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Single-Cell Technologies for Studying the Evolution and Function of Mitochondrial DNA Heteroplasmy in Cancer

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Abstract

The mitochondrial genome, which encodes genes essential for respiration and cellular homeostasis, is the target of abundant and highly diverse somatic alterations in cancers. Somatic alterations to mitochondrial DNA (mtDNA) nearly always arise heteroplasmically, producing heterogeneous ensembles of mtDNA within single cells. Here, we review new insights derived from exponential increases in genomic sequencing data that have uncovered the nature of, selective pressure for, and functional consequences of cancer-associated mtDNA alterations. As many discoveries have been limited by their ability to determine cell-to-cell variation in mtDNA genotype, we describe a new generation of single-cell sequencing approaches that resolve otherwise indeterminate models of mtDNA heteroplasmy. In tandem with novel approaches for mtDNA editing and modeling of mutations, these advances foreshadow the quantitative dissection of dosage-dependent mtDNA phenotypes that underlie both tumor evolution and heterogeneous response to therapies.

INTRODUCTION

Mitochondrial DNA (mtDNA) acquires mutations and copy number alterations during tumorigenesis and subsequent tumor evolution (Gorelick et al. 2021, Ju et al. 2014, Kim et al. 2022, Reznik et al. 2016, Stewart et al. 2015). The cancer metabolism and cancer genomics literature is replete with evidence that such somatic mtDNA alterations, private to the tumor and not evident in the germline, arise in at least 50% of cancers (Gorelick et al. 2021, Hertweck & Dasgupta 2017, Ju et al. 2014, Kim et al. 2022, Stewart et al. 2015, Yuan et al. 2020). Sequencing technologies have driven basic discoveries on the selective pressure for acquiring somatic mtDNA alterations, their function or lack thereof once acquired, and their role in therapeutic response. More recently, discoveries implicating mtDNA in new cancer biology (Imanishi et al. 2011, Lin et al. 2022, Mahmood et al. 2024, Sun et al. 2018, Tan et al. 2015, Tigano et al. 2021, Victorelli et al. 2023, Vyas et al. 2016) have combined with technological innovations in both mtDNA engineering (Bacman et al. 2013; Cho et al. 2022; Gammage et al. 2014, 2018a,b; Gaude et al. 2018; Guo et al. 2022; Mok et al. 2020; Lee et al. 2021, 2023; Lim et al. 2022; Wei et al. 2024) and single-cell sequencing (Baysoy et al. 2023, Lareau et al. 2023b, Mimitou et al. 2021) to open new avenues for understanding the impact of somatic mtDNA alterations on both mitochondrial function and tumor evolution.

This review summarizes the existing observations on the prevalence, function, and intratumoral heterogeneity of somatic mtDNA alterations in cancer. Complementing prior reviews on the patterns of mtDNA mutations across populations, we focus on novel sequencing approaches for studying and leveraging intracellular mtDNA heterogeneity (Nitsch et al. 2024, Stewart & Chinnery 2015, Vyas et al. 2016, Wallace 2010, Weinberg & Chandel 2009). We conclude by describing several key unresolved questions relating mitochondrial genetic variation to tumorigenesis and evolution and the potential for significant progress with new sequencing technologies.

STRUCTURE, FUNCTION, AND HETEROPLASMY OF MITOCHONDRIAL DNA

Human mtDNA is a highly polyploid, ~16.5-kb circular genome encoding the respiratory complexes and associated translational machinery responsible for executing oxidative phosphorylation (OXPHOS), including 13 protein subunits, 2 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) (Anderson et al. 1981). In combination with over 1,000 nuclear-encoded proteins that ultimately translocate to the mitochondria (Anderson et al. 1981, Harbauer et al. 2014, Rath et al. 2021), mtDNA-derived proteins take part in a multifaceted array of cellular processes such as cellular metabolism, cellular signaling, formation of iron–sulfur clusters, and apoptosis (Spinelli & Haigis 2018, Stewart & Chinnery 2015).

OXPHOS is an integral metabolic pathway composed of five protein complexes (four of which, Complexes I, III, IV, and V, are composed in part by mtDNA-encoded subunits). Complexes I through IV couple the oxidation of NADH and $FADH_2$ to the reduction of molecular oxygen and the translocation of protons across the inner mitochondrial membrane, generating a chemiosmotic gradient that is dissipated by Complex V (ATP synthase) to generate ATP (Bonora et al. 2012, Hatefi 1985, Papa et al. 2012, Senior 1988). Decades of cellular biochemistry have demonstrated that this set of biochemical reactions generates the majority of cellular ATP (Mitchell 1961), which is ultimately essential to cellular homeostasis and physiology (Hatefi 1985, Spinelli & Haigis 2018, Watt et al. 2010, Wilson 2013). At the same time, OXPHOS underpins an intact tricarboxylic acid (TCA) cycle and subsequently acts on and produces metabolites, including NAD(H) and various reactive oxygen species (ROS), whose abundance dictates key downstream cellular processes (Chandel 2014, Hamanaka & Chandel 2010, Martínez-Reyes & Chandel 2020). Disruption of OXPHOS function perturbs these metabolite pool sizes and

therefore elicits downstream consequences of cellular metabolism; for example, disruption of Complex I leads to the accumulation of NADH and downregulation of the NAD^+:NADH ratio (Stein & Imai 2012). Therefore, Complex I malfunction reduces the thermodynamic favorability of NADH-dependent reactions (Bekiaris & Klamt 2023) and alters cellular redox balance, thereby increasing ROS production, which promotes hypoxic activity, cell proliferation, and tumorigenicity (Indo et al. 2007, Schieber & Chandel 2014, Sharma et al. 2011).

