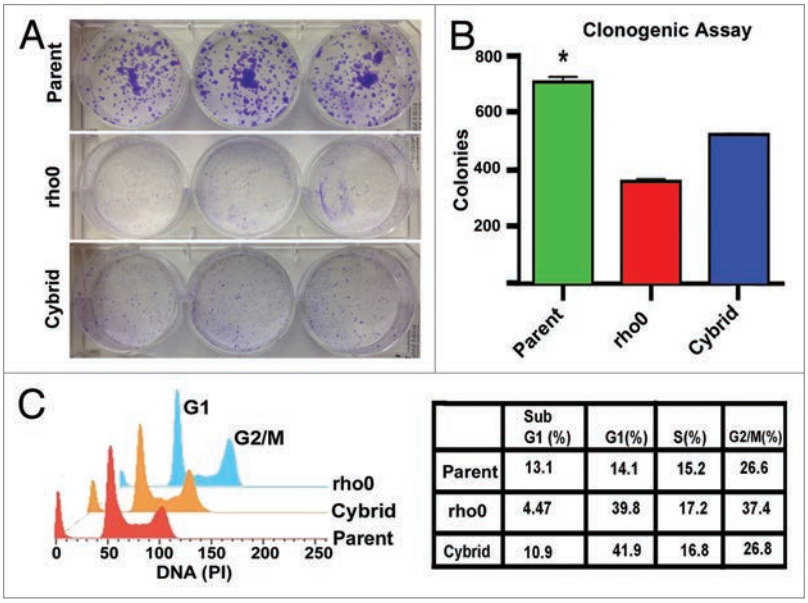
Elevated levels of DNA damage in rho0 cells led us to inquire if p53-regulated DNA repair and apoptosis were affected upon depletion of mtDNA. Immunoblot analyses showed downregulation of the tumor suppressor protein p53 in rho0 cells compared with parent cells (**Fig. 4E**). Cybrids revealed a restoration of p53 protein levels to those found in parent cells, suggesting that mtDNA may regulate the nuclear expression of p53. Importantly, the levels of the anti-apoptotic protein survivin as well as that of hypoxia-inducible factor (HIF)-1α, which are negatively regulated by p53,[27] were substantially elevated in rho0 cells compared with parent cells (**Fig. 4E**), suggesting a reduction in the activity of p53 in rho0 cells.

Electron transport chain (ETC) inhibition is sufficient for phenocopying rho0 cells

Since the mitochondrial genome encodes several components of the ETC, we next asked if the aberrant mitotic phenotypes in rho0 cells were due to the dysfunctional ETC in these cells. To test this, we examined the consequence of Complex I inhibition in parent cells, since Complex I is the most upstream in the ETC, and its inhibition would shut down the entire ETC. After a 24-h treatment of parent cells with 100 μM of rotenone, a mitochondrial Complex I inhibitor, we quantitated centrosome amplification, multipolarity, spindle abnormality, and mitotic index in all 3 cell types (**Fig. 5A–E**). Similar to rho0 cells, rotenone-treated parent cells showed a significantly higher extent of CA (64%) than parent cells alone (10%) (**Fig. 5A**). Moreover, multipolarity, spindle abnormality, and mitotic index in rotenone-treated parent cells all correlated with levels observed in rho0 cells (**Fig. 5A–E**). Immunoblotting of γH2AX also revealed increased DNA damage after rotenone treatment (**Fig. 4C**). In addition, PLK4 and Aurora A were upregulated upon rotenone treatment as compared with parent cells (**Fig. 5F**), albeit not to the same extent as in rho0 cells. Taken together, these results suggested that a defective ETC plays an important role in the genesis of aberrant phenotypes of rho0 cells.

