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## Mitohormesis, UPR<sup>mt</sup>, and the complexity of mitochondrial DNA landscapes in cancer

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### Abstract

The discovery of the Warburg effect - the preference of cancer cells to generate ATP via glycolysis rather than oxidative phosphorylation has fostered the misconception that cancer cells become independent of the electron transport chain (ETC) for survival. This is inconsistent with the need of ETC function for the generation of pyrimidines. Along with this misconception, a large body of literature has reported numerous mutations in mitochondrial DNA (mtDNA) further fueling the notion of non-functional ETC in cancer cells. More recent findings, however, suggest that cancers maintain oxidative phosphorylation capacity and that the role of mtDNA mutations in cancer is likely far more nuanced in light of the remarkable complexity of mitochondrial genetics. This review aims at describing the various model systems that were developed to dissect the role of mtDNA in cancer, including cybrids, and more recently mitochondria-nuclear exchange (MNX) and conplastic mice. Further, we put forward the notion of mtDNA landscapes, where the surrounding non-synonymous mutations and variants can enhance or repress the biological effect of specific mtDNA mutations. Notably, we review recent studies describing the ability of some mtDNA landscapes to activate the mitochondrial unfolded protein response (UPR<sup>mt</sup>), but not others. Further, the role of the UPR<sup>mt</sup> in maintaining cancer cells in the mitohormetic zone to provide selective adaptation to stress is discussed. Among the genes activated by the UPR<sup>mt</sup>, we suggest that the dismutases SOD2 and SOD1 may play key roles in the establishment of the mitohormetic zone. Finally, we propose that using a UPR<sup>mt</sup> nuclear gene expression signature may be a more reliable readout than mtDNA landscapes given their diversity and complexity.

### Keywords

mitochondria; mtDNA; UPR<sup>mt</sup>; mitohormesis; cancer; ROS; SIRT3; SOD1; SOD2; HSP60; metastasis

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The role of mitochondrial DNA (mtDNA) in cancer has been the focus of extensive study over the years. The Warburg effect, characterized by a preference for glycolysis, suggests that cancer cells become unreliant on oxidative phosphorylation for the generation of ATP. Given that the activity of the mitochondrial electron transport chain (ETC) is required for the

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generation of pyrimidines, which are essential for the growth of rapidly dividing cells, a complete lack of ETC activity is incompatible with the growth of cancer (1). Compelling evidence for the necessity of mitochondria in tumor formation was provided by studies in which tumor cells depleted of mtDNA and engrafted in mice, only formed tumors after acquiring host mtDNA via whole mitochondria transfer(2,3). Using similar experimental systems, it was recently demonstrated that the acquisition of mitochondria by mtDNA depleted tumors cells is dependent on the acquisition of OXPHOS for pyrimidine synthesis, not ATP generation(4). Acquisition of mtDNA through exosomes or whole organelle transfer has also been shown as a resistance mechanism by breast, blood, and brain tumors in response to targeted, systemic, or radiotherapy(5–7).

Further, the confusing nature of the role of mtDNA in cancer biology is in part due to the complexity of its genetics. As a result of their bacterial origin, the mitochondria of eukaryotic cells contain their own circular genome. In humans, mtDNA is 16.5 kb in length and encodes 22 tRNAs, 2 rRNAs, and 13 proteins. These proteins encode core subunits of complexes I, III, IV, and V of the ETC. The mitochondrial genome is found in multiple copies per mitochondrion and thereby hundreds to thousands of copies per cell. The numerous copies of mtDNA per cell confer mitochondrial genetics with a level of complexity that requires specialized nomenclature. Specifically, homoplasmy denotes when the same sequence of mtDNA is present in all strains of mtDNA in a cell. In contrast, heteroplasmy, refers to a state in which mixed mtDNA sequences or mutations are present. Heteroplasmy at a specific locus is defined as a percentage of abundance relative to the wild type sequence (Cambridge reference mitochondria sequence). The genetics of mtDNA are further complicated by the fact that mitochondria are dependent on genes encoded by the nuclear genome for their function. Many subunits of the ETC are encoded in the nuclear genome. Additionally, proteins necessary for the maintenance, replication, and transcription of the mitochondrial genome are encoded by the nuclear DNA. For example, transcription of the mitochondrial genome is dependent on POLRMT, a nuclear encoded polymerase found on chromosome 19. In fact, ~99% of the mitochondrial proteome is encoded by nuclear genes. To demonstrate the influence of the mitochondrial genome alone on a given process, it must be experimentally separated from the effect of the nuclear genome.

However, the mitochondrial genome has proved difficult to edit genetically despite advanced using nucleases such as mitochondrial zinc finger nucleases (mtZFN) and mitochondrially targeted transcription activator-like effector nucleases (mitoTALENs) (8–10). The current inability to effectively deliver exogenous RNA into mitochondria has prevented mtDNA engineering from benefiting from the CRISPR revolution. Many in the field are actively trying to adapt the CRISPR/Cas9 system for mtDNA editing with focus on the introduction of gRNAs into the mitochondria(10). Further development of the molecular toolkit to edit the mitochondrial genome has the potential to transform our understanding of mtDNA and the therapeutic interventions against mitochondrial diseases.