Each cell contains hundreds to thousands of mtDNA copies, and thus germline and somatic mutations and also structural variants (Hjelm et al. 2023, Phillips et al. 2017) typically occur as mixtures with the wild-type allele in a phenomenon termed heteroplasmy (Stewart et al. 2015). Heteroplasmy and mitochondrial copy number are dynamic and vary across healthy and malignant tissues at both the intracellular and intercellular levels (Stewart & Chinnery 2015). Typically, heteroplasmy arises via the accumulation of somatic mutations, which accrue with age (Glynos et al. 2023, Green et al. 2023, Gupta et al. 2023, Kennedy et al. 2013, Michikawa et al. 1999) and are the result of replication errors (Ju et al. 2014). Bulk data have established that ROS are not major contributors to the clonally expanded mtDNA pool, although recent duplex sequencing has identified that ROS-induced mtDNA mutations do arise but are dynamically cleared with age (Sanchez-Contreras et al. 2023).

Most of the natural somatic variation in healthy tissues has no measurable clinical impact, whereas hundreds of pathogenic germline variants, which are inherited through the maternal germline, can result in variably penetrant multisystem metabolic dysfunction, known as mitochondrial disease (Gorman et al. 2015). The most common mtDNA allele causative of mitochondrial disease is the m.3243G>A mutation, which at high heteroplasmy causes mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (Elliott et al. 2008, Li et al. 2022). At lower heteroplasmic doses, however, it leads to intermediate to no clinical phenotypes (Gupta et al. 2023). Mechanistically understanding how pathogenic mtDNA alleles elicit extreme symptoms of disease remains a major challenge, but recent advances in mitochondrial genome engineering (Bacman et al. 2013; Gammage & Minczuk 2018; Gammage et al. 2014, 2018b; Lee et al. 2021; Mok et al. 2020; Wei et al. 2024) show promise in untangling the relevant pathways.

TUMOR-ASSOCIATED SOMATIC VARIATION IN MITOCHONDRIAL DNA

Tumors accumulate a diverse repertoire of somatic alterations to mtDNA. We summarize the various categories of these alterations below and describe where possible their incidence and patterns of heteroplasmy.

Single-Nucleotide Variants

The vast majority of somatic mtDNA mutations in tumors arise as single-nucleotide variants (SNVs) in either the non-protein-coding control region (where they are predominantly C>T on the light strand, T>C on the heavy strand) or the protein/tRNA/rRNA-coding regions (where the mutation signature reverses and arises primarily as C>T on the heavy strand, T>C on the light strand) (Gorelick et al. 2021, Ju et al. 2014, Stewart et al. 2015, Yuan et al. 2020). Comparative analysis of the incidence of SNVs indicates that, while synonymous mutations demonstrate no preference as to which genes they affect (consistent with their likely neutral impact), nonsynonymous mutations are enriched in Complex III, and intact Complex III is required for tumor cell viability (Martínez-Reyes et al. 2020). Interestingly, certain mutations that are known to be pathogenic from the germline mitochondrial literature (including the MELAS variant m.3243A>G) have been recurrently observed in some tumors (Gopal et al. 2018,

Gorelick et al. 2021). In general, the function of the vast majority of SNPs, especially those affecting the non-protein-coding control region, rRNAs, and tRNAs, is unknown.

Insertions or Deletions

Small insertions or deletions (indels) that disrupt the protein-coding reading frame are commonly observed in nuclear DNA–encoded tumor suppressor genes and have recently been described in tumor mtDNA (Gorelick et al. 2021). A recent analysis of off-target mtDNA reads from bulk exome sequencing in The Cancer Genome Atlas samples established that ~10% of solid tumors harbor a truncating (either nonsense or frame-disrupting indel) mtDNA variant and that these mutations predominantly affect Complex I (Gorelick et al. 2021). Such indels are largely absent from the germline, with rare exceptions (Alston et al. 2010). Comparative analysis of the mutation rate across the four OXPHOS complexes composed of mtDNA-encoded subunits further indicated that Complex V was under negative selection for truncating variants relative to other complexes. Truncating indels in mtDNA further arose at specific homopolymeric loci and preferentially affected specific tumor lineages, including colorectal, thyroid, and renal cell cancers (Gorelick et al. 2021, Ju et al. 2014, Yuan et al. 2020). Interestingly, these cancers have distinct mitochondrial histologies, impeding our understanding of the cause of tissue-specific mtDNA mutation burdens. For example, thyroid cancers such as Hürthle cell carcinoma (HCC; now alternatively known as oncocytic carcinoma of the thyroid) are characterized by the formation of oncocytic cells, leading to abundant and large mitochondria (Stojadinovic et al. 2001). On the other hand, colorectal cancers do not commonly display oncocytic features, implying that the morphologic adaptation to truncating variants in colorectal cancers is not homogeneous across tissue lineages.