Discussion

Recent discoveries of mtDNA mutations in a variety of human diseases and cancers have generated an upsurge of interest in mitochondrial gateways to human pathologies. Human mtDNA contains a single control region called the D-loop that controls mtDNA replication and transcription of mtDNA-encoded OXPHOS genes. Mutation in the D-loop region have been reported in most tumors examined to date.[3,8,9] Mutations



	Sub G1 (%)	G1(%)	S(%)	G2/M(%)
Parent	13.1	14.1	15.2	26.6
rho0	4.47	39.8	17.2	37.4
Cybrid	10.9	41.9	16.8	26.8

Figure 3. mtDNA depletion decreases cell proliferation and induces G_2/M stall. (**A**) Visual representation of a clonogenic assay performed with parental, rho0, and cybrid cell lines. One hundred (100) cells of each cell line were seeded in triplicate 3.5-mm wells and grown for 10 d. Paraformaldehyde fixing followed by crystal violet staining allowed visualization of colonies formed. (**B**) Bar graph representation of number of colonies formed in parental, rho0, and cybrid cell lines. Colonies were arbitrarily defined as at least 50 cells. (*P < 0.05 compared with Rho0 and cybrids using 2 sample t test). (**C**) Three-dimensional DNA histograms representing cell-cycle kinetics of parental, rho0, and cybrid cell lines. The x-axis shows DNA amounts representing different cell cycle phases; the y-axis shows the number of cells containing that amount of DNA; and the z-axis shows the differing 143B cell lines. Table representing the percentages of cells in each cell cycle for parental, rho0, and cybrid cell lines.

in the D-loop region result in altered binding affinities of the nuclear proteins involved in mtDNA replication and transcription, leading to the depletion of mtDNA content.[28] Consistent with this notion, our laboratory reported a near absence of mtDNA-encoded cytochrome c-oxidase subunit II expression in more than 40% of breast and ovarian tumors.[29] Other laboratories have also reported a decrease in mtDNA content in breast,[30] renal,[31] hepatocellular,[32] gastric,[33] and prostate tumors.[10] Reduced mtDNA is shown to decrease mtOXPHOS activity in renal tumors.[34] A study also demonstrated that decrease in mtDNA content correlates with tumor progression and prognosis in breast cancer patients. Indeed our studies demonstrate that reduced mtDNA content leads to tumorigenic phenotype in vitro and in vivo.[35] These studies suggest that mtDNA plays an important role in tumorigenesis and may contribute to the Warburg effect. However, mechanisms involved in the tumorigenesis process are not yet completely understood. Herein, using in vitro models of mtDNA depletion, we have uncovered a novel biologic relationship between depletion of mtDNA and induction of CA, conceivably leading to chromosome missegregation, which results in low-grade aneuploidy, nuclear genomic instability, cumulative mutations, and tumor progression. Utilizing the rho0/cybrid cell approach, our study shows that mtDNA depletion in osteosarcoma (parent cells) induces a significant increase in CA that results in spindle multipolarity. It is noteworthy that ~75% of mitotic rho0 cells display bipolar and tripolar spindles (**Fig. 2C**). Assuming that a significant proportion of the bipolar spindles are actually pseudo-bipolar, due to supernumerary centrosome clustering, it is clear that the predominantly low-grade multipolarity, in concert with some pseudobipolarity in rho0 cells, could cause CIN. By promoting low-grade aneuploidy, CA fosters karyotypic heterogeneity or plasticity, as cancer cells need to reshuffle their genomes to acquire and establish superlative chromosomal compositions conducive to better growth in a given environment (e.g., faster anchorage- and mitogen-independent growth).[13-15,36-38]

In consonance with earlier studies, we found that depletion of mtDNA decreases cell proliferation.[39] Our data suggest that rho0 cells underwent a G_2/M stall as seen by fluorescence-activated cell sorting (FACS). Although these findings at the outset seem counterintuitive, since rho0 cells are known to be more tumorigenic, recent reports indicate that mtDNA defects influence the tumorigenic process not by fueling cell proliferation, but rather by inducing genomic instability and enhancing cell survival.[5] In support of this notion, rho0 cells have been proven to be tumorigenic in the more applicable in vivo mice models.[35] Furthermore, rho0 cells had a ~3-fold smaller subG_1 fraction (cells with <2N DNA, which are likely apoptotic) as compared with the parent cells, suggesting their increased survival.