## **Cybrids as a tool to test the role of mtDNA in cancer**

The challenges in manipulating mtDNA with the traditional approaches of molecular biology has led to the development of transmitochondrial cytoplasmic hybrid (cybrid)

technology. This technique relies on the generation of  $\rho^0$  cells devoid of mtDNA by prolonged culture with ethidium bromide (Fig. 1A). The resulting cells serve as the donor of the nuclear genome. Donor cells of mtDNA are enucleated and the fusion of both creates cybrids with the nuclear and mitochondrial genomes of experimental interest (Fig. 1A).

Using cybrids, the Hayashi group was the first to demonstrate that mtDNA alone could impact the metastatic behavior of cells(11). In this seminal work, cybrids were generated between non-metastatic P29 and highly metastatic A11 murine cancer cells. In tail vein injections and spontaneous metastasis assays, highly metastatic A11 mtDNA imparted a metastatic phenotype in cybrids with P29 nuclei. Conversely, non metastatic P29 mtDNA blunted the metastatic nature of A11 nuclei. The authors ascribed this effect to mutations in ND6 (G13997A and 13885insC) present in the mitochondrial genome of A11 cells. However, while frameshift and missense mutations represent a possible mechanism to explain this phenotype, additional differences in the mitochondrial genomes of the two cells cannot be excluded.

Following this original study, multiple others followed, providing countless examples of the effect of mtDNA on invasion and metastasis. Cybrids generated between invasive triple negative MDA-MB-231 breast cancer cells and fetal fibroblasts showed that the mtDNA of MDA-MB-231 confers metastasis (12). The authors postulated that the metastatic conferring capacity of MDA-MB-231 mtDNA is due to pathogenic mutations in ND4 (C12084T) and ND5 (A13966G) (12). Cybrids with the mtDNA of MDA-MB-435 cells, which carry a pathogenic A12308G mutation in the tRNA<sup>Leu(CUN)</sup> gene, were also shown to promote metastasis *in vivo* (13). Patient derived mtDNA with an A3243T mutation in tRNA<sup>Leu(UUR)</sup> conferred invasive and metastatic properties to 143 osteosarcoma cells(14). Cybrids of A549 cells paired with the mtDNA of lung adenocarcinoma patients with missense and nonsense mutations in ND6, also showed enhanced invasion, migration, and mitochondrial superoxide production(15). In addition, mtDNA of highly metastatic B82M fibrosarcoma containing a ND6 13885insC mutation conferred cybrids with metastatic capacity *in vivo* (16). In this study, mtDNA sequencing of NADH dehydrogenase (ND) genes in 115 patients with non-small cell lung carcinoma (NSCLC) identified 14 single nucleotide variants (SNVs) computationally predicted to decrease complex I function (16). These SNVs in ND genes were significantly more abundant in patients with distant metastasis, providing evidence that mtDNA mutations are associated with metastasis (16). Further supporting a mechanistic link between complex I function and metastasis, is the observation that manipulation of complex I activity by subunit overexpression or knockdown affected metastasis *in vivo*(17). Since the majority of proteins encoded in mtDNA are subunits of complex I, a large portion of the mitochondrial genome affects complex I function. Using cybrids, it was demonstrated that mild mutations in complex I (G3460A in ND1, G11778A in ND4, and T14484C in ND6) conferred enhanced tumorigenicity in nude mice compared to parental cells(18). Cybrids with the G8363A mutation tRNA<sup>Lys</sup>, a rare and severe mtDNA mutation clinically implicated in Leigh syndrome, cardiomyopathy, and MERRF-like syndrome, were also generated(19). Interestingly, of all ten cybrids and cell lines generated in this study, G8363A tRNA<sup>Lys</sup> cybrids had the highest levels of mitochondrial superoxide but were not tumorigenic in nude mice(18). These data suggest that mild mtDNA mutations may be more potent cancer drivers than severe mtDNA mutations. One potential explanation for these

observations is that on one hand, severe mtDNA mutations may compromise mitochondrial function to an extent that is incompatible with tumor formation. Milder mtDNA mutations, on the other hand, do not severely compromise mitochondrial function but still result in elevated ROS, which act as a pro-tumorigenic signaling molecule.

### Clinical evidence supporting a role of mtDNA in cancer

In addition to studies using cybrids, clinical evidence further supports the influence of mtDNA mutations on cancer etiology and progression. In a cohort of 1,675 tumor samples across 30 different cancer types, 1,907 somatic mtDNA substitutions were identified (20). Similarly, somatic mtDNA mutations were found in approximately three quarters of a cohort of breast cancer patients(21). The majority of mutations described in these patients were found in genes encoding subunits of complex I (21). In renal oncocytoma patients, loss of function mtDNA mutation of complex I was identified as a driver event that proceeds chromosome 1 loss and cyclin D1 overexpression(22). In response to complex I mtDNA mutations, cancer cells of these patients showed adaptive upregulation of glutathione biosynthesis. This metabolic rewiring may function to enhance oxidative stress capacity. The compensatory changes of nuclear gene expression in response to mtDNA mutation highlight the cross talk between the mitochondria and the nucleus.