Copy Number Alterations

A number of groups have investigated the incidence of changes in total mtDNA abundance in tumors (Hosgood et al. 2010, Kim et al. 2024, Reznik et al. 2016, Sun et al. 2018). The basic principle underlying these analyses is that total read depth can often be used as a surrogate measure of copy number. Therefore, the read depth of mtDNA relative to a region of known copy number in the nuclear genome can be used to determine the absolute copy number of mtDNA. Along these lines, studies utilizing both amplification-free whole-genome sequencing (to assay absolute mtDNA copy number) and exome sequencing (to assay relative mtDNA copy number) across cohorts of tumors have observed heterogeneous changes heavily dependent on cancer type, with approximately half of all tumor lineages (best exemplified by *VHL*-null clear cell renal cell carcinoma) demonstrating concerted loss of mtDNA copy number relative to adjacent normal tissue (Reznik et al. 2016). In contrast, relatively few diseases show an increase in copy number, although there is evidence of this effect in lung squamous cell carcinoma, pancreatic adenocarcinoma, and chronic lymphocytic leukemia (Yuan et al. 2020).

Deletions of Mitochondrial DNA/Structural Variants

Although comparatively less well studied than SNVs, large deletions of the mitochondrial genome have been identified as causes of mitochondrial disease (Lestienne & Ponsot 1988, Rotig et al. 1989, Shoffner et al. 1989, Zeviani et al. 1988). These deletions frequently involve the 4,977–base pair (bp) common deletion (Bai et al. 1997, Holt et al. 1988, Lee et al. 1994) and are due to errors in replication-dependent repair (Persson et al. 2019, Phillips et al. 2017). In various cancers, such as breast, colorectal, hepatocellular, and gastric, the common deletion is a frequently observed alteration found to be under strong negative selection, indicated by the comparatively rare occurrence of homoplasmy (Chen et al. 2011, Kotake et al. 1999, Tseng et al.

2006, Wu et al. 2005). Apart from the common deletion, other notable lengths of deletions have been reported, ranging from as short as 2 bp in the ND1 gene to 2,583 bp in 16S rRNA (Burgart et al. 1995, Habano et al. 1999, Horton et al. 1996, Savre-Train et al. 1992, Temperley et al. 2003). A consequence of such large deletions is reductions in mtDNA genome size, which were long believed to confer a replicative advantage to cells, and which have been associated with increased generation of ROS (Diaz et al. 2002, Ga Cortopassi et al. 1992, Moraes et al. 1999).

Nuclear Mitochondrial DNA and Somatic Insertions into the Nuclear Genome

Advances in the sequencing of mtDNA have revealed that small fragments of mtDNA have been transferred over to the noncoding regions of the nuclear genome. These are referred to as nuclear mitochondrial DNAs (NUMTs) and are substantially conserved across species (Hazkani-Covo et al. 2010). Originally, the transfer events (denoted numtogenesis) were regarded as ancient translocation events with no clinical impact, but later they were observed to be an ongoing process, disrupting protein-coding genes and ultimately causing disease (Richly & Leister 2004, Turner et al. 2003, Xue et al. 2023). Somatic transfer events have been identified in ∼2% of all cancer genomes and preferentially affect skin, lung, and breast tissue lineages (Ju et al. 2015, Wei et al. 2022, Yuan et al. 2020). The fragments that translocate to the nuclear genome range from tens of bases to the entire 16-kb genome and most commonly involve the regulatory D-loop region (Ju et al. 2015, Wei et al. 2022, Yuan et al. 2020). Tumors with missense mutations in nuclear DNA repair oncogenes have elevated rates of numtogenesis, suggesting that genomic instability may increase susceptibility to somatic translocation events (Wei et al. 2022, Yuan et al. 2020). However, future studies investigating the landscape of NUMTs across cancers and their associated mechanisms are needed to discern their phenotypic impact.

INTRATUMORAL EVOLUTION, SELECTION, AND FUNCTION OF MUTANT MITOCHONDRIAL DNA HETEROPLASMY IN CANCER

The vast majority of somatic mtDNA mutations in tumors arise heteroplasmically, affecting just a fraction of the total pool of mtDNA copies in a given cell in the tumor. This empirical observation derives from a basic fact about de novo mtDNA mutations: They are initially present in just a single mtDNA copy and therefore must undergo progressive expansion to achieve sufficient heteroplasmy to be technically detectable and elicit a cellular phenotype. Such expansion is driven by a variety of selective forces as well as neutral drift (Burgstaller et al. 2018, Wonnapinij et al. 2008).