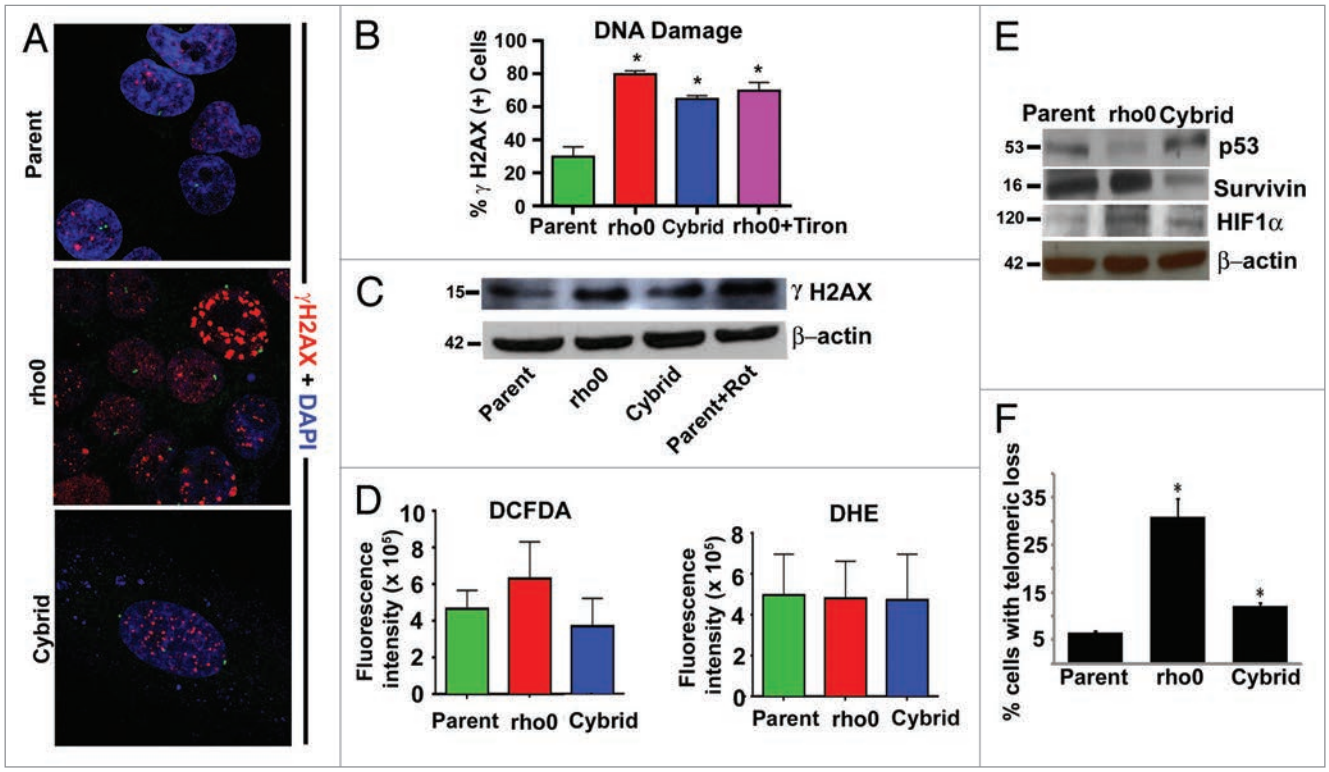


Figure 4. DNA damage and oxidative stress in parental, rho0, and cybrid cells. (**A**) Confocal micrographs of 143B cell lines stained with γH2AX (red; γ-tubulin, green; DAPI, blue) to view DNA damage, centrosomes, and DNA, respectively. (**B**) Bar graph representation of γH2AX positive cells. A total of 500 cells were counted (*$P < 0.05$ compared with parental cells). (**C**) Immunoblotting for γH2AX. β-actin was used as a loading control. (**D**) Bar graph representation of DCFDA and DHE fluorescein measurement in 143B cell lines. Cells were treated with 5 μM of DCFDA or 5 μM DHE. DCFDA detects hydrogen peroxides, and DHE detects superoxides . (**E**) Western blot detection of antiapoptotic and survival genes. Immunoblotting for Survivin, HIF-1α, and p53 in parental, rho0, and cybrid cells lines. β-actin was used as a loading control. (**F**) Percentage of cells showing telomere shortening using FISH (*$P <$ 0.05 compared with parental cells).