Using RNA sequencing data from 344 patients with breast cancer, some of whom had patient matched normal samples, 9,063 SNVs and 84 insertions or deletions (INDELs) were detected in tumor mtDNA(23). Of the identified somatic variants/mutations, 95% were typical of replication-coupled mtDNA substitutions. Further, the number of identified mtDNA variants/mutations was significantly correlated with the age of patients, suggesting that replication induced mtDNA changes that accumulate with age are largely responsible for the mtDNA landscapes seen in breast cancer patients (23). In diffuse large B-cell lymphomas, tumor-normal pairs identified private constitutional variants and somatic mtDNA mutations in a mutational spectrum consistent with the replication associated mode of mutagenesis(24). In a panel of colorectal cancer cell lines, mtDNA mutations were found in 70% of samples(25). The majority of these somatic mutations were homoplasmic and were transitions at purines, the preferred target of free radicals, indicating ROS induced mtDNA mutation(25). Therefore, oxidation and replication induced mutagenesis may be considered to be the two main sources of mtDNA damage.

Using whole-genome sequencing of 1,916 patients with matched normal and tumor samples across 24 different cancer types, a study identified distinct mutational patterns present in the mitochondrial genomes of tumor cells (26). This study identified 2,350 tumor-specific somatic mtDNA mutations present across both the coding and non-coding regions of the mitochondrial genome, though the majority resided in RNA coding regions. When metastatic or recurrent tumor samples were compared to primary tumors, there was a significant increase in the proportion of mutations residing in RNA encoding regions of the genome (91% metastatic, 96% recurrent, 87% primary)(26). These data suggest a selection for mtDNA mutations in coding regions of the mitochondrial genome in cancer progression. Tumor specific mtDNA mutations were observed across all electron transport chain complexes, but a significant proportion of these mutations were found in genes encoding

subunits of Complex I. Interestingly, several of these mutations in complex I were recurrent or mutually exclusive, suggesting certain mutations provide an adaptive advantage that promotes tumorigenesis. Analysis of the mitochondrial genomes of normal samples in this study identified 1,154 normal-cell heteroplasmies(26). The identified heteroplasmic sequences were found throughout the mitochondrial genome but the vast majority of the variants/mutations described were in the non-coding D-Loop, which is important for mtDNA replication and transcription. Compared to tumor specific mtDNA mutations, normal cell heteroplasmies were significantly less abundant in RNA encoding regions of the mitochondrial genome (69% normal, 87% primary tumor). Further, tumor mtDNA had significantly more nonsense mutations, missense mutations, insertions, and deletions than normal cells (26).

Additional insight into the changes in the mitochondrial genomes of cancer cells during metastatic progression is gained from a rapid autopsy study of 10 prostate cancer patients with primary tumors, soft tissue metastases, bone metastases, and normal tissue analyzed by mtDNA sequencing(27). This study found that bone metastases had significantly more somatic mtDNA mutations than both the primary tumor and soft tissue metastases. In 70% of patients, a G10398A missense mutation in the ND3 gene of complex I was found exclusively in bone metastases (27). This observation argues that specific mtDNA mutations provide an adaptive advantage to form secondary lesions in particular target organs. In an epidemiological study, the 10398A allele was found to be an independent risk factor for invasive breast cancer in African-American but not Caucasian women(28). Significant increased risk of breast cancer and squamous cell carcinoma of the esophagus was also seen with the 10398A in an Indian cohort with the mtDNA haplogroup N(29). Further studies using cybrids generated with the mtDNA of African Americans show that cells with the 10398A allele have resistance to apoptosis, decreased mitochondrial membrane potential, increased ROS production, enhanced colony formation capacity, and increased metastatic capacity in tail vein injection experiments(30). These data suggest that specific mtDNA mutations can significantly modulate cellular phenotypes resulting in enhanced tumorigenicity and disease progression. Other reviews discussing the influence of mtDNA in cancer provide further insight into the well-established, albeit confusing nature of this phenomenon(31–40).

### **Are mitochondrial unfolded protein response activating-mtDNA landscapes rather than individual mtDNA mutations the answer?**

Our group recently performed mitochondrial DNA sequencing in order to determine whether common mtDNA mutations can distinguish invasive from non-invasive breast cancer cell lines (41). Specific missense mutations, A8860G in ATP8 and A15380G in CYB, were present in all cell lines at homoplasmic levels. However, no common mutations were exclusively seen in invasive cell lines. Additionally, neither the number nor location of mtDNA mutations/variations identified invasive from non-invasive cell lines. Unexpectedly, we found that the only commonality in the mtDNA of invasive cells was the presence of at least one variant with high levels of heteroplasmy(41).