A key observation from the germline mitochondrial disease literature is that phenotypes arising from heteroplasmic mtDNA mutations are sensitive to heteroplasmic dosage. This dosage sensitivity reconciles (at least in part) the contrasting prevalence of pathogenic germline mtDNA mutation carriers [estimated at 1 in 200 (Elliott et al. 2008, Gupta et al. 2023)] relative to the prevalence of mitochondrial disease [approximately 1 in 5,000 (Gorman et al. 2016)]. Thus, inheritance of pathogenic mtDNA mutations is relatively common, whereas clinical manifestation of disease is not. In reality, pathogenic mtDNA variants are often inherited at low heteroplasmies, resulting in cells with an mtDNA pool of majority wild-type molecules and thus unaffected mitochondrial function. However, as mutant heteroplasmy increases, OXPHOS defects prevail and consequently cause disease phenotypes (Rossignol et al. 2003).

A relatively narrow window of evidence suggests that select tumor-associated somatic mtDNA variants elicit heteroplasmy-dependent phenotypes. Only Complex I truncating mutations, but not truncating mutations in other complexes, demonstrated elevated levels of heteroplasmy relative to silent mutations in the same complex (Gorelick et al. 2021). Interestingly, high-heteroplasmy truncating variants (now irrespective of the complex affected) demonstrated a

lineage-agnostic transcriptional signature that was attenuated or absent in low-heteroplasmy samples (Gorelick et al. 2021).

The most striking example of a heteroplasmy-sensitive metabolic phenotype is found in HCC. HCC tumors are morphologically characterized by the accumulation of abundant, dysfunctional mitochondria, and early studies suggested that somatic mutations to mtDNA could be driving this accumulation (Máximo et al. 2002). More contemporary genomic studies identified putatively causative mtDNA variants in ~50% of cases, including homoplasmic Complex I truncating variants and a novel, nearly pathognomonic hotspot mutation in *MT-TL1* (m.G3244A) (Ganly et al. 2018, Gopal et al. 2018). Three recent studies (from our group and others) have characterized these tumors using a variety of metabolomic approaches and demonstrated in cell lines, patient-derived xenograft models, and human tumor specimens that HCC tumors have large-magnitude drops in citrate and *cis*-aconitate pool sizes (Frank et al. 2023, Ganly et al. 2018, Gopal et al. 2018). Although the cause of these large drops in TCA metabolite pool sizes is not known, one speculative hypothesis is that HCC tumors (and potentially those from other forms of thyroid cancer) shunt the vast majority of glucose carbon away from the TCA cycle and adapt to the drop in glucose-derived TCA carbon through glutamine anaplerosis. Corroborating such hypotheses, however, requires as-yet-unavailable isotope tracing experiments in model systems or patients.

The above studies indirectly suggest that somatic mtDNA mutations, especially truncating mutations, could functionally impact tumor cell phenotypes. Still, because they are observational in nature (focusing on human samples), causal conclusions have been challenging to draw. Mechanistic studies utilizing chemical inhibition of OXPHOS complexes and mouse models of mtDNA mutagenesis have demonstrated the functional significance of OXPHOS dysfunction (and, by proxy, mtDNA mutations) on cancer cell metabolism and consequent tumor cell growth. Patient-derived cancer cell lines with endogenous Complex I mutations increased biguanide sensitivity, reducing tumor growth (Birsoy et al. 2014). These effects depended on cells being maintained in a low-glucose environment, mimicking the reduced glucose concentrations (Abbott et al. 2024) in the microenvironment and interstitial fluid surrounding tumor cells. In an osteosarcoma cell line (143B) devoid of Complex III (essential for both ubiquinol oxidation and NAD^+ regeneration), tumor growth was inhibited in both the presence and absence of exogenous NAD^+, suggesting that ubiquinol oxidation is essential to cancer cell proliferation (Martínez-Reyes et al. 2020). Using a mouse model of accelerated mtDNA mutagenesis ($PolgA^{mut/mut}$), Smith et al. (2020) determined that OXPHOS deficiencies observed in colon cancers were due to somatic mtDNA mutations and, more specifically, that age-associated mtDNA mutations in precancerous colonic crypts could metabolically facilitate subsequent tumorigenesis.

Direct evidence of the oncogenic effects of specific mtDNA alleles, however, has been limited, in significant part because effective tools to engineer putatively functional mtDNA alleles into cancer cells have (until recently) been unavailable. Early technologies, such as mitochondrially targeted zinc-finger nucleases (mtZFNs), relied on the introduction of double-stranded breaks to eliminate preexisting heteroplasmic mtDNA mutations (Gammage et al. 2014). When applied to generate isogenic cell lines differing only by their mutant heteroplasmy, this approach determined that the oxygen consumption rate (a measure of mitochondrial function) decreased proportionally with heteroplasmic dosage (Gaude et al. 2018). However, mtZFNs remained unable to introduce nucleotide changes at specific mitochondrial alleles. Recently, DddA-derived cytosine base editors (DdCBEs) have overcome this obstacle, enabling precise editing of the mitochondrial genome to induce point mutations (Mok et al. 2020). In brief, the mechanism of this base editor relies on an interbacterial toxin (DddA), which catalyzes the deamination of a cytosine, resulting in C>T/G>A mutations (Mok et al. 2020). Two DddA halves are ligated to a transcription activator-like effector (TALE), which targets the flanking regions of the target

mtDNA site, ensuring editing precision. DdCBE-engineered Complex I truncating alleles (at positions homologous to those observed as hotspots in human cancer samples) generated subtle but significant changes in metabolism and redox balance in mutant melanoma cells (Mahmood et al. 2024). Interestingly, these subtle changes nevertheless elicited a profound change in the immune microenvironment that ultimately poised tumors bearing mutant mtDNA to respond to checkpoint blockade therapies. Importantly, these effects were heteroplasmy-dependent (i.e., proportional to the abundance of mutant mtDNA), with high-heteroplasmy samples demonstrating more potent metabolic and microenvironmental phenotypes.

