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The second genome: Effects of the mitochondrial genome on cancer progression

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Abstract

The role of genetics in cancer has been recognized for centuries, but most studies elucidating genetic contributions to cancer have understandably focused on the nuclear genome. Mitochondrial contributions to cancer pathogenesis have been documented for decades, but how mitochondrial DNA (mtDNA) influences cancer progression and metastasis remains poorly understood. This lack of understanding stems from difficulty isolating the nuclear and mitochondrial genomes as experimental variables, which is critical for investigating direct mtDNA contributions to disease given extensive crosstalk exists between both genomes. Several in vitro and in vivo models have isolated mtDNA as an independent variable from the nuclear genome. This review compares and contrasts different models, their advantages and disadvantages for studying mtDNA contributions to cancer, focusing on the mitochondrial-nuclear exchange (MNX) mouse model and findings regarding tumor progression, metastasis, and other complex cancer-related phenotypes.

The vast majority of genetic studies in cancer focus on the nuclear genome, where numerous genetic and epigenetic alterations that contribute to tumorigenesis and metastasis have been characterized (Esquela-Kerscher & Slack, 2006; Goldberg et al., 2003; Lee et al., 1996; Lee & Welch, 1997; Phillips et al., 1996; Seraj, Samant, Verderame, & Welch, 2000; Steeg & Theodorescu, 2007; Weinstein & Joe, 2006; Welch et al., 1994). While nucleus-focused studies have provided critical insights into cancer initiation and progression, they, for the most part, have failed to consider the second genome harbored in eukaryotic cells: the mitochondrial genome. Otto Warburg's description of cancer cell aerobic glycolysis nearly a century ago set the stage for subsequent studies that have linked cancer and altered mitochondrial function (Brandon, Baldi, & Wallace, 2006; Chandra & Singh, 2011; Vyas, Zaganjor, & Haigis, 2016), but much has yet to be learned about mitochondrial contributions to cancer.

One of the challenging aspects in studying mitochondrial contributions to cancer is its inextricable link to the nuclear genome. At fewer than 17,000 base pairs, mitochondrial DNA (mtDNA) encodes only a fraction of the molecules required to carry out all the

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Conflict of interest

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physiological functions in which the organelle is involved (Pagliarini et al., 2008; Taanman, 1999). Many of the other molecules necessary for mitochondrial function are encoded in nuclear DNA (nDNA), and accordingly oncogenic mutations in nDNA have significant impacts on mitochondrial biology (Nagarajan, Malvi, & Wajapeyee, 2016). Therefore, in order to study direct contributions of mtDNA on cancer and metastasis, mtDNA and nDNA must be isolated as separate experimental variables. To isolate mtDNA from nDNA contributions in vivo, we generated mitochondrial nuclear exchange (MNX) mice, in which mtDNA from various mouse strains can be combined with nDNA of other mouse strains (Kesterson et al., 2016). In this review, we detail how aberrant mitochondrial function contributes to tumor progression and metastasis, and how MNX mice have been utilized to clarify novel roles of mtDNA in cancer and metastasis as well as complex cancer-related phenotypes.

1. Mitochondrial evolution and genetic variation

Phylogenetic analyses suggest that mitochondria originate from a bacterium that developed an endosymbiotic relationship with the ancient unicellular host that phagocytosed it (Andersson et al., 1998; Ferla, Thrash, Giovannoni, & Patrick, 2013; Fitzpatrick, Creevey, & McInerney, 2006; Sassera et al., 2011; Wang & Wu, 2015; Yang, Oyaizu, Oyaizu, Olsen, & Woese, 1985). Although the identity of the precise bacterial ancestor remains controversial (Martijn, Vosseberg, Guy, Offre, & Ettema, 2018), the remains of unique bacterial traits, such as formylated proteins (Carp, 1982; Zhang et al., 2010), make clear the roots of mitochondria in bacterial ancestry. Extensive co-evolution between mitochondria and their hosts have resulted in an organelle that is central not only to its canonical role in metabolism and energy production but also to cell signaling, regulation of apoptosis, and many other critical cellular functions as well (Chandel, 2014; Martinou & Youle, 2011).

Another relic from the mitochondrial bacterial ancestor is the relatively small, circular mtDNA genome. The genome, which is composed of ~16,500 base pairs in humans and 16,300 base pairs in mice, encodes for 22 tRNAs, 2 rRNAs, and 13 proteins (Anderson et al., 1981). These proteins are all part of the electron transport chain (ETC) that resides within mitochondria, and include NADH dehydrogenase (ND)1, ND2, ND3, ND4, ND4L, ND5, and ND6 (complex I); cytochrome B (CYB, complex III); cytochrome c oxidase (CO)I, COII, and COIII (complex IV); and ATP synthase subunits 6 and 8 (ATP6 and ATP8, complex V) (Chomyn et al., 1986, 1985; Macreadie et al., 1983). Other components of the ETC, as well as machinery for mtDNA replication, transcription, and other critical mitochondrial functions are encoded in nDNA.

Counterintuitively, given the importance of the ETC for cellular energy and viability, the mutation rate of mtDNA is relatively high (Brown, George, & Wilson, 1979; Parsons et al., 1997). This high mutation rate, however, serves as a mechanism by which selective pressures can induce evolutionary adaptations. Accordingly, selection of mtDNA variants within ancient human populations enabled evolutionary adaptations to the various climates that those populations encountered while migrating to different regions of the planet. The selective pressure of climate on mtDNA evolution is evident in phylogenetic analysis of human mtDNA variants, where related variants, known as haplogroups, cluster according to

geographic location (Balloux, Handley, Jombart, Liu, & Manica, 2009; Wallace, 2015). The first haplogroup, termed L0, originated in Africa, and the divergence of additional haplogroups began in Africa 130,000–170,000 years ago. Two haplogroups, M and N, diverged from the L3 haplogroup in Africa and went on to populate the rest of the world (Wallace, 2015). Divergence of mtDNA variants has been documented in wild and inbred laboratory mice as well, where introductions of the latter into relatively clean, controlled laboratory environments over the last century have likely played a major role in mtDNA evolution (Goios, Pereira, Bogue, Macaulay, & Amorim, 2007).

The necessity of mtDNA adaptive evolution to climate is a logical one, considering that different climates are accompanied by different metabolic demands. Indeed, haplotype-defining mtDNA variants alter mitochondrial functions in ways that would aid in adaptation to new climates. For example, coupling efficiency, the efficiency with which the ETC generates proton gradients for ATP production by complex V, varies by haplotype, where more efficient haplotypes burn fewer calories per ATP generated and vice versa. Therefore, haplogroups in which more heat is generated per ATP are advantageous in cold climates, while haplotypes with better coupling efficiencies are more beneficial in warm climates (Kazuno et al., 2006; Wallace, 2015).

While high mtDNA mutation rates confer advantages for evolutionary adaptation, they also have the potential to increase the risk of incurring mutations that damage the ETC. This risk is largely mitigated by mtDNA copy number and inheritance during cell division. Eukaryotic cells can contain thousands of mitochondria per cell, and within each mitochondrion resides several copies of mtDNA (Fernandez-Vizarra, Enriquez, Perez-Martos, Montoya, & Fernandez-Silva, 2011; Robin & Wong, 1988; Shuster, Rubenstein, & Wallace, 1988). Therefore, unlike nDNA mutations that persist in all cell progeny, mtDNA mutations are limited to single organelles. This results in mixtures of normal and mutant mtDNA copies in each cell, a phenomenon collectively known as heteroplasmy.

Heteroplasmy and the nature of mtDNA inheritance limit the effects of both germline and somatic mtDNA mutations. mtDNA is exclusively inherited from maternal oocytes. During mitotic cell division, mitochondria and the mtDNA copies they harbor are distributed randomly (and unevenly) among daughter oocytes, and rounds of division and mtDNA replication lead to oocytes with various heteroplasmic ratios (Wallace & Chalkia, 2013). Oocytes enriched with mtDNA mutations that alter global mitochondrial function in the cell are selectively eliminated in the ovary, preventing dissemination of the mutations (Fan et al., 2008; Stewart et al., 2008). Similarly, mtDNA with somatic mutations are often outnumbered by normal mtDNA and are further diluted during cell division.