As our laboratory has a long-standing interest in the mitochondrial unfolded protein response (UPR<sup>mt</sup>), we hypothesized that perhaps the mtDNA mutational landscape of invasive cells differentially activates this pathway. The UPR<sup>mt</sup> is a multibranching signaling pathway that promotes mitochondrial fitness through induction of nuclear encoded protein quality control programs, antioxidant machinery, mitochondrial biogenesis, and mitophagy and is activated by perturbations to mitochondrial homeostasis (42–51). Similarly, others have postulated that the ability of mtDNA mutations to promote cancer initiation and progression is mediated through adaptive nuclear gene expression patterns, as reviewed elsewhere in detail(43,44,46,47,49,51–58). In agreement with our hypothesis, we found that activation of the UPR<sup>mt</sup> was a defining characteristic of invasive cell lines and inhibition of the UPR<sup>mt</sup> abolished invasion (41). Importantly, this observation was confirmed using a set of 143B/206 osteosarcoma cybrids and hybrids in which the mtDNA of CyB or COX1 cybrids was fused with mtDNA of another patient were also used in this study(59). mtDNA sequencing of cybrids and their respective hybrids revealed minor differences in heteroplasmy within the pairs. Despite sharing the same nuclear genome and having the same mtDNA mutation as their respective cybrid, both hybrid cells were significantly less invasive and showed reduced activation of the UPR<sup>mt</sup> (41). These results suggest that the effects of a given ROS-generating mutation on UPR<sup>mt</sup> activation and invasion is dependent on the surrounding mtDNA landscape in which it resides.

This observation is consistent with the finding that the 10398A allele described above is only associated with cancer in certain ethnic groups. Human populations are characterized by mtDNA haplotypes which mirror our evolution out of Africa(60). A result of sequential accumulation of mtDNA variants through maternal lineage, these haplotypes differ in polymorphisms across the mitochondrial genome. The ethnic differences in the effect of a given mtDNA mutant on clinical phenotypes is a result of the mtDNA haplotype in which that mutation resides.

The ability of mtDNA haplotype to influence cancer behavior is highlighted by a number of clinical studies. Patients with mtDNA haplotype M exhibited significant increased risk of breast cancer, with M sub-haplotype D5 having the most pronounced risk(61). Interestingly, despite increased breast cancer incidence, haplotype M had significantly lower rates of metastasis than haplotype N. This study also reported a significant increased risk of thyroid cancer in haplotype D4a(61). In hepatocellular carcinoma patients, polymorphisms in the D-Loop (315insC and 16263T) were independent predictors of increased tumor-free survival(62). In gastric cancer, mtDNA haplotype N is associated with better survival(63). In gastroenteropancreatic neuroendocrine neoplasm patients, three SNVs in the D-Loop were significant predictors of survival with 16257A, 150T, and 151T alleles doing worse(64). It is not yet known how these alleles affect nuclear gene expression in these cancer types. It is likely, however that these alleles modulate nuclear gene expression. In an elegant study utilizing nuclear matched cybrids with different levels of heteroplasmy of a single mtDNA tRNA<sup>LEU</sup> 3242A>G, it was demonstrated that nuclear gene expression was significantly affected in phases corresponding to the heteroplasmic levels of the point mutant(65).



These studies highlight the ability of mtDNA landscapes to influence clinical outcomes in cancer patients. Collectively, this body of work suggests that mild differences in mtDNA landscapes can modulate the effect of specific mtDNA mutations.

## Mimicking the effect of haplotype using mice with mtDNA from different genetic backgrounds

One of the most significant advances in dissecting the effect of mtDNA in cancer has arisen from the generation of mitochondrial-nuclear exchange (MNX) mice. Using pronuclear exchange, MNX mice are generated with the nuclear genome of an inbred mouse strain in the background of mtDNA from other inbred mouse strains (Fig. 1B). When crossed to mammary specific oncogenic drivers, MNX mice with FVB/NJ nuclear DNA and BALB/cJ or C57BL/6J mtDNA had significantly different tumor latency, metastatic tumor size, and metastatic tumor burden in comparison to wildtype FVB/NJ strain mice(66,67). mtDNA backgrounds also significantly affected spontaneous tumor formation in aged mice of 9 different MNX strains(68). Genomic analyses of MNX mice generated with different mitochondrial pairings from FVB/NJ, C3H/HeN, C57BL/6J, and BALB/cJ mice also revealed selective differential DNA methylation and nuclear gene expression in strains with identical nuclear genomes but differing mtDNA (69). The observed epigenetic and transcriptional changes in MNX mice highlight the ability of mtDNA to influence nuclear gene expression. Considering the crosstalk between UPR<sup>mt</sup> activation and chromatin and epigenetic modification(70–74), it is possible that the UPR<sup>mt</sup> may underlie the reported changes in MNX mice.