Collectively, the above studies describe mounting evidence of functional and heteroplasmy-dependent effects of mtDNA alterations in cancer.

SINGLE-CELL METHODS FOR STUDYING MITOCHONDRIAL GENOMES

Most of our understanding of how mtDNA genotypes evolve in cancer has been derived from bulk sequencing, obscuring the intercellular variability within mitochondrial genomes. However, a suite of technologies (**Table 1**) have emerged that enable genotyping of mtDNA while measuring DNA, RNA, epigenetic modifications, protein abundance, and other biomolecules at single-cell resolution (Nitsch et al. 2024). While in most cases not intentionally designed to study mtDNA, a common by-product of these assays has been the inadvertent generation of abundant mtDNA-derived sequencing data, catalyzing entirely new lines of inquiry into mitochondrial biology and somatic evolution. Below, we inexhaustively summarize some of the key conceptual, biological, and technical innovations that have derived from single-cell sequencing of mtDNA in tumors and other related settings.

Technologies for Single-Cell Sequencing

As most of the mitochondrial genome is transcribed, recent work has shown that single-cell RNA sequencing (scRNA-seq) technologies could be used to genotype mtDNA (Ludwig et al. 2019). The earliest technologies demonstrating this capacity were from plate-based chemistries, including extensions of the Smart-seq2 protocol (Ludwig et al. 2019, Velten et al. 2021). In recent years, 3′-biased droplet scRNA-seq protocols have become more widely used for profiling thousands of cells in a single reaction, but the coverage and quality of mtDNA genotyping inferences from commercial scRNA-seq kits are limited. However, recent methods, such as mitochondrial alteration enrichment from single-cell transcriptomes to establish relatedness (MAESTER), enhance commercially available 3′ platforms by enriching for mitochondrial transcripts from pooled primers that increase coverage nearly 50-fold (Miller et al. 2022).

As the mitochondrial genome is packaged without nucleosomes or histones, transposases such as Tn5 can access native mtDNA without modifications. In particular, the single-cell assay for transposase-accessible chromatin by sequencing (scATAC-seq) utilizes the hyperactive Tn5 transposase to cut accessible chromatin regions throughout the nucleus, ultimately yielding a highly uniform sampling of mtDNA (Buenrostro et al. 2015). While the primary goal of scATAC-seq has been the study of epigenomic heterogeneity in single cells, ATAC-seq chemistries readily allow for studying mitochondrial genetic variation alongside nuclear regulatory landscapes. In the initial versions of the widely used droplet-based protocols, an upstream nuclei extraction step was used, thereby depleting mitochondria from reactions. To overcome this, the mitochondrial scATAC-seq protocol (mtscATAC-seq) optimized lysis and buffer conditions for permeabilization of whole cells (instead of only nuclei) to retain cytoplasmic mitochondria, resulting in 25–30% of molecules from this reaction mapping to the mitochondrial genome. Of note, an additional fixation step was

Table 1 Summary of single-cell multiomics technologies described to detect mitochondrial variants

Technology	Reference	Transcriptome	Epigenome	Proteome	Mitochondrial DNA	Mitochondrial RNA	Nuclear mutations
Smart-seq2	Ludwig et al. 2019	×				×	×
Mitochondrial alteration enrichment from single-cell transcriptomes to establish relatedness (MAESTER)	Miller et al. 2022	×				×	
Mitochondrial single-cell assay for transposase-accessible chromatin by sequencing (mtscATAC-seq)	Lareau et al. 2021		×		×		
ReDeeM	Weng et al. 2024	×	×		×		
Genotyping of targeted loci with single-cell chromatin accessibility (GoT-ChA)	Myers et al. 2022		×		×		×
Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq)	Stoeckius et al. 2017	×		×		×	
Assay for transposase-accessible chromatin with select antigen profiling by sequencing (ASAP-seq)	Mimitou et al. 2021		×	×	×		
DOGMA-seq	Mimitou et al. 2021	×	×	×	×	×	
PHAGE-ATAC	Fiskin et al. 2022		×	×	×		

used to isolate mitochondria to their original cell during pooled permeabilization, collectively resulting in a 50-fold increase in coverage compared to the commercial workflow (Lareau et al. 2021, 2023b). ReDeeM (Weng et al. 2024) later improved mtDNA coverage threefold by specifically enriching mtDNA within sequencing libraries. Though these data demonstrate a clear ability to transpose native mtDNA, other data indicate that only a subset of mitochondrial genomes may be accessible. Specifically, recent data from single-cell examination of the mitochondrial genome uncovered previously unappreciated heterogeneity in mtDNA nucleoid packaging, regulated by nuclear-encoded mitochondrial transcription factor A (TFAM) (Isaac et al. 2024). High TFAM levels were shown to reduce the proportion of accessible nucleoids. Considering that TFAM levels differ across tissues and disease states (Hsieh et al. 2021, Kang et al. 2018), this raised the question of whether inaccessible nucleoids are inactive or if they serve as a genetic cache, becoming accessible when required.