Just as mtDNA variations in organismal populations allow adaptations to new environments, they also allow cancer cells to adapt to dynamic microenvironments during tumorigenesis and metastasis (Brandon et al., 2006; Wallace, 2012, 2016). Metabolic adaptations are critical to tumor cell growth, as tumorigenesis can produce extreme microenvironmental alterations involving hypoxia and limited availability of other nutrients. Metastasis can induce even more extreme changes, where tumor cells must adapt to entirely new extracellular environments in order to colonize distant tissues. As discussed below, these

adaptations to these dynamic microenvironments involve mutations in both mtDNA and nDNA.

2. Mitochondria and cancer

The importance of mitochondrial contributions to tumorigenesis and metastasis is underscored by recent observations that whole mitochondria are transferred from normal cells to tumor cells (Dong et al., 2017; Lu et al., 2017; Pasquier et al., 2013; Tan et al., 2015). Furthermore, Ishikawa and colleagues demonstrated that transferring mitochondria from tumor cells with high metastatic potential to tumor cells with low metastatic potential enhances the metastatic potential of the recipient cells and vice versa (Ishikawa et al., 2008). The interplay between oncogenic signaling and mitochondrial function is complex and, as described below, involves and extends beyond the role of mitochondria as the main energy producers of the cell.

2.1 The Warburg effect

Earliest indications of mitochondrial involvement in cancer came with Otto Warburg's discovery of aerobic glycolysis, commonly known as the Warburg effect, in cancer cells in the 1920s (Warburg, Wind, & Negelein, 1927). This discovery apparently contradicted Louis Pasteur's observation in 1861 that the presence of oxygen promotes more rapid cell division and inhibits fermentation in yeast, indicating that cancer cells displayed altered metabolism relative to healthy cells. Many groups have since described the Warburg effect in cancer cells, although it is not a universal trait among all tumor cell types (Dang, 2010; Herst & Berridge, 2007; Suganuma et al., 2010; Vander Heiden & DeBerardinis, 2017).

Warburg's explanation of aerobic glycolysis was predicated on the idea that mitochondrial oxidative phosphorylation (OXPHOS) was irreversibly defective in cancer cells, forcing the cells to perform glycolysis to produce ATP (Warburg, 1956a, 1956b). While impaired OXPHOS can underlie aerobic glycolysis (Lopez-Rios et al., 2007; Owens, Kulawiec, Desouki, Vanniarajan, & Singh, 2011), such defects are not necessarily irreversible. For example, Fantin et al. showed that knocking down lactate dehydrogenase A (LDH-A) stimulated ATP generation through OXPHOS in cancer cells that preferentially rely on aerobic glycolysis for energy production, demonstrating that neoplastic cells maintain the capacity to perform OXPHOS (Fantin, St-Pierre, & Leder, 2006). Furthermore, upregulated OXPHOS has been observed in multiple cancer types, further exemplifying that OXPHOS too can be utilized in oncogenic metabolism (Birkenmeier et al., 2016; Caro et al., 2012; Jones et al., 2016; Lagadinou et al., 2013; Viale et al., 2014; Whitaker-Menezes et al., 2011).

Many studies since Warburg's observations have provided mechanistic insights into aerobic glycolysis in cancer cells. Aerobic glycolysis provides a pathway through which cancer cells can continue to grow in hypoxic conditions. Hypoxic tumor microenvironments result from rapid tumor cell growth that can outpace vascularization (Chen et al., 2018; Semenza, 2013) and OXPHOS, due to reliance on oxygen reduction to produce ATP, is inefficient under hypoxic conditions. Although OXPHOS generates more ATP molecules per cycle ($n = 36$) than does glycolysis ($n = 2$), glycolysis produces ATP more rapidly than does OXPHOS,

which is another aspect of glycolysis that benefits rapidly dividing cells (Pfeiffer, Schuster, & Bonhoeffer, 2001).

Aerobic glycolysis, like many other aspects of tumor cells, can be heterogeneous, enabling dynamic metabolic symbiosis within growing tumors. One example of metabolic symbiosis through heterogeneous aerobic glycolysis can occur between tumor cells in vascularized, oxygenated microenvironments and tumor cells in hypoxic environments (Corbet et al., 2018). Here, oxygenated tumor cells undergo OXPHOS and allow glucose from circulation to travel to tumor cells in hypoxic regions. In return, lactate from the glycolytic tumor cells travels back to the tumor cells undergoing OXPHOS, in which it can be converted to pyruvate by LDH-B and enter the mitochondria for OXPHOS and ATP generation (Bonuccelli et al., 2010; Griguer, Oliva, & Gillespie, 2005). Similarly, tumor cells can induce a glycolytic phenotype in surrounding fibroblasts and use the resulting lactate for OXPHOS in a phenomenon known as the reverse Warburg effect (Bonuccelli et al., 2010; Pavlides et al., 2009).

In addition to providing a metabolic advantage to tumor cells, aerobic glycolysis can modulate the microenvironment in ways that promote tumor progression and metastasis. For example, excess lactate production results in an acidic microenvironment, which has been shown to promote invasion and metastasis (Gatenby & Gawlinski, 1996). While tumor cells can accrue mutations to persist in such an acidic microenvironment, other cells that could limit tumor growth, such as immune cells, undergo apoptosis in low pH microenvironments (Park, Lyons, Ohtsubo, & Song, 1999). Similarly, high rates of glucose uptake for glycolysis competitively inhibit functionality of activated T cells that also undergo glycolysis, preventing elimination of tumor cells by the immune system (Cham, Driessens, O'Keefe, & Gajewski, 2008; Chang et al., 2013; Macintyre et al., 2014).

Multiple mutations and epigenetic alterations underlying the Warburg effect have been described. One such mechanism is hexokinase II expression in hepatomas, as opposed to hexokinase IV expression that occurs in normal hepatic cells. Hexokinase II has a lower K_m than hexokinase IV, and can reside on the outer mitochondrial membrane (OMM) where it can use outgoing OXPHOS-generated ATP to phosphorylate glucose to glucose-6-phosphate and initiate glycolysis (Bustamante & Pedersen, 1977). Impairment of OXPHOS due to mutations in nDNA and mtDNA-encoded ETC components can drive aerobic glycolysis in cancer cells as well. Mutations in the mtDNA displacement (D)-loop, a major control locus for mtDNA replication and transcription (Taanman, 1999), downregulate transcription of mtDNA-encoded ETC components by lowering mtDNA copy number and transcription efficiency (Coskun, Beal, & Wallace, 2004; Lee et al., 2004). Similarly, mutations in mtDNA and nDNA genes encoding proteins involved in mtDNA maintenance and the ETC abrogate OXPHOS (Gasparre et al., 2007; Lopez-Rios et al., 2007; Owens et al., 2011; Singh, Ayyasamy, Owens, Koul, & Vujcic, 2009).

2.2 Reactive oxygen species

Mitochondria, in addition to NADPH oxidase, are the major source of reactive free radicals in cells. These include reactive oxygen species (ROS), a misnomer which also encompasses hydrogen peroxide as well as superoxide and hydroxide radicals, as well as reactive nitrogen

species, such as nitric oxide and nitric dioxide radicals (Wiseman & Halliwell, 1996). ROS, in particular, are generated frequently in mitochondria as byproducts of OXPHOS, where electron carriers of the ETC can transfer unpaired electrons in oxygen in the mitochondrial matrix. Mitochondria are equipped with a number of nDNA-encoded antioxidants, including superoxide dismutase, glutathione, thioredoxin, and peroxiredoxins, to protect mitochondrial DNA, proteins, and lipids from ROS-mediated oxidative damage (Hanschmann, Godoy, Berndt, Hudemann, & Lillig, 2013).

Increased ROS levels are frequently seen in cancer cells and, as with aerobic glycolysis, increases are often due to OXPHOS impairment. More specifically, mutations of ETC components that abrogate the flow of electrons through the ETC often induce increased levels of ROS (Mattiuzzi et al., 2004). These mutations can impact the ability of ETC complexes to accept electrons, leaving the electrons on electron carriers and making them available for ROS generation (Ishii et al., 1998; Senoo-Matsuda et al., 2001).