At the same time, activation of the UPR<sup>mt</sup> in the absence of underlying pathology prolongs lifespan and delays the onset of age related diseases(71,73,75,76). To reconcile the longevity effect of the UPR<sup>mt</sup> and its potential ability to promote tumor progression, we hypothesize the following. In the setting of spontaneous tumor formation, MNX mice carrying mtDNA landscapes that activate the UPR<sup>mt</sup> may show a delay in aging and age related diseases, such as primary tumor formation. Once a tumor is established, however, activation of the UPR<sup>mt</sup> may inadvertently promote tumor progression, since the same beneficial effect of the UPR<sup>mt</sup> that confers longevity, may be hijacked by cancer cells to promote their growth and survival.

Additional *in vivo* evidence of the ability of mtDNA to influence nuclear gene expression and complex disease phenotypes like aging and cancer have emerged from conplastic mice. Taking advantage of the maternal inheritance of mtDNA, conplastic mice are generated by crossing female mice with a mitochondrial genome of interest to male mice with the nuclear genome of interest. Female offspring are continually backcrossed to male mice with nuclear genome of interest for a minimum of ten generations to create conplastic mice with mtDNA and nuclear DNA of interest (Fig. 1C). Conplastic mice with C57BL/6 nuclear genomes paired with C57BL/6 mitochondria (BL/6<sup>C57</sup>) or NZB/OlaHsd mitochondria (BL/6<sup>NZB</sup>) were used in a comprehensive study of healthspan (77). While the divergence of mtDNA genome between these mice is comparable to that seen between Eurasian and African human mitochondrial genomes, the authors found that BL/6<sup>NZB</sup> mice had a significant extension in

median but not maximal lifespan(77). Interestingly, analysis of mitochondrial ROS between conplastic strains at a young age showed that BL/6<sup>NZB</sup> actually had higher levels. However, in older BL/6<sup>NZB</sup> mice, there was no age-related increase in ROS as seen in BL/6<sup>C57</sup> mice(77). The observation that younger BL/6<sup>NZB</sup> mice have higher ROS levels than BL/6<sup>C57</sup> is seemingly at odds with their more youthful aging profile. This observation, however, is consistent with the phenonemon of mitohormesis which has been shown to promote health and longevity in model organisms. The findings from MNX and conplastic mice and the studies describing the influence of human mtDNA haplotype suggest that different haplotypes have different susceptibilities to cancer(66,77,78).

## Mitohormesis regulates cancer metastasis

Mitohormesis describes the paradoxical effect of moderate mitochondrial stress that activates persistent cytoprotective mechanisms and results in enhanced mitochondrial stress resistance (79,80). Mitochondrial ROS has been demonstrated to be an important inducer of mitohormesis and promote longevity and healthspan in *C. elegans* and yeast(81–85). The UPR<sup>mt</sup> is a key effector pathway of mitohormesis and is necessary for longevity induction by mitohormesis in worms and flies (70–72,75,86–88)(Figure 2A, 2B). One possible consequence of increased ROS levels at young age in BL/6<sup>NZB</sup> mice is induction of mitohormesis and activation the UPR<sup>mt</sup>. In this scenario, mitohormetic activation of the UPR<sup>mt</sup> would protect against subsequent age related increases in ROS. However, while mitohormesis has been most extensively studied in lower model organisms such as flies, worms, and yeast, only recently has mitohormesis been demonstrated in mice. Most notably, the Shadel group generated an inducible and reversible SOD2 knockdown mouse (iSOD2-KD), and showed that mitohormesis is induced in mice by transient SOD2 knockdown during embryogenic development(89). Upon SOD2 knockdown, embryos experienced significantly more oxidative stress, which led to induction of mitohormesis that persisted when SOD2 knockdown was reversed. Mitohormetically primed mice were characterized by enhanced mitochondrial biogenesis, upregulation of antioxidant defenses, and reduced ROS. Significant overlap was seen between the signature of these mice and the mitoprotective outcomes of UPR<sup>mt</sup> activation. Taken together, these studies demonstrate that mitohormesis does in fact occur in mammals and has profound implications for health and age-related diseases. Additionally, these studies implicate ROS and the UPR<sup>mt</sup> as important components of mitohormetic signaling in mammals.

Given our interest in the UPR<sup>mt</sup> and cancer, we recently tested whether mitohormesis occurred in tumors and if so whether mitohormesis influences phenotypic cancer cell behavior or disease progression. Having demonstrated that activation of the UPR<sup>mt</sup> promotes invasion of cancer cells(41), we hypothesized that mitohormetic activation of the UPR<sup>mt</sup> may serve to promote tumor metastasis and disease progression. To test these hypotheses, the murine breast cancer model (MMTV-rTA/NeuNT) was used. We found that primary tumors contain two sub-populations of cancer cells based on mitochondrial ROS levels. We isolated these two sub-populations and found that the ROS+ population is significantly more invasive *in vitro* and metastatic *in vivo*(90). The ROS+ population of cancer cells had significant activation of the UPR<sup>mt</sup> (Fig. 2B, 2C). Consistent with mitohormesis, basal activation of the UPR<sup>mt</sup> persisted in ROS+ cells and these cells were resistant to subsequent



oxidative stress(90). These data suggest that oxidative stress in the primary tumor primes a subpopulation of cancer cells to upregulate the UPR<sup>mt</sup> through mitohormetic signaling. As a result, these cells are more invasive and metastatic. These observations were further validated in human breast cancer cell lines(90).