Further multimodal analyses that build upon the mtDNA and accessible chromatin profiles in mtscATAC-seq include those that incorporate genotype profiling through nuclear somatic mutation capture [genotyping of targeted loci with single-cell chromatin accessibility (GoT-ChA)] (Myers et al. 2022) and proteogenomic analyses using surface/intracellular protein detection such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) (Stoeckius et al. 2017), ATAC with select antigen profiling by sequencing (ASAP-seq) (Mimitou et al. 2021), and DOGMA-seq (Mimitou et al. 2021). Alternatively, PHAGE-ATAC (Fiskin et al. 2022), which uses M13 bacteriophages to genetically encode and display peptide sequences, overcomes the need for oligonucleotide conjugates to antibodies.

Finally, recent work has shown the feasibility of using direct library preparation (DLP+) single-cell whole-genome sequencing (scWGS) to profile both mitochondrial and nuclear genomes from the same cell (Kim et al. 2024). DLP+ is an amplification-free scWGS protocol that provides single-cell DNA sequencing with analogous high-resolution images that quantify cell size (Laks et al. 2019). As a consequence, DLP+ is currently the only single-cell sequencing technology able to directly assay mtDNA copy number at single-cell resolution, and it does so with concurrent measurements of mtDNA and nuclear DNA genotype (Kim et al. 2024).

Application: Single-Cell Mitochondrial DNA Sequencing as a Tool for Lineage Tracing

Due to their propagation during cell divisions, the age-related accumulation of somatic mutations in nuclear DNA (nuDNA) and mtDNA has been exploited as a tool for lineage tracing. In addition to mapping out clonal dynamics and cellular ancestry (Sankaran et al. 2022), somatic mutations can be interpreted as a form of genetic barcoding and have allowed for identifying mutations leading to tumor initiation and subsequent progression (Aaltonen et al. 2020). Historically, such inferences had predominantly been made from somatic mutations arising in nuDNA due to its abundance and well-established role in driving oncogenic processes, as mutations that confer selective (or at least neutral) advantages tend to have an equal segregation inheritance pattern (Mitchell et al. 2022, Spencer Chapman et al. 2021). However, the higher somatic mutation rate of mtDNA compared to nuDNA (Taylor et al. 2003), possibly owing to limited repair mechanisms (Copeland 2012, Druzhyna et al. 2008, Liao et al. 2022), coupled with the high rate of mitochondrial turnover and mosaic clonal expansion of variants, makes for easier lineage tracing and differentiation between clonal populations (Weng et al. 2024). These unique aspects of the mitochondrial genome allow for resolving cell lineages from deconvolving bulk heteroplasmy values to resolve potential models of clonality (**Figure 1**). Additionally, the high copy number of mtDNA allows for sensitive variant detection for clonal tracing without prior knowledge, which may be advantageous over analogous scWGS techniques (Penter et al. 2021).