ROS can augment tumor progression and metastasis through oxidative damage of macromolecules. mtDNA, given its proximity to ROS and lack of protection by histones, is particularly susceptible to ROS-mediated oxidative damage, and resulting mutations can contribute to increased ROS production and aerobic glycolysis as discussed above (Liemburg-Apers, Willems, Koopman, & Grefte, 2015; Lu, Sharma, & Bai, 2009). nDNA can sustain oxidative damage by ROS as well, which can result in mutations with the potential to further promote tumor progression and metastasis (Sallmyr et al., 2008).

In addition to its role as a genomic mutagen, ROS can also promote tumorigenesis by acting as mitogenic signaling molecules. More specifically, hydrogen peroxide is required for multiple signaling pathways that stimulate cell growth and division, including those involved in cytokine, insulin, and growth factor signaling as well as NF- κ B signaling (Chandel, Trzyna, McClintock, & Schumacker, 2000; Krieger-Brauer & Kather, 1992; Lo & Cruz, 1995; Schreck, Rieber, & Baeuerle, 1991; Sundaresan, Yu, Ferrans, Irani, & Finkel, 1995). ROS-mediated signal augmentation is achieved through oxidation of thiol-containing cysteine residues within protein tyrosine phosphatase active sites, which promotes phosphorylation and activation of signaling molecules (Cunnick, Dorsey, Mei, & Wu, 1998; Denu & Tanner, 1998; Lee, Kwon, Kim, & Rhee, 1998). Depending on the target proteins, such alterations can provide mitogenic signals to promote tumor growth. Indeed, ROS-mediated oxidation of cysteine residues in proteins such as PTEN and Src provides mitogenic signals in cancer (Lee et al., 2002; Leslie et al., 2003).

While limited ROS concentrations can provide oncogenic signals through mutagenic and mitogenic means, excessive ROS concentrations and corresponding oxidative damage can induce cell death (Redza-Dutordoir & Averill-Bates, 2016). Therefore, tumor cells must maintain a balanced concentration of ROS to take advantage of their oncogenic properties without undergoing apoptosis. To achieve this balance, expression of antioxidant pathway molecules is often upregulated in tumor cells (DeNicola et al., 2011). This point is particularly important in metastasis, where treatment with antioxidants has actually been shown to cause increased metastatic efficiency, presumably due to attenuation of increased

ROS levels associated with the metabolic demands of metastasis (Le Gal et al., 2015; Piskounova et al., 2015).

2.3 Oncometabolites

Just as nDNA-encoded components are instrumental for mtDNA maintenance and mitochondrial function, so too are mtDNA and mitochondrial functions critical for epigenetic nDNA organization. nDNA organization is in large part mediated by specific chemical modifications to deoxynucleotides as well as histone tails, where dynamic enzymatic modifications allow chromatin loosening and condensation based on the transcriptional requirements of the cell. These reactions require availability of substrates such as acetyl groups, which are in part provided by mitochondrial metabolism. For example, acetyltransferases can acquire acetyl groups from acetyl-coenzyme A (CoA), which is generated as a substrate for the tricarboxylic acid (TCA) cycle in mitochondria (Wellen et al., 2009).

In addition to participating in crosstalk with the nucleus in normal physiological states, mitochondrial metabolites function in oncogenic mitochondrial-nuclear crosstalk. The most well-characterized examples of this crosstalk involve molecules that structurally resemble α -ketoglutarate (α -KG), a metabolite in the TCA cycle. Aside from the TCA cycle α -KG serves as a substrate for dioxygenases, a superfamily of enzymes whose functions include epigenetic modifications and chromatin remodeling (Gerken et al., 2007). Mutations in isocitrate dehydrogenase, succinate dehydrogenase, and fumarate hydratase, which have been documented in several cancers (Alam et al., 2005; Baysal et al., 2000; Gimm, Armanios, Dziema, Neumann, & Eng, 2000; Parsons et al., 2008), result in excesses of metabolites that resemble α -KG (2-hydroxyglutarate, succinate, and fumarate, respectively). These metabolites can compete with α -KG for binding at dioxygenase active sites, inhibiting dioxygenase function. In some cancers, this results in epigenetic silencing of gene expression via hypermethylation at CpG islands (Noushmehr et al., 2010).

2.4 Mitochondrial morphology

Mitochondria, rather than existing as static organelles, exhibit dynamic morphologies that are dependent upon cellular status. Mitochondrial morphology is governed by two main processes: fusion and fission. Fusion is mediated by GTPases mitofusin 1 (Mfn1), Mfn2, and optic atrophy 1 (Opa1), where Mfn1 and 2 facilitate OMM fusion and Opa1 facilitates inner mitochondrial membrane (IMM) fusion to merge multiple mitochondria into a single, tubular organelle (Cipolat, Martins de Brito, Dal Zilio, & Scorrano, 2004; Santel & Fuller, 2001). Fission is mediated by dynamic related protein 1 (Drp1), a GTPase that binds receptors on the OMM and constricts the membrane to fragment a single mitochondrial network into multiple organelles (Smirnova, Shurland, Ryazantsev, & van der Bliek, 1998).

Mitochondrial structure has major impacts on mitochondrial function, and as such mitochondrial structure is dependent on the needs of the cell. Mitochondrial fusion is generally associated with OXPHOS, which is demonstrated by the observation that cells in nonfermentable conditions tend to display elongated mitochondrial networks (Egner, Jakobs, & Hell, 2002; Rossignol et al., 2004). These networks are thought to occur because fusion

results in relatively large numbers of mtDNA copies in mitochondrial networks, enhancing production of mtDNA-encoded components of the ETC (Chen, Chomyn, & Chan, 2005; Chen et al., 2010). Oxidative stress also promotes mitochondrial fusion, perhaps as a mechanism to disperse ROS that are produced as byproducts of OXPHOS (Shutt, Geoffrion, Milne, & McBride, 2012). Conversely, mitochondrial fission is typically observed upon inhibition of OXPHOS, and fission decreases OXPHOS coupling efficiency (Wikstrom et al., 2014).

Aberrations in mitochondrial morphology have been reported in cancer cells. Skewing toward mitochondrial fission is often seen in cancer cells (Hagenbuchner, Kuznetsov, Obexer, & Ausserlechner, 2013; Inoue-Yamauchi & Oda, 2012; Kashatus et al., 2015; Rehman et al., 2012; Wan et al., 2014; Zhao et al., 2013), a logical observation given that OXPHOS is downregulated in many tumor cell types and fission is associated with OXPHOS inhibition (Wikstrom et al., 2014). While mitochondrial fission is not a universal trait of tumor cells (von Eyss et al., 2015), it can be critical to tumor progression and metastasis, as Drp1 inhibition and Mfn2 overexpression can impair tumor cell growth (Inoue-Yamauchi & Oda, 2012; Rehman et al., 2012) and upregulated Drp1 expression has been associated with a migratory phenotype in tumor cells (Ferreira-da-Silva et al., 2015).

The impacts of morphology on mitochondrial function extend beyond metabolism. Mitochondrial fission confers several other advantages to tumor cells. One advantage is resistance to apoptosis, a hallmark of tumor cells. Mitochondria play integral roles in initiation of apoptosis. In response to various signals, pro-apoptotic Bcl-2 family members Bax and Bak oligomerize to induce OMM permeabilization (MOMP) which, in turn, results in cytochrome c release into the cytoplasm, leading to activation of proteolytic caspases that execute apoptotic pathways. In addition to upregulated expression of anti-apoptotic members of the Bcl-2 family (Strasser, Harris, Bath, & Cory, 1990; Tsujimoto, Finger, Yunis, Nowell, & Croce, 1984), increased mitochondrial fission functions as a mechanism by which tumor cells escape apoptosis, where hyperfragmentation inhibits Bax interactions with the OMM (Renault et al., 2015).