Mechanistically, we showed that UPR<sup>mt</sup> activation was sufficient to promote invasion and that the SIRT3 axis of the UPR<sup>mt</sup> was necessary for invasion and metastasis. This axis of the UPR<sup>mt</sup>, mediated by the mitochondrial sirtuin SIRT3, is responsible induction of antioxidant programs, mitochondrial biogenesis, and mitophagy (Fig. 2A). In this study, genetic knockdown of SIRT3 inhibited invasion and metastasis in *in vitro* and *in vivo* model systems(90). Using a 7-gene signature of the UPR<sup>mt</sup> we found that patients with UPR<sup>mt</sup>-HIGH tumors show significantly worse clinical outcomes including metastasis(90). Importantly, UPR<sup>mt</sup>-HIGH patients had gene expression programs consistent with mitohormesis. Collectively, these studies indicate that while mitohormesis promotes health and longevity in the absence of underlying pathology, it is perniciously used by cancer cells to promote tumor progression.

### SOD1 as an additional key mediator of the UPR<sup>mt</sup>

Given the role of ROS in activation of mitohormesis and the UPR<sup>mt</sup>, we have shown that superoxide dismutases, SOD1 and SOD2, are upregulated by the UPR<sup>mt</sup>(90). The mitochondrial matrix localized SOD2 is part of the SIRT3 axis of the UPR<sup>mt</sup> and plays an important role in mitochondrial antioxidant capacity (Fig. 2A). The cytosolic, nuclear, and mitochondrial intermembrane space localized SOD1 is upregulated by mitochondrial stress and is part of the UPR<sup>mt</sup>, though which axis it belongs to is currently unknown (Fig. 2A). In the context of cancer, SOD2 is initially downregulated upon tumor initiation and re-activated later during cancer progression and metastasis by the SIRT3 axis of the UPR<sup>mt</sup>. We previously proposed a SOD2 to SOD1 switch during tumor initiation(91,92). We found that SOD1 levels are increased in breast cancer cells with decreased SOD2 levels(92). We showed that the majority of the superoxide activity in a panel of breast cancer cell lines is due to SOD1 activity. In addition, we found that the mitochondrial fraction of SOD1 is increased in breast cancer cells. Moreover, inhibition of SOD1 led to a selective increase in mitochondrial superoxide levels in cancer cells compared to the normal mammary epithelial cells MCF10A. SOD1 inhibition also led to an increase in mitochondrial fragmentation and altered mitochondrial morphology(92). These findings suggest that SOD1 is essential for mitochondrial integrity in the presence of increased oxidative stress in cancer cells.

We recently sought to better understand the role of SOD1 in cancer. To test the role of SOD1 *in vivo*, we crossed the SOD1 knockout mice with the inducible ErbB2 and Wnt transgenic models of mammary tumors. We found that in both models, SOD1 is necessary for oncogene-driven proliferation (Fig. 2D). Congruent with our previous findings that SOD1 inhibition does not affect normal mammary epithelial cells *in vitro*, we found that absence of SOD1 did not affect normal proliferation during different stages of mammary gland development *in vivo*(93). Additionally, SOD1 knockout mice do not have any phenotypic differences in other proliferative tissues such as the skin or the intestine. We hypothesized that the specific requirement for SOD1 in cancer cells but not normal highly proliferative

tissues may be due to differences in mitochondrial superoxide levels. We found that the proliferation driven by pregnancy does not increase the level of mitochondrial superoxide, while tumors exhibit remarkably high levels of mitochondrial superoxide, and the high ROS sub-population from tumors show higher expression of SOD1(93). This correlated with SOD1 immunohistochemistry staining in which SOD1 was not detected in normal mammary ducts or proliferating mammary glands during pregnancy but was detected in tumors(93). Our results suggest that in the absence of SOD1, malignant cells are unable to cope with elevated superoxide levels associated with transformation and undergo oncogene-induced senescence and apoptosis. In addition, breast cancer patients with high SOD1 levels have poor clinical outcomes.

Our findings concur with other groups that have demonstrated the efficacy of inhibiting SOD1. One of the first studies linking SOD1 to cancer demonstrated that SOD1 is important for the viability of leukemia cells(94). Following this finding, the Varmus group identified SOD1 as a selective therapeutic target in a high throughput small molecules screen of approximately 200,000 compounds in human lung adenocarcinomas(95,96). Their top small molecule, LCS-1 showed efficacy at killing only lung cancer cells, and had no effect on normal lung epithelial cells *in vitro*. We have shown that LCS-1 treatment also selectively inhibits the growth of mammary tumor cells (93,97) . LCS-1 has also been tested in a model of neck and head cancer(98). Previously, SOD1 has also been pharmacologically inhibited with copper chelators, such as diethyldithiocarbamate (DDC) and tetrathiomolybdate (TM) (99). ATN-224, a TM derivative, and was shown to have efficacy in promoting cell death in lung cancer cells lines and an experimental model of non-small cell lung cancer(100). Collectively, these studies suggest that SOD1 is vital for cancer cells and may be a potential, selective therapeutic target.