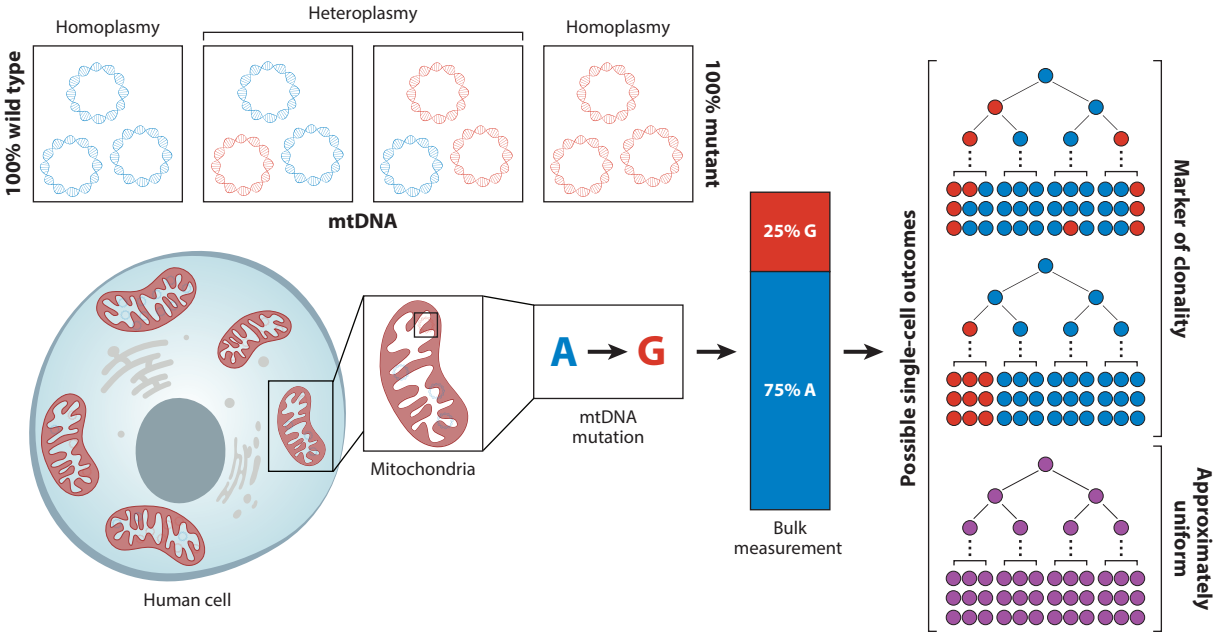

Figure 1

Enhanced detection and tracing of mitochondrial DNA (mtDNA) variations in clonal populations using single-cell sequencing. Each cell exhibits variations in mitochondrial copy number and the number of mitochondria per cell. Somatic variants in mtDNA can manifest in different forms: completely absent (wild-type homoplasmy), present alongside wild-type copies (heteroplasmy), or present in all mtDNA within a single cell (mutant homoplasmy). While bulk measurements capture variant proportions across cell populations, single-cell measurements resolve the complex distribution of mtDNA alleles both within and between cells, enabling the reconstruction of clonal subpopulations.

The potential for somatic mtDNA variants to serve as lineage tracers in humans was initially observed by mtDNA variant expansion in the human colon (Greaves et al. 2006, Taylor et al. 2003). Immunohistochemical (IHC) staining for the mtDNA-encoded Complex IV enzyme cytochrome *c* oxidase revealed clonal patches in colonic crypts. Laser microdissection and targeted polymerase chain reaction sequencing of cells within these clonal patches verified that the lack of staining was associated with loss-of-function mutations occurring at high heteroplasmy originating from the crypt stem cell compartment. Numerous subsequent studies across various tissues—liver (Fellous et al. 2009), prostate (Blackwood et al. 2011), esophagus (Nicholson et al. 2012), lung (Teixeira et al. 2013), breast (Cereser et al. 2018), and endometrium (Tempest et al. 2020)—further demonstrated comparable expansions of mtDNA mutant cells with altered protein levels detected through either IHC or immunofluorescence of cytochrome *c* oxidase. These studies underscored the utility of mtDNA variants for lineage tracing but were unable to discover mtDNA variants in an unsupervised manner, motivating the development of single-cell genomics tools.

Due to sample availability and ease of processing, hematopoietic malignancies have been explored most thoroughly for subclonal structure via somatic mtDNA mutations. For example, in acute myeloid leukemia (Xu et al. 2019), mtDNA tracing has validated known lineage hierarchies and helped characterize clonal evolution and diversity of preleukemic hematopoietic stem cells and their descendants. In chronic lymphocytic leukemia (CLL) (Penter et al. 2021), where mtDNA mutations remain stable over many years without significant selective pressures, this approach revealed nongenetic aspects of disease progression through altered chromatin states. On the other hand, chemotherapy and relapse led to significant shifts in the mtDNA mutational

profile and clonality in CLL, suggesting that mtDNA tracing could be used to predict prognosis, treatment resistance, and response. Similarly, in addition to identifying transcriptomic and epigenomic changes relative to subclonal structures in multiple myeloma (Poos et al. 2023), multimodal longitudinal analysis found many mtDNA mutations and copy number variants resulting in drug-resistant subclones, thereby pinpointing potential therapeutic targets. In sum, these emerging single-cell multiomics tools have enabled integrated analyses of mtDNA and cell states measured by all layers of the central dogma.

Application: Single-Cell DNA Sequencing and Functional Consequences of Cell-to-Cell Variation in Mitochondrial DNA Copy Number and Heteroplasmic Dosage

Despite the growing number of studies describing patterns of cell-to-cell variation in mtDNA genotype, comparatively little work has been done to understand intercellular variation in mtDNA copy number. Recent work using DLP+ enabled an interrogation of the association between intercellular variation in mtDNA copy number and heteroplasmy and nuclear DNA copy number profiles, as well as single-cell transcriptional phenotypes. These analyses revealed that cell-to-cell variation in mtDNA copy number in tumors was large, with the range of single-cell mtDNA copy number in a typical cell line, patient-derived xenograft, or tumor varying by thousands of mtDNA copies. This substantial mtDNA copy number variation was largely ascribable to three distinct phenomena: biophysical changes in cell size, whole-genome doubling (WGD) events in the nuclear genome, and ongoing tumor evolution/aneuploidy. In particular, WGD, which affects ~30% of advanced cancers (Bielski et al. 2018), was associated with a near-stoichiometric doubling of mtDNA copy number. Furthermore, intratumoral variation in both germline and somatic mtDNA alleles was associated with a heteroplasmy-dependent change in mtDNA copy number and a heteroplasmy-dependent transcriptional phenotype characterized by upregulation of mtDNA-encoded, but not necessarily nuclear DNA–encoded, OXPHOS gene expression. These data indicated that both the heteroplasmic dosage of an mtDNA variant and the absolute copy number of that variant are determinants of phenotype. What remains unclear is the sequencing resolution required to determine heteroplasmy within a single mitochondrion. Consequently, one cannot yet ascertain how co-occurring mitochondrial variants influence the function of an individual mitochondrion, as opposed to understanding how variants shared across multiple mitochondria within a single cell impact overall cellular function.