Along with a G_2/M arrest, we found increased DNA damage in rho0 cells. Interestingly, γH2AX staining and immunoblotting confirmed higher double-stranded DNA breaks in not only rho0 cells, but also in cybrids when compared with parent cells. Thus, repletion of mtDNA into rho0 does not fully reverse the extent of DNA damage. This trend was also observed in the case of telomeric loss (**Fig. 4F**). Since the cybrid cell technology is frequently used to study specific mtDNA mutations in many human diseases,[40] it is pertinent to recognize the significant DNA damage in cybrid cells when evaluating the role of their targeted mutations. Despite increased DNA damage, we found that p53 was downregulated in rho0 cells, while cybrid cells maintained it to the same level as parent cells (**Fig. 4E**). Many studies have shown evidence of forward communication between the nuclear genome and its effect on mitochondrial counterpart.[25] Our result implicates the relatively lesser-known role of the mitochondrial genome in retrograde inter-genomic cross-talk, from mtDNA to nuclear DNA. The decreased p53 levels in rho0 cells showed a concomitant increase in molecules (PLK4, Cyclin E, and Aurora A) that are crucial players in centrosome-duplication events. Our findings are in complete concordance with studies that have shown p53 downregulation results in upregulation of PLK4, Cyclin E, and Aurora A to aid in centrosome amplification.[41-43] We have recently demonstrated that a brominated noscapine analog-mediated DNA damage induces centrosome amplification via a PLK4-dependent mechanism.[44] It is noteworthy that the rho0 cells do not completely revert back to parent cells. The source of the cybrid cells are the rho0 cells. Since rho0 cells have adapted themselves to surviving without the mitochondrial DNA, it is likely that cybrids recruit compensatory mechanisms and pathways to adapt to the loss of mitochondrial DNA gene products. Also, generation of rho0 cells leads to remodeling of metabolism and genomic instability in the nucleus, thus repletion of mtDNA in cybrids precludes reversal to "near" parental cells.

Furthermore, since the role of OXPHOS dysfunction in rho0 cells is well known and mimics hypoxia seen in tumor cells, we explored the status of HIF-1α and the anti-apoptotic protein, survivin. Indeed, both HIF-1α and survivin were increased in the mtDNA-depleted cells, with restoration seen in the cybrids. Although mechanistically not explored in these cells, p53 and HIF-1α have been shown to interact with and affect each other's activity.[27] These results again implicate the role of mitochondria-to-nucleus communication in cancer development. Lastly, we show that inhibition of Complex I of the ETC in the parent cells resulted in a rho0 phenotype, suggesting its role as well as that of the OXPHOS system in centrosome amplification, spindle multipolarity, and DNA damage.

Taken together, our results implicate the role of mtDNA depletion and mitochondrial dysfunction in an aggressive tumorigenic phenotype through centrosome amplification, spindle abnormalities, and p53 downregulation. While more needs to be learned about the mechanisms' underpinnings in these phenotypes, the findings herein represent an important role of mtDNA in maintaining centrosomal homeostasis and normal spindle architecture. In addition, further investigation of centrosome amplification in parent, rho0, and cybrid cell lines in an in vivo