Another mechanism by which skewed mitochondrial fission can be oncogenic is through induction of increased mitophagy. Mitophagy is the process by which mitochondria are cleared from the cell, often due to damage and dysfunction. Dysfunctional mitochondria tend to have depolarized membranes due to inability of the ETC to generate proton gradients across the IMM, and this depolarization allows Pink1 kinase to accumulate at the OMM and phosphorylate mitochondrial surface proteins (Matsuda et al., 2010; Narendra et al., 2010). This results in recruitment and activation of Parkin (Okatsu et al., 2015), an E3 ligase that ubiquitinates mitochondrial surface proteins, resulting in degradation of the proteins and targeting of the mitochondria for autophagic membranes (Chan et al., 2011; Sarraf et al., 2013). Fission can potentiate mitophagy simply by decreasing mitochondrial size, and increased mitophagy can help established tumors adapt to new environments and engender therapeutic resistance (Hu et al., 2012). The relationship between mitophagy and cancer is complex, however, as mitophagy can also inhibit tumor growth (Lee et al., 2012; Tay et al., 2010), indicating that mitophagy can be pro- or anti-tumorigenic depending on the dynamic needs of the tumor.

Mitochondrial spatial dynamics are critical to tumor cell progression and metastasis as well (Attanasio et al., 2011; Caino et al., 2016; Desai, Bhatia, Toner, & Irimia, 2013). One of the most well-documented examples was demonstrated by Altieri and colleagues, who showed that Akt reactivation in tumor cells treated with a phosphoinositide 3-kinase (PI3K) inhibitor results in translocation of mitochondria to the cortical cytoskeleton (Caino et al., 2015). This translocation resulted in increased lamellipodia dynamics and focal adhesion complex turnover, which combined to augment tumor cell migration and invasion (Caino et al., 2015). Notably, Mfn1 and OXPHOS inhibition ameliorated both mitochondrial translocation as well as increased tumor cell migration and invasion, suggesting respiration is critical for both organellar translocation as well as the resulting phenotypes (Caino et al., 2015). Mitochondrial translocation is also dependent on syntaphilin (SNPH), where ubiquitinated SNPH inhibits mitochondrial dynamics and translocation (Caino et al., 2016; Seo et al., 2018).

2.5 mtDNA mutations and haplotype predispositions

Interestingly, the mtDNA mutations that contribute to the altered, oncogenic functionality of mitochondria described above can occur throughout the mitochondrial genome. Thus, a somatic mutation in a region defining a particular haplotype can “convert” the sequence to another haplotype, thereby possibly confusing interpretation of haplotype-dependent susceptibility (Brandon et al., 2006, 2005). While incidence of the latter observation may be overestimated due to sequencing errors, as parallel sequencing of normal and tumor tissue from the same individual is not performed in most studies, its occurrence has been definitively demonstrated (Parrella et al., 2001). Large insertions or deletions that give rise to changes in conserved amino acids can have drastic impacts on mtDNA and mitochondrial function, e.g., truncation of ETC components that may be important for early tumorigenesis. Mutagenic conversions that match non-self mtDNA haplotype sequences, however, may be important for metabolic adaptations to dynamic tumor microenvironments, much like divergent mtDNA haplotypes were important for adaptations to new climates in ancient peoples.

Understanding how mtDNA haplotype variants contribute to tumorigenesis and cancer progression is important for two main reasons. One reason is that individuals with particular mtDNA haplotypes have increased predispositions for developing certain cancers relative to individuals with other mtDNA haplotypes (Brinker et al., 2017; Bussard & Siracusa, 2017; Feeley et al., 2015). Indeed, adaptive advantages that mtDNA variants confer can also resemble oncogenic mitochondrial function discussed above (Ross et al., 2001; van der Walt et al., 2003). Better understandings of these predispositions can enable more effective cancer screening and prevention. The second reason why a better understanding of how mtDNA variants contribute to cancer is critical is because it can precipitate development of therapeutic interventions that block the ability of tumors to adapt to changing microenvironments, which may halt tumor growth and prevent therapeutic resistance.

Since nDNA-encoded components are instrumental to mtDNA maintenance and mitochondrial function, querying direct contributions of mtDNA to cancer requires separating nDNA and mtDNA as isolated variables. As outlined below, there are several

ways these variables can be isolated in vitro in mouse and man. However, corresponding in vivo studies in humans present major ethical barriers. These studies can be performed in vivo in mouse models, but traditional backcrossing on female genetic backgrounds to obtain conplastic mice with nDNA from one mouse strain and mtDNA from another can introduce confounding recombinations in nDNA. To address this issue, we generated MNX mice by exchanging embryonic pronuclei among mouse strains (Fetterman et al., 2013; Kesterson et al., 2016), and the resulting model has enabled and will continue to enable novel insights on direct mtDNA contributions to cancer and other complex phenotypes that can interact with the disease.

3. Studying direct mtDNA contributions to disease

The unique characteristics of mtDNA relative to its nuclear counterpart make mtDNA genetic manipulation and corresponding functional studies challenging. For example, the presence of hundreds to thousands of mtDNA molecules within single cells makes alteration of all mtDNA copies extremely difficult. Therefore, traditional approaches to mtDNA engineering would likely result in heteroplasmy, and random distribution of altered mtDNA among daughter cells would produce variable heteroplasmic ratios among manipulated cells, confounding effects of the alteration.

Although methods for mtDNA genetic engineering are being explored (Gammage, Rorbach, Vincent, Rebar, & Minczuk, 2014; Hashimoto et al., 2015; Jo et al., 2015; Patananan, Wu, Chiou, & Teitell, 2016; Reddy et al., 2015; Trifunovic et al., 2004), existing in vitro and in vivo models for studying mtDNA contributions to disease are predicated on transferring mtDNA molecules with polymorphisms/mutations of interest to cells with the same nDNA as cells containing control mtDNA. These models have enabled studies elucidating mtDNA contributions to mitochondrial physiology and disease such as those discussed above. Current efforts, including our generation of the MNX mouse model, are focused on improving these models, with the goals of facilitating a better understanding of mtDNA biology and harnessing gained knowledge to develop improved therapies for diseases to which mtDNA contributes.

3.1 Cybrids

One of the first major innovations that enabled interrogation of direct mtDNA impacts on phenotypic traits was the development of cytoplasmic hybrid, or cybrid, cells. Cybrids are generated by fusing nucleated cells with enucleated cells (often platelets), resulting in transfer of cytoplasmic contents of the enucleated cell, including mtDNA, to the nucleated cell. This is as opposed to hybrid cells, which are the products of fusing nucleated cells.

The first cybrid cells that were produced were generated using nucleated cells that were replete with mtDNA, resulting in heteroplasmic mixtures of enucleated cell and nucleated cell mtDNA (Bunn, Wallace, & Eisenstadt, 1974). While cybrids have proven useful (Wallace, Bunn, & Eisenstadt, 1975), more direct experiments querying how mtDNA sequence variants influenced phenotypic traits required generation of cybrids homoplasmic for those mtDNA sequences. This was accomplished by the development of rho-null (ρ^0) cells, which are nucleated cells lacking a mitochondrial genome.

The feasibility of mtDNA depletion was demonstrated by the observation that yeast naturally reduced mtDNA copy numbers under conditions that favored glycolysis over OXPHOS (Wilkins, Carl, & Swerdlow, 2014). Subsequently, several methods by which mtDNA can be artificially depleted from cells have been developed. The first was incubation with ethidium bromide (EtBr), a positively charged compound that can enter negatively charged mitochondrial matrices and intercalate into mtDNA to inhibit its replication. After several decades of work the first human ρ^0 cell line was derived from the 143B osteosarcoma cell line in 1989 (King & Attardi, 1989). Since that time additional human ρ^0 cell lines, and comparatively less toxic methods to generate them, have been produced as reviewed elsewhere (Wilkins et al., 2014).