FUTURE POSSIBILITIES AND OPEN QUESTIONS

The Prognostic and Predictive Value of Somatic Mitochondrial DNA Alterations in Cancer Therapy

Perhaps the most critical and open question related to somatic mtDNA alterations is their role in mediating disease progression, therapeutic response, and overall clinical outcome. A limited number of studies have carried out prognostic analyses, identifying associations between overall survival and the presence of pathogenic mtDNA mutations (Fendt et al. 2020, Gorelick et al. 2021, Hong et al. 2023, Qi et al. 2016). A more clinically relevant question is whether mtDNA mutations prime tumors to intrinsically respond to or resist certain cancer therapies. Data from our group suggest that, at least in murine models of melanoma, mtDNA mutations poise tumors to demonstrate improved responses to immunotherapy and that this effect ultimately arises from the disruption of cellular redox balance. Interestingly, human patients with melanoma demonstrate similar tumor transcriptional phenotypes and associations between response and mtDNA mutation status, motivating a more general (and as yet not complete) survey of the association of somatic

mtDNA mutations and therapeutic response across cancer types. Two major obstacles stand in the way of such an analysis: (*a*) the paucity of mtDNA coverage in targeted clinical sequencing data and (*b*) incomplete or small clinicogenomic corpora with adequate treatment response information.

Heteroplasmic Dosage as a Determinant of Phenotype

In the germline, pathogenic mtDNA alleles often result in disease phenotypes in a heteroplasmic-dosage-dependent manner (Gupta et al. 2023). Importantly, germline disease models have illustrated that the threshold for disease does not follow an all-or-nothing model in which disease phenotypes emerge only at a certain threshold, reaching a maximal state (Choi et al. 2010, Picard et al. 2014). Instead, disease phenotypes depend on the heteroplasmy dosage of mutation alongside likely nuclear genetic modifiers. Interestingly, recent work suggests that certain cell lineages do not tolerate pathogenic alleles even at vanishingly low heteroplasmy (Lareau et al. 2023a), implying that cells harbor a mechanism both for sensing and for eliminating low-heteroplasmy mutations. These data raise the very likely possibility, supported by a variety of relatively crude analyses from bulk data (Gorelick et al. 2021, Qi et al. 2016), that the phenotypes produced by heteroplasmic mtDNA alleles in cancer are equally dependent on heteroplasmic dosage. Single-cell technologies will prove useful in further teasing apart this relationship, providing parallel readouts of cell state measures (e.g., gene expression) and mtDNA genotype. Prospective single-cell capture that optimizes mitochondrial read depth is paramount in establishing this relationship, as relying on retrospective single-cell data does not yield sufficiently high coverage in the mitochondrial genome to provide confidence in genotype.

Interactions with Other Cancer Drivers and the Mito-Nuclear Germline

A major conclusion of the cancer genomics era is that most cancers carry more than one functional driver and that the selective pressure for a given driver mutation depends sensitively on preexisting somatic alterations, environmental exposures, ongoing mutagenesis, and therapeutic treatment. Despite the numerous genomic studies focused on identifying somatic mtDNA mutations, there has been no systematic analysis of the co-occurrence or mutual exclusivity of somatic mtDNA alterations and other driver alterations. Such an analysis would likely identify specific genomic contexts in which mtDNA dysfunction is under positive selection and thereby prioritize disease models for subsequent characterization. A generalization of this analysis, including both somatic alterations and the mito-nuclear germline, could reveal the role of inherited mtDNA haplotypes (and mutations that disrupt those haplotypes) in cancer.

Lineage Tracing in Solid Tumors

While somatic mtDNA mutations revealed subclonal architectures in hematologic cancers, their application in solid tumors has not been as extensively explored. We suggest that this is due to (*a*) single-cell isolation being less straightforward for epithelial tissues; (*b*) sample collection being more invasive for solid tumors, thereby dramatically reducing the availability of longitudinal samples; and (*c*) blood, as compared to epithelial tissues, accumulating mtDNA mutations at shorter timescales and in greater abundance. For these reasons, additional effort is needed to establish the utility of mtDNA tracing in solid tumors, especially given the critical clinical relevance of determining which subpopulations can metastasize from solid tumors.

CONCLUSION

The unique heteroplasmic manifestation of mtDNA alterations, affecting a fraction of the total population of mtDNA in any given cell, has major implications for studies that assess their

evolution and function. Bulk measurements of genotype, which have led to profound advances in our understanding of nuclear DNA evolution in cancer, are inadequate for analogous studies in mtDNA. In turn, single-cell measurements (of RNA and DNA) are proving to be powerful tools for studying both the functional consequences of mtDNA alterations and somatic evolution at large. We anticipate outsized returns from future cohorts and technologies that quantify mtDNA mutations in tumors that will further refine emerging evidence of mtDNA influencing therapeutic outcomes and tumor evolution.

DISCLOSURE STATEMENT

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