## Concluding remarks

The controversy over the role of mtDNA in cancer arises in large part from two factors. First, the misconception that cancer cells become independent of mitochondrial oxidative phosphorylation; and second, the remarkable complexity of mitochondrial genetics where in addition to heteroplasmy, distinguishing driver mutations/variants from passenger mutations/variants is extremely challenging. In light of the most recent studies, particularly those in MNX and conplastic mice, the picture that emerges is that the impact of mtDNA on cancer may be best explained if the respective contribution of the baseline (wild type) mtDNA landscape is separated from the contribution of acquired mutations and variants during transformation. This potential scenario of multiple levels of mtDNA complexity is illustrated in Figure 2E. The observations described in the experimental models described in this review suggest that different haplotypes have different susceptibilities to cancer(66,77,78). This represents Level 1 of mtDNA complexity. In other words, an individual with haplotype A has a higher risk of developing cancer than an individual with haplotype B or C (Fig. 2E). If those two individuals were to acquire the exact same mtDNA driver mutation X, the aggressiveness of the tumor would be altered by the specific haplotype in which it arises (Fig. 2E). This represents Level 2 of mtDNA complexity. Evidence for the existence of driver mtDNA driver mutations come from a study sequencing renal oncocytoma tumor (101). In this study, it was demonstrated that mtDNA mutations in complex I were early

genetic events that contributed to tumor formation. While some mutations were recurrent, there were a number of ways by which these tumors mutated *amtDNA* encoded complex I subunit(101). This suggests that mutation of complex I, regardless of the particular mutation, is a driver *mtDNA* mutation. The emergence of additional stochastic mutations and variants may further modulate the impact of the mutation and haplotype on cancer, positively or negatively. These modifier variants and mutations represent Level 3 of *mtDNA* complexity (Fig. 2E). Level 4 of *mtDNA* complexity, arises from the combinatorial effect of the heteroplasmic frequencies of multiple modifiers and drivers (Fig. 2E).

Ultimately, we propose that the resulting combinatorial size and diversity of potential *mtDNA* landscapes can be divided into those that activate the  $UPR^{mt}$  and those that do not (Fig. 2E).  $UPR^{mt}$  activating landscapes enable increased mitochondrial fitness and adaptation to stress thereby providing a selective advantage to the cells carrying them. In agreement with this possibility, using a 7 gene  $UPR^{mt}$  signature, we show that patients with  $UPR^{mt}$  positive tumors have worst outcome and increased metastasis.

Finally, if this multi-layered model of *mtDNA* complexity is correct, the lack of consensus about which *mtDNA* mutations/variants influence metastasis may be reconciled. It is likely that all previous reports are true but the we have failed to appreciate the complex *mtDNA* landscapes in which these reported *mtDNA* mutations/variants lie. This may mean, however, that finding driver *mtDNA* mutations in cancer may be nearly impossible. We propose that a nuclear gene  $UPR^{mt}$  signature can be used as a tool to bypass the complexity of *mtDNA* landscapes and our current inability to dissect the effect of a given *mtDNA* mutation/variant in cancer.

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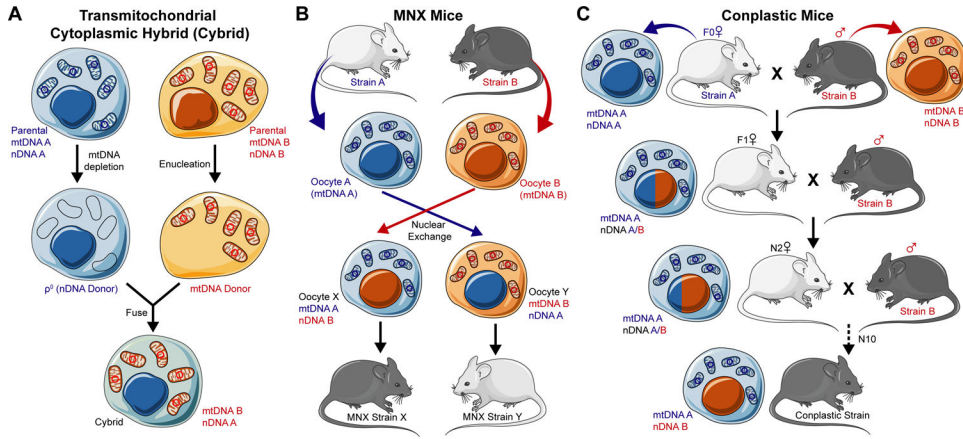