Rather than outright destroying mtDNA, agents used to generate ρ^0 cell lines inhibit its replication. Therefore, as cell division progresses in the presence of the agent, mtDNA is diluted among daughter cells until no mtDNA remains. Mitochondrial remnants can still be found in ρ^0 cells, but their functionality is severely altered (Swerdlow et al., 1996). Most notably, since they lack the mtDNA-encoded components of the ETC they are incapable of OXPHOS and rely solely on glycolysis for energy production. In addition, upon complete depletion of mtDNA, human ρ^0 cells become auxotrophic for uridine and pyruvate due to lack of ETC function and glycolytic oxidation-reduction requirements, respectively (Gregoire, Morais, Quilliam, & Gravel, 1984; King & Attardi, 1989, 1996).

The use of ρ^0 cell lines has been critical for clarifying many aspects of mitochondrial biology, from establishing potential links between mtDNA sequence variants and phenotypes to uncovering how mitochondrial dysfunction contributes to various diseases. For example, cybrid studies using mtDNA from patients with Leber's hereditary optic neuropathy (LHON), which is associated with mutations in mtDNA ND genes encoding complex I components, revealed connections between mutant mtDNA and deficiencies in oxygen consumption and complex I function (Baracca et al., 2005; Jun, Trounce, Brown, Shoffner, & Wallace, 1996). Cybrid studies have been useful for investigating mtDNA contributions to nonclassical mitochondrial diseases as well. Such is the case with Parkinson's disease, for which cybrid studies have demonstrated that mtDNA is at least partially responsible for decreases in complex I function that have been documented in the disease (Esteves et al., 2008, 2010; Gu, Cooper, Taanman, & Schapira, 1998; Swerdlow et al., 1998).

Cybrid studies have also been instrumental for demonstrating how nDNA and mtDNA backgrounds (as well as crosstalk between nDNA and mtDNA) influence phenotypic manifestations of mitochondrial diseases. For example, cybrids have been used to show that variable OXPHOS kinetics among mtDNA haplotypes contribute to discrepant susceptibilities of those haplotypes to LHON (Pello et al., 2008). In addition, the potency with which the mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS)-associated A3243G inhibits cytochrome oxidase function is dependent on the nDNA background of the ρ^0 cell line used to study it (Dunbar, Moonie, Jacobs, & Holt, 1995). Furthermore, Picard et al. elucidated a molecular mechanism underlying the heterogeneity with which MELAS presents clinically by using cybrid cell lines to show that

various heteroplasmic ratios of the A3243G mutation resulted in differential transcriptomic profiles from both nDNA and mtDNA (Picard et al., 2014).

While cybrids have proven to be useful tools for studying mitochondrial biology and mtDNA contributions to disease, they do have several important limitations. In addition to the caveat of all in vitro models, namely that observations may not recapitulate what occurs in normal physiological settings, only limited ρ^0 cell lines have been created and performing experiments in the most relevant cell type may be challenging (Wilkins et al., 2014). Most ρ^0 cell lines that have been created are derived from tumor cells as well, which may further confound results due to nDNA instability and corresponding potential expression level alterations of nDNA-encoded mitochondrial components (as well as other nDNA-encoded genes). Finally, although it is not an issue limited to cybrid models, it is extremely difficult to associate single nucleotide polymorphisms (SNP) with phenotypic observations. mtDNA tends to harbor variability in wide ranges of nucleotides among individuals, and mtDNA SNPs are extremely difficult to isolate given the challenges associated with mtDNA engineering. This lack of ability to isolate SNPs is confounding because, as demonstrated using cybrid models themselves (Pello et al., 2008), the functional consequences of SNP are influenced by the mtDNA haplotypes in which they exist. Microheteroplasmy, which is the presence of relatively small proportions (1–2%) of mutant mtDNA molecules that can be difficult to detect, can influence phenotypic traits as well (Smigrodzki & Khan, 2005). These limitations notwithstanding, cybrids still serve as valuable tools for understanding mtDNA biology.

3.2 Transmitochondrial mouse models

To enable examination of mtDNA contributions to disease in vivo, mtDNA transgenic mice were created. Transmitochondrial mice, often referred to as “mito-mice,” were first generated using microinjection of mitochondria containing mtDNA of interest into zygotes followed by implantation into nDNA-matched females (Irwin, Johnson, & Pinkert, 1999; Pinkert, Irwin, Johnson, & Moffatt, 1997). Subsequent techniques harnessed advancements that were used in the generation of cybrids and ρ^0 cells. More specifically, ρ^0 embryonic stem (ES) cells were made using rhodamine 6G, fused with cytoplasts containing the mtDNA of interest, and either injected into zygotes or co-cultured with blastocysts, after which the chimeric germ cells were implanted into nDNA-matched females (Inoue et al., 2000; Irwin et al., 1999; Marchington, Barlow, & Poulton, 1999; Sligh et al., 2000). Although first generation progeny from each of these methods contained heteroplasmic ratios of transgenic and endogenous mtDNA, homoplasmic transmitochondrial mice were attainable by breeding transmitochondrial females with nDNA-matched males (Sligh et al., 2000).

Given the ability to transfer any desired mtDNA into ρ^0 mouse ES cells, transmitochondrial mice provide an ideal model to study physiological effects of pathogenic mtDNA. Several homoplasmic transmitochondrial mouse models have been successfully created, the first being mice containing mtDNA conferring chloramphenicol resistance (CAP^R) (Levy, Waymire, Kim, MacGregor, & Wallace, 1999; Marchington et al., 1999). These mice were derived by fusing CAP^R cytoplasts harboring the T2433C mtDNA 16S rRNA mutation with

mouse ES cells in culture (Blanc, Wright, Bibb, Wallace, & Clayton, 1981; Bunn et al., 1974; Levy et al., 1999), and the resulting progeny displayed striking pathogenic phenotypes including growth retardation and in utero or perinatal lethality (Sligh et al., 2000). Similarly, transmitochondrial mice containing mtDNA with a 4696 base pair mtDNA deletion have been generated, and these mice display mitochondrial dysfunction in multiple tissues prior to mortality, often due to renal failure (Inoue et al., 2000).

Another way in which the transmitochondrial mouse model has been used to study effects of mtDNA variants is through generation of xenomitochondrial mice. Several xenocybrids, including human ρ^0 cells containing primate mtDNA or mouse ρ^0 cells containing *Rattus norvegicus* mtDNA, have been created (Dey, Barrientos, & Moraes, 2000; Kenyon & Moraes, 1997; McKenzie & Trounce, 2000; Yamaoka et al., 2000). Each displayed significant OXPHOS defects, likely owing to incompatibility of mismatched nDNA and mtDNA-encoded ETC components (Barrientos, Kenyon, & Moraes, 1998; Dey et al., 2000; McKenzie & Trounce, 2000; Yamaoka et al., 2000). McKenzie et al. combined the xenocybrid and transmitochondrial mouse models to generate xenomitochondrial mice, in which mtDNA from *Mus spretus* and *Mus dunni* mice was transferred to ρ^0 *Mus musculus domesticus* ES cells (McKenzie, Trounce, Cassar, & Pinkert, 2004). OXPHOS was largely unaltered in the xenomitochondrial mice, but increased glycolysis indicated that the xenomitochondrial cells were more glycolytic than their wild-type counterparts (McKenzie et al., 2004).

Despite the great potential of transmitochondrial mouse models, their utility has been limited by one major factor: a paucity of mutant mtDNA sequences to study. Few natural murine pathogenic mtDNA mutations are known, and current inability to engineer pathogenic mtDNA mutations hampers the ability to create pathogenic mtDNA. This may soon change, as random mtDNA mutagenesis has been achieved through homozygous knock-in of a proofreading-deficient mtDNA polymerase γ subunit, Polga (Trifunovic et al., 2004), and directed mtDNA mutagenesis using mitochondria-targeted restriction endonucleases, transcription activator-like effector nucleases (mitoTALEN), zinc finger nucleases (mtZFN), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 hold promise (Gammage et al., 2014; Hashimoto et al., 2015; Jo et al., 2015; Reddy et al., 2015).