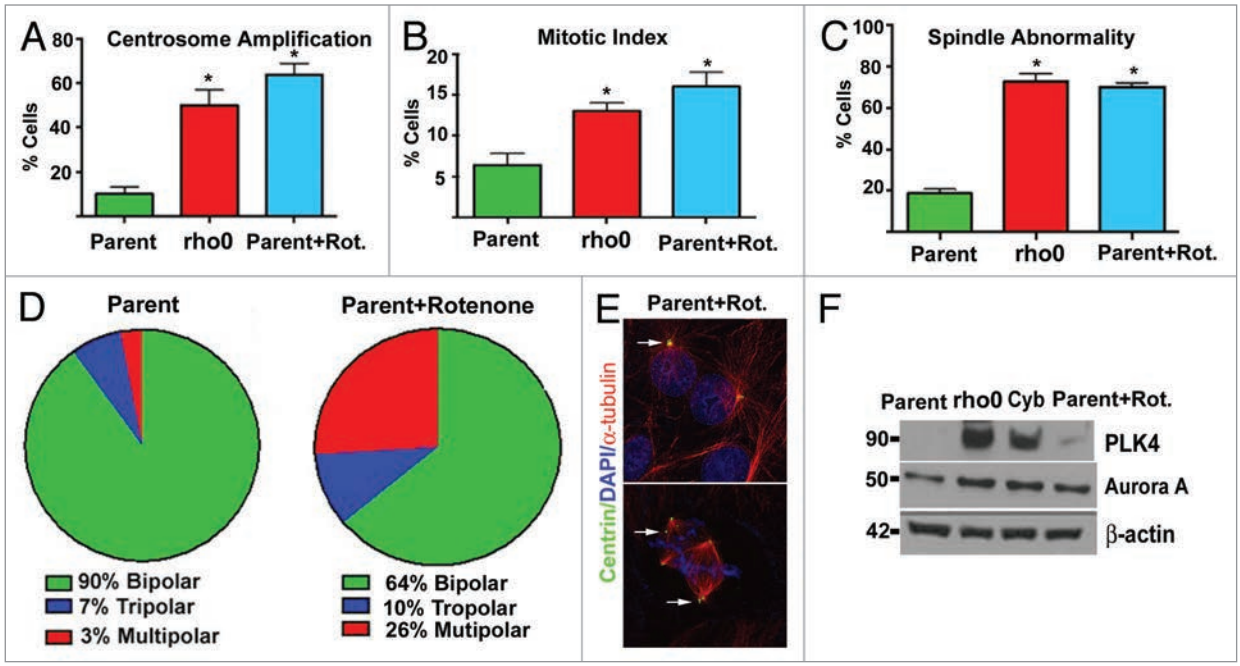


Figure 5. Inhibition of mitochondrial Complex I induces rho0 phenotype in parental cells. (**A**) Bar graph representation of centrosome amplification in parental cell line treated with rotenone. (**B**) Bar graph representation of mitotic index in parental cell line treated with rotenone. (**C**) Bar graph representation of spindle abnormalities seen in parental cell line treated with rotenone. All parameters shown represent 500 counted cells. (*$P < 0.05$ compared with parental cells using 2 sample *t* test). (**D**) Pie chart quantitation of parental cells (control) and parental cells treated with 100 mM of the Complex I inhibitor rotenone for 24 h. (**E**) Representative picture of parent cells treated with rotenone showing centrosome amplification and formation of multipolar spindle. Arrows show centrosome amplification. (**F**) Western blot analysis of centrosome amplification genes PLK4 and Aurora A. β-actin was used as a loading control.

model will provide additional evidence on the role of mitochondrial genome in regulating mitotic fidelity. As more knowledge accumulates on the role of mtDNA in malignant transformation, mitochondria-targeted therapies can be explored as an alternative, or in supplement, to current cancer therapeutics. Since our study demonstrates a major role of mtDNA-encoded proteins in CA, targeting these mitochondrial proteins may serve as a useful therapeutic intervention for reducing CA and avoiding low-grade aneuploidy and aggressive tumor phenotype.