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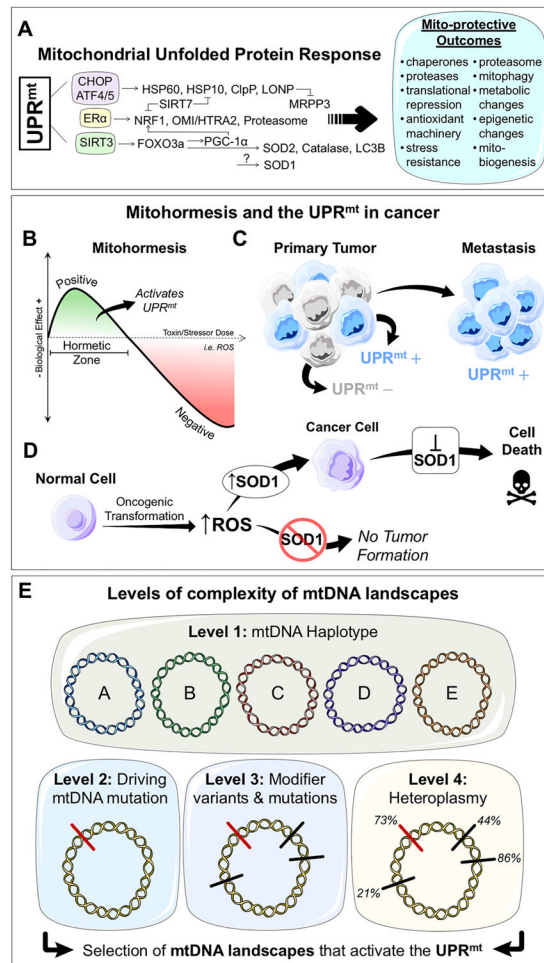
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**Figure 1. Cellular and animal models to study the role of mtDNA in cancer**

**A.** Transmittochondrial cytoplasmic hybrid (Cybrid) technology. Cells with nuclear DNA of interest (nDNA A) have mtDNA depleted to generate p<sup>0</sup> cells. Cells with mitochondrial DNA of interest (mtDNA B) are enucleated to generate an mtDNA donor. p<sup>0</sup> and the mtDNA are fused to generate a cybrid with nDNA A and mtDNA B. **B.** MNX mice are generated by enucleating the oocytes of inbred mouse strains (Strain A and B, oocytes A and B). Nuclei from these oocytes are exchanged into opposite oocytes generating oocyte X with mtDNA A and nDNA B and oocyte Y with mtDNA B and nDNA A. These oocytes are placed in pseudopregnant females to generate MNX strains X and Y. **C.** Conplastic mice are generated by the continual backcrossing of females with mtDNA of interest (mtDNA A) to male mice with nDNA of interest (nDNA B) for at least 10 generations until conplastic mice are generated with mtDNA A and nDNA B. (modified from LES LABORATOIRES SERVIER)<sup>TM</sup> available under a Creative Commons 3.0 Unported License.



**Figure 2. The mitochondrial unfolded protein response, mitohormesis, and mtDNA landscape complexity in cancer.**

**A.** The mammalian mitochondrial unfolded protein response (UPR<sup>mt</sup>). Different axes of the UPR<sup>mt</sup> are activated in parallel by perturbations to mitochondrial stress leading to collective mito-protective outcomes. **B.** Dose response curve of a mitohormetic response to a given stressor or toxin (i.e. ROS). At low levels of toxin/stressor, within the hormetic zone, positive biological responses are seen – such as activation of the UPR<sup>mt</sup>. At higher doses, negative biological responses are seen. **C.** Within the primary tumor, UPR<sup>mt</sup> negative (–) and positive (+) populations can be identified. UPR<sup>mt</sup>+ populations are characterized by mitohormesis and show increased invasive and metastatic capacities. **D.** The role of SOD1, a member of the UPR<sup>mt</sup>, in cancer. Oncogenic transformation of a normal cell results in the increase of reactive oxygen species (ROS). In the absence of SOD1, tumor formation is blocked. SOD1 inhibition selectively kills established cancer cells. **E.** Levels of complexity of mtDNA landscapes in cancer. Level 1 of complexity is the mtDNA haplotype with its unique set of variants and mutations. Level 2 of complexity is the acquisition of a driving mtDNA mutation in cancer. This mutation will manifest differently given the mtDNA haplotype in which it resides. Level 3 of complexity is the additional variants and mutants gained. These additional mtDNA differences can act as modifiers of both the driving mutation and the mtDNA haplotype. Level 4 of complexity is heteroplasmy. The

heteroplasmic frequencies of each driving mutation and modifier variants/mutations will also impact the effect of mtDNA on cancer. We propose that cancer cells will select mtDNA landscapes that activate the UPR<sup>mt</sup> to promote primary tumor formation and metastasis. (modified from LES LABORATOIRES SERVIER)<sup>TM</sup> available under a Creative Commons 3.0 Unported License.

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