3.3 Conplastic mouse models

The most traditional genetic way in which to study the physiological consequences of mtDNA variation is the conplastic mouse. Leveraging exclusive maternal inheritance of mtDNA, conplastic mice are generated by breeding female mice with mtDNA of interest with male mice harboring the nDNA background of interest. Female F₁ progeny, containing the desired mtDNA and equal contributions of nDNA from maternal and paternal sources, are then backcrossed with male mice containing the original paternal nDNA. This cross is performed for at least 10 generations, resulting in conplastic mice with mtDNA from one inbred mouse strain and 99.9% nDNA from another (Markel et al., 1997).

Rather than studying the effects of known pathogenic mtDNA in vivo as with transmitochondrial mice, conplastic mouse models examine how natural variations in mtDNA impact mitochondrial function and downstream phenotypes. Such a model is highly

relevant to the human condition, where divergent mtDNA haplotypes differentially influence mitochondrial function and can consequentially predispose individuals to disease (Canter, Kallianpur, Parl, & Millikan, 2005; Liu et al., 2003). Ibrahim and colleagues, in an exhaustive phylogenetic study, demonstrated that mtDNA divergence has also occurred in inbred laboratory mouse strains, where 50 of 52 strains tested contained mtDNA that diverged from a single *Mus musculus domesticus* female ancestor (Yu et al., 2009). These mtDNA variants, although not directly analogous to human mtDNA haplotypes, provide a platform with which conplastic models can be used to understand how mtDNA variants influence mitochondrial function and relevant phenotypes in vivo.

Conplastic mice have been used to demonstrate previously unappreciated mtDNA contributions to complex phenotypic traits as well as diseases. For example, using conplastic mice with mtDNA from the FVB/NJ and NZB/BlNJ strains on the C57BL/6J nDNA background alongside wild-type C57BL/6 mice, Hirose et al. demonstrated that a SNP in ATP8, the only SNP between FVB/NJ and C57BL/6J mtDNA, altered microbial profiles present in the intestine (Hirose et al., 2017). While previous associations between human mtDNA haplotypes and intestinal microbiota had been established (Ma et al., 2014), the conplastic model allowed for a direct association without the confounding factor of disparate nDNA backgrounds. The relevance of conplastic models to the human condition has also been demonstrated by the observation that various mouse mtDNA backgrounds impart differential susceptibility to experimental autoimmune encephalomyelitis (EAE) (Yu et al., 2009). EAE is a model that recapitulates the etiology and symptoms of multiple sclerosis (MS) (Constantinescu, Farooqi, O'Brien, & Gran, 2011), and consistent with the finding that mtDNA influences EAE in conplastic mice human mtDNA variants have been associated with MS susceptibility (Yu et al., 2008).

Conplastic mice also provide a useful model for understanding the consequences of mtDNA replacement, which is critical given recent interest in using mitochondrial replacement to prevent mitochondrial diseases in humans (Craven et al., 2010; Ma et al., 2015). Accordingly, a comprehensive study by Latorre-Pellicer et al. demonstrated how complex the ramifications of mtDNA replacement are, as a multitude of phenotypes, most notably those involving metabolism and aging, were altered upon replacement of C57BL/6JOLA^{Hsd} mtDNA with that of NZB/Ola^{Hsd} on the C57BL/6JOLA^{Hsd} nDNA background (Latorre-Pellicer et al., 2016). Such changes are indicative of the complexity involved in nuclear-mitochondrial crosstalk. Changes in sperm motility among conplastic strains independent of ATP production or polymorphism load (number of SNP per mtDNA) exemplify this complexity as well (Tourmente et al., 2017).

Although conplastic mouse models largely eliminate disparate nDNA backgrounds as confounding variables in mtDNA studies, numerous backcrosses necessary to derive the mice are accompanied by a higher probability for introduction of nDNA recombination that muddles comparisons between conplastic mice and their wild-type counterparts. The backcrosses are also relatively time-consuming, which can make studies using conplastic strains long and expensive.

3.4 The MNX mouse model

To circumvent the issues associated with the approaches above, we generated MNX mice. Unlike transmitochondrial and conplastic mouse models, which require cybrids and extensive backcrossing, respectively, MNX mice with unaltered nDNA (i.e., not exposed to mutagens) and homoplasmic mtDNA (also not mutagen-exposed) are generated relatively rapidly via pronuclear transfer.

To begin, super-ovulated dams with the desired mtDNA are mated with nDNA-matched males. The resulting embryos are harvested from the oviducts, pronuclei are isolated from embryos of each strain using micropipettes, and the extracted pronuclei are transferred to embryos of the other strain. The embryos are then implanted into pseudopregnant females and brought to term, after which F₁ females are bred with nDNA-matched males to propagate the MNX strain. Successful mtDNA transfer and homoplasmy are evaluated using restriction fragment length polymorphisms (Kesterson et al., 2016).

In addition to the relatively rapid time in which MNX mice can be generated, the major advantage that the MNX model offers over transmitochondrial and conplastic models is reduced potential for introducing confounding variables. For example, many of the transmitochondrial mouse models that have been created remain heteroplasmic, which makes direct attributions of observed phenotypes to a particular mtDNA sequence impossible. Off-target effects from using rhodamine 6G to generate cybrids for transmitochondrial mouse production may influence observed phenotypes as well. In conplastic models extensive backcrossing involves nDNA recombination that could alter nuclear-mitochondrial crosstalk, which, in addition to mtDNA variants, could influence observed phenotypes. Thus, we believe that the MNX model has significant advantages for probing novel aspects of mtDNA and mitochondrial biology.

The impetus for generating the MNX mouse model was rooted in a study by Kent Hunter and colleagues that aimed to determine the impact of genetics on tumor latency and metastatic efficiency (Lifsted et al., 1998). They crossed females from 27 different inbred mouse strains to male FVB/N mice harboring a transgene encoding the oncogenic polyomavirus middle T antigen (PyMT) under the control of the mouse mammary tumor virus (MMTV) promoter (FVB/N-TgN(MMTV-PyMT)). Expression of the oncogenic transgene results in spontaneous mammary gland tumors that readily metastasize to the lungs, providing an excellent model for studying both tumor growth and dissemination (Guy, Cardiff, & Muller, 1992). Crossing the inbred strains with the FVB/N-TgN(MMTV-PyMT) mouse revealed striking ranges in latency to tumor outgrowth as well as metastatic burden among the strains, demonstrating the influence of genetic factors on tumor progression and metastasis (Lifsted et al., 1998).

Since establishing a role for mouse genetic backgrounds in tumor latency and metastatic efficiency, Hunter and colleagues employed backcrossing and global genetic screens to identify nDNA-encoded metastasis modifiers, (Faraji et al., 2014; Ha, Long, Cai, Shu, & Hunter, 2016). These modifiers exemplify the complexities of the biological processes underlying metastasis, as they encode proteins whose functions range from immune cell interactions to circadian rhythm maintenance (Faraji et al., 2014, 2012; Ha et al., 2016).

Importantly, the results have also uncovered homologous metastasis modifiers in human breast cancer, demonstrating the relevance of the model to human disease (Herschkowitz et al., 2007; Hsieh, Look, Sieuwerts, Foekens, & Hunter, 2009; Pfefferle et al., 2013).

Given maternal transmission of mtDNA and the Hunter group's original experimental design, crossing females from various strains to male FVB/N-TgN(MMTV-PyMT) mice, we hypothesized that mtDNA was contributing to the observed phenotypes as well. To test this hypothesis, we crossed male FVB/N-TgN(MMTV-PyMT) mice with wild-type female FVB/NJ mice (FF mice, Table 1) as well as two female MNX strains containing FVB/NJ nDNA: one with mtDNA from C57BL/6J mice and another with mtDNA from BALB/cJ mice (FC and FB mice, respectively, Table 1) (Feeley et al., 2015). Our results were strikingly similar to those reported by Lifsted et al., where FC mice displayed longer tumor latency and lower metastatic burden and FB mice had shorter tumor latency and more metastatic burden than FF mice (Table 1) (Feeley et al., 2015; Lifsted et al., 1998). This observation, for the first time in a spontaneous tumor model, showed that mtDNA does indeed influence tumor progression and metastasis.