Materials and Methods

Cell culture, chemicals, and reagents

143B human osteosarcoma cells (parent cells) were used in the present study. 143B rho0 (mtDNA-depleted) and 143B cybrid cell lines were derived from the parent 143B cell line. A genetic method involving expression of the HSV 12.5 gene was used to generate 143B rho0 cells.[7] Complete depletion of mtDNA in this cell line was achieved by transient expression of HSV UL12.5 followed by clone isolation. Cybrids of the rho0 cells were constructed as described.[7] Platelets were isolated from normal human blood and fused with rho0 cells using polyethylene glycol 1500. The resulting cybrid cells were selected in a uridine-free culture medium. Single cybrid clones were isolated by ring cloning. The rho0 and cybrid status was confirmed by COX II immunoblotting. Cell lines were grown in Dulbecco modified Eagle medium with 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin (Invitrogen). Rho0 cells were supplemented with 50 μg/ml uridine (Sigma). All cell lines were maintained in a humidified 5% CO_2 atmosphere at 37 °C. DCFDA was from Sigma. DHE, 4,5-dihydroxy-1, 3-benzenedisulfonic acid disodium salt monohydrate (tiron) and rotenone were from Fisher Scientific. Primary antibodies directed against PLK4, Cyclin E, β-actin, Aurora A, and survivin were from Cell Signaling; centrin-2 and γ-tubulin were from Santa Cruz Biotechnology, Inc; HIF-1α and p53 were from BD Biosciences; γ-H2AX was from EMD Millipore, and α-tubulin from Sigma. 4',6-Diamidino-2-phenylindole, Dihydrochloride (DAPI) was from Invitrogen. Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. Alexa-488 and 588 conjugated secondary antibodies were from Invitrogen.

Clonogenic survival assay

Cells were seeded at 100 cells/well into triplicate 3.5-mm wells with corresponding cell media. After 10 d, media was aspirated, and colonies were fixed with 4% formaldehyde, stained with crystal violet, and counted. A colony was arbitrarily defined to consist of at least 50 cells.

Measurement of ROS

Cells were seeded in 96-well black optical bottom Nunc plates and incubated for 24 h at 37 °C. The medium was then aspirated and cells were pre-treated with 5 μM of either DHE (oxidized by ROS into ethidium bromide) or DCFDA (oxidized by ROS to DCF) for 30 min at 37 °C.[45] Cells were then washed with PBS, and ROS was measured using a fluorescent SpectraMAX Plus384 microplate reader (Molecular Devices).

Cell cycle analysis by flow cytometry

Flow-cytometric evaluation of cell cycle status was performed using FlowJo software. Cells were grown for 24 h, harvested and centrifuged, washed twice with ice-cold PBS, and fixed in 70% ethanol overnight in 4 °C. Propidium iodide (0.1% in 0.6% Triton X-100 in PBS) was then added followed by analysis on a FACS Canto flow cytometer.

Immunoblot analysis

Western blots were performed as described earlier.[44] Briefly, proteins were resolved by PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked in tris-buffered saline containing 0.05% Tween-20 and 5% fat-free dry milk and incubated first with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce Biotechnology). β-actin was used as loading control.

Immunofluorescence microscopy

Cells were grown on glass coverslips for immunofluorescence microscopy. After treatment with drugs or media for 24 h, cells were fixed with cold (–20 °C) methanol for 10 min in 4 °C, washed twice with PBS, and blocked by incubating with 2% BSA/PBS at room temperature for 1 h. Primary antibodies (1:2000 dilution) were incubated with the coverslips for 45 min at 37 °C. The cells were washed 10× with PBS at room temperature before incubating at 37 °C with a 1:2000 dilution of conjugated secondary antibodies. Cells were washed 5s with PBS and then mounted with Prolong-Gold antifade reagent that contained DAPI (Invitrogen).

Statistical analysis

All experiments were repeated at least 3 times. Data were expressed as mean ± standard deviation. Statistical analysis was performed using Student *t* test. The criterion for statistical significance was $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This study was supported by grants to R.A. from the National Cancer Institute at the National Institutes of Health (R00CA131489, R01 CA169127). Research in K.K.S. laboratory is supported by Veterans Administration grant 1I01BX001716 and National Institute of Health R01CA121904. We thank Dr Bhupendra Singh for helping us with **Figure 4F**.

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