It is important to emphasize that mtDNA polymorphisms are likely metastasis *modifiers* rather than drivers *per se*. mtDNA encodes quantitative trait loci (QTL) that combine with both nuclear and mitochondrially encoded genes to regulate complex diseases like cancer and disease severity (Cookson, Liang, Abecasis, Moffatt, & Lathrop, 2009; Hunter, Amin, Deasy, Ha, & Wakefield, 2018; Zhang et al., 2018). QTL are a group of alleles that influence a particular phenotype or trait (Abiola et al., 2003). Measurable trait differences can be influenced by the combined interactions of multiple different polymorphisms present within the genome, as well as environmental factors. This is exemplified by observations that nDNA backgrounds influence the same mtDNA in different ways (Dunbar et al., 1995), and that mtDNA polymorphisms do not tend to exhibit strong maternal inheritance patterns. Instead, nuclear-mitochondrial crosstalk as well as individual responses to changing environmental factors are more likely influenced by mitochondrial polymorphisms.

While pulmonary metastatic burden differed between FF, FB and FC strains, the number of metastases was not significantly different among the strains (Table 1) (Feeley et al., 2015; Lifsted et al., 1998). This observation suggested that the mtDNA polymorphisms among the strains (Fig. 1) affected tumor cell growth at the metastatic site, potentially through differences in ability to adapt to dynamic metabolic demands at the new site. Accordingly, tumor cells from FC mice displayed significantly higher basal oxygen consumption rates (OCR) than did tumor cells from FB or FF mice, but significantly lower reserve capacity than those observed in the other strains (Feeley et al., 2015). Since reserve capacity has been linked with ability to adapt to metabolic stress (Dranka, Hill, & Darley-Usmar, 2010), such as that associated with metastasis and colonization of distant tissue sites, diminished reserve capacity and consequential difficulty in adapting to metabolic stress may have been responsible for the decreased metastatic burden in FC mice relative to the other strains.

Metabolic alterations owing to mtDNA polymorphisms among the MNX strains may have contributed to the noted differences in tumor latency as well. Tumor cells from FC mice had higher ratios of OCR to extracellular acidification rates (ECAR) than did the other strains,

suggesting an increased dependence on OXPHOS as opposed to glycolysis in tumor cells from those mice relative to the other strains (Feeley et al., 2015). A more respiratory metabolic profile could hinder adaptation to rapid tumor cell growth and associated hypoxia, which could slow tumor outgrowth. Tumor cells from FC mice were capable of glycolysis, however, even exhibiting the highest ECAR among the strains (Feeley et al., 2015), but transitioning from relying more on OXPHOS than glycolysis to a more glycolytic phenotype may slow metabolic adaptation and tumor outgrowth.

Another interesting observation that has been made using the MNX mouse model is that mtDNA influences on tumor progression and metastatic efficiency are oncogenic driver-dependent. This was demonstrated by crossing female FF, FB, and FC mice with male FVB/N-Tg(MMTVneu) mice over-expressing wild-type Her2/neu oncogene under the control of the MMTV promoter (Brinker et al., 2017). While the results between FF and FC mice were like those seen in the PyMT model, Her2 FB mice had unexpectedly longer tumor latency and lower metastatic burden than FF mice (Table 1). In both MNX crosses, numbers of pulmonary metastases were significantly lower relative to FF mice (Table 1) (Brinker et al., 2017). Importantly, the Her2 study also definitively demonstrated that observed changes in tumor progression were directly attributable to mtDNA and not another cytoplasmic element among the MNX mice, as crossing male FB and FC mice with female FVB/N-Tg(MMTVneu) mice resulted in female offspring whose tumor latency did not significantly differ (Brinker et al., 2017).

That mtDNA impacts tumor progression and metastasis in an oncogenic driver-dependent manner is novel but not necessarily surprising. PyMT and Her2 expression induce two distinct breast cancer subtypes (Pfefferle et al., 2013). Therefore, it stands to reason that nuclear-mitochondrial crosstalk may be altered in each tumor type. Indeed, oncogene-specific signaling between the nucleus and mitochondria has been demonstrated. For example, c-Myc and mammalian target of rapamycin (mTOR) drive transformation of many tumor cell types, and while both modulate expression of nDNA-encoded mitochondrial components the targets and modulatory mechanisms differ between the signaling molecules. c-Myc increases mitochondrial biogenesis in part by upregulation transcription of hundreds of nDNA-encoded mitochondrial genes (Li et al., 2005), while mTOR increases mitochondrial biogenesis through specific transcriptional upregulation of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α) and Yin Yang 1 (YY1) and resulting increased translation of mitochondrial mRNAs through inhibition of 4E binding proteins (4E-BPs) (Cunningham et al., 2007). Oncogenes can also manipulate mitochondrial biology by controlling mitochondrial dynamics (Caino et al., 2016; Kashatus et al., 2015; Serasinghe et al., 2015). This too exhibits oncogene specificity, as oncogenic K-Ras promotes mitochondrial fission through Drp1 phosphorylation (Kashatus et al., 2015; Serasinghe et al., 2015), while c-Myc promotes mitochondrial fusion (von Eyss et al., 2015).

It is important to consider that in the MNX mouse breast cancer studies detailed above mtDNA substitutions were made not only in the tumor cells but also in the rest of the cells in the mice as well. This raises the possibility that mtDNA effects on the observed phenotypes were products of both tumor cell intrinsic as well as tumor cell extrinsic mechanisms. Addressing this possibility, unpublished work from our laboratory demonstrates that

transplantation of tumor cells with canonical mtDNA into nDNA-matched (i.e., syngeneic) MNX mice with various mtDNA backgrounds alters metastatic efficiency (A. E. Brinker & D. R. Welch, in preparation), indicating that mtDNA also influences metastasis through tumor cell extrinsic mechanisms. Preliminarily, ROS neutralization normalized differences in metastatic efficiency in these studies, suggesting mtDNA-mediated ROS production from non-tumor cells impacts metastatic efficiency (A. E. Brinker & D. R. Welch, in preparation).

One way in which mtDNA could regulate complex, polygenic phenomena such as tumor progression and metastasis in tumor cell intrinsic and extrinsic manners is through modulation nDNA epigenetics and subsequent gene expression (Bellizzi, D'Aquila, Giordano, Montesanto, & Passarino, 2012; Smiraglia, Kulawiec, Bistulfi, Gupta, & Singh, 2008; Xie et al., 2007). To test this possibility for the first time in vivo, we performed integrative global genomic analyses to look for variations in nDNA methylation and gene expression in brain tissue from MNX mice derived from the BALB/cJ, C57BL/6J, FVB/NJ, and C3H/HeN backgrounds (Table 1) (Vivian et al., 2017) as well as histone marks (J. T. McGuire & D. R. Welch, in preparation). Three-way comparisons including wild-type “parental” strains and MNX mice revealed specific contributions of mtDNA to variations in both nDNA methylation as well as gene expression, and in some cases increased methylation status corresponded with downregulated gene expression as expected. While these observations were made using brain tissue, which fails to exactly recapitulate epigenetic landscapes most relevant for the mammary tumors used for our studies and their corresponding sites of metastasis (lung and lymph node), the results solidify epigenetic modulation as a mechanism by which mtDNA impacts tumor progression and metastasis.

Rather than global alterations, changes in DNA methylation among the MNX strains were limited to specific loci. The mechanism for such specificity is unclear given mtDNA-mediated epigenetic regulation is thought to occur through contributions to global metabolite pools that can serve as substrates for epigenetic-modifying enzymes (Lozoya et al., 2018; Wellen et al., 2009). One potential mechanism may be mtDNA-directed localization of DNA methyltransferases (DNMTs) that could differ among mtDNA backgrounds. Another intriguing possibility that oncogene-directed localization of DNMTs, in concert with variable contributions to global epigenetic substrates by different mtDNA backgrounds, serves as a mechanism by which mtDNA impacts on tumor progression and metastasis are oncogene dependent.

Modulating the immune system could exert tumor cell intrinsic (e.g., antigen presentation or checkpoint regulator expression) or extrinsic effects on tumor progression and metastasis (e.g., immune cell differentiation or effector function). The immune system plays a complex, dichotomous role in cancer. Recognition of inflammatory danger signals and mutated host peptides can instigate immune-mediated tumor cell clearance, but induction of immune tolerance by tumor cells and other immune cells promotes immune evasion and tumor growth (Mittal, Gubin, Schreiber, & Smyth, 2014). Anti-tumor immunotherapy, a booming area of cancer therapy that has provided some promising results (Couzin-Frankel, 2013), leverages both aspects of this relationship (Brahmer et al., 2012; Hodi et al., 2010; Porter, Levine, Kalos, Bagg, & June, 2011; Topalian et al., 2012).

Immune cell activation and differentiation impose dynamic metabolic demands, and differential responsiveness to those demands by polymorphic mtDNA could alter overall immune responses to inflammatory stimuli (Buck, Sowell, Kaech, & Pearce, 2017). The dynamic metabolic requirements required for immune cell function are exemplified by effector and memory T cells, both of which are critical for anti-tumor immunity. Memory T cells, which persist after primary immune responses to surveil for reemergence of cognate foreign or mutated antigens, tend to display mitochondrial fusion and rely on OXPHOS to fuel cell functions. Effector T cells, which are short-lived, inflammatory cells that respond to acute immune insult, tend to display mitochondrial fission and rely on aerobic glycolysis to fuel cell functions (Buck et al., 2016). Because many tumor cell types also perform aerobic glycolysis, glucose consumption by tumor cells represents a tumor cell intrinsic mechanism by which mtDNA could modulate immunity, where glucose depletion by tumor cells can inhibit effector T cell function (Cham et al., 2008; Chang et al., 2013; Macintyre et al., 2014).

Several studies using MNX mice have implicated that mtDNA (in) directly influences the immune system. Fetterman et al. used MNX mice with C57BL/6J and C3H/HeN genetic backgrounds to test mtDNA impacts on atherosclerosis and cardiac valve regurgitation (Fetterman et al., 2013), both pathologies which have been associated with macrophage infiltration (Brands et al., 2013). ROS production and membrane polarization in cardiomyocytes or mice harboring C57BL/6J mtDNA were more susceptible to both phenotypes than cells/mice containing C3H/HeN mtDNA (Table 1) (Fetterman et al., 2013). Similarly, Betancourt et al. demonstrated that mtDNA influences aspects of atherogenic diet-induced non-alcoholic fatty liver disease (NAFLD), as MNX mice with C57BL/6 and C3H/HeN mtDNA displayed intermediate phenotypes relative to their wild-type counterparts (Betancourt et al., 2014). Aberrant macrophage function has also been associated with NAFLD (Chatterjee et al., 2013; Huang et al., 2010; Lanthier et al., 2011; Rivera et al., 2007). Furthermore, initial studies have revealed mtDNA-dependent differences in immune cell differentiation and proportion (T. C. Beadnell & D. R. Welch, unpublished data).

Finally, mtDNA may also influence tumor progression and metastasis in a tumor extrinsic manner by altering commensal microbiota. mtDNA haplotypes have been associated with commensal microbial profiles (Hirose et al., 2017; Ma et al., 2014), and both cancer and cancer therapy responsiveness have been linked to microbiota (Farrell et al., 2012; Routy et al., 2018; Vetizou et al., 2015; Viaud et al., 2013; Wang et al., 2012). The relationship between mtDNA and commensal microbes is multi-faceted and complex. One facet is host metabolism, which affects many aspects of host physiology and can therefore impact commensal microbes in a multitude of ways. Bacterial products, by processes such as quorum sensing, can affect host mitochondria as well, which could dictate commensal microbe colonization in a mtDNA-dependent manner (Tao et al., 2016). Additionally, given the cyclical nature of immune system interactions with microbiota (Maynard, Elson, Hatton, & Weaver, 2012), mtDNA-mediated immune system dynamics may alter microbiota and, conversely, mtDNA-mediated microbial colonization could modulate immune system maturation and/or evolution. Indeed, ongoing work indicates that intestinal microbiota

differs among MNX mouse strains (S. J. Manley & D. R. Welch, in preparation), suggesting that mtDNA influences commensal microbe colonization in the MNX mouse model.

4. Concluding remarks and remaining questions

Querying direct, physiologically relevant mtDNA contributions to phenotypes and diseases is extremely difficult in humans due to confounding nDNA heterogeneity, but cybrids and mouse models have proven indispensable for studying such contributions. The MNX mouse model is the first in which other confounding variables such as mutagens, heteroplasmy, and nDNA recombination have been eliminated, making it quite useful for in vivo modeling of mtDNA biology. Accordingly, MNX mice have been utilized to demonstrate novel mtDNA impacts on tumor initiation, tumor outgrowth, metastasis, and other cancer-relevant phenotypes including epigenetic, immune, and commensal microbial profiles (Table 1).

While the MNX mouse model has enabled many novel insights into mtDNA biology, its utilization has led to as many, if not more, questions about how such a relatively small genome can impact such complex, multigenic phenotypes. Of them all, perhaps an overarching question is the most intriguing: what mtDNA-regulated signals mediate influence over complex phenotypes? Work to this point has implicated ROS and metabolic substrates as potential mediators of metastatic potential and epigenetic landscapes, respectively. Are there other signals? What are they? Recently, it was discovered that mtDNA encodes small non-coding RNA (sncRNA) derived from coding or tRNA genes (Barrey et al., 2011; Ro et al., 2013; Sripada et al., 2012). Some of these sncRNA are associated with complex phenotypes such as cancer (Magee, Telonis, Loher, Londin, & Rigoutsos, 2018; Telonis et al., 2015; Telonis & Rigoutsos, 2018). Could polymorphic sncRNA differentially affect mtDNA contributions to complex phenotypes? MNX mice provide an ideal model with which to investigate these and many other questions.

In addition to shedding light on novel aspects of mtDNA biology, results from MNX mouse studies carry the potential to inform mtDNA-related disease prevention and therapeutic strategies in the future. In particular, since that the MNX mouse model more closely recapitulates *pre-existing* [emphasis added] mtDNA variants as opposed to somatic mtDNA mutants, MNX mice can be used to understand how mtDNA variation impacts basal mitochondrial function and how variable mitochondrial function confers disease susceptibility as a QTL. Exploration into these topics will be highly relevant to understanding how human mtDNA haplotypes impart disease susceptibility. The outcome could be valuable for individualized screening and/or therapeutic strategies. Opportunities like these may soon be made reality given increasing appreciation for, and study of, mtDNA as a genomic contributor to disease.

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Fig. 1. mtDNA SNP among MNX mouse strains. SNP relative to FVB/NJ mtDNA are indicated in boxes, with initial letters indicating mouse strains as in Table 1 and amino acid changes indicated in parentheses where applicable. mtDNA map was adapted from SnapGene® Viewer 4.1.6, and mtDNA sequences were obtained from NCBI Nucleotide at the following GenBank accessions: B: [AJ512208.1](#), C: [DQ106412.1](#), F: [EF108338.1](#), H: [EF108335.1](#).

Table 1

MXN mice and corresponding mtDNA-directed phenotypes.

nDNA	mtDNA	Abbreviation	Tumor latency ^{a,b}	Metastatic Size ^{a,b}	Metastatic number ^{a,b}	Epigenetic changes ^c	Cardiac volume overload ^d
FVB/NJ	BALB/cJ	FB	PyMT: ↓ Her2: ↑	PyMT: ↑ Her2: ↑	PyMT: NS Her2: ↓	Yes	ND
FVB/NJ	C57BL/6J	FC	PyMT: ↑ Her2: ↑	PyMT: ↓ Her2: ↑	PyMT: NS Her2: ↓	Yes	ND
C57BL/6J	C3H/HeN	CH	ND	ND	ND	Yes	Resistant
C3H/HeN	C57BL/6J	HC	ND	ND	ND	Yes	Sensitive

^aFeeley et al. (2015).
^bBrinker et al. (2017).
^cVivian et al. (2017).
^dFetterman et al. (2013).

Phenotypes are relative to wild-type strains with matching nDNA. ND, not done; NS, not significant.