MitoPerturb-Seq identifies links between nuclear and mitochondrial genomes in single cells

The replication, transcription and quality-control of mitochondrial DNA are tightly regulated by nuclear-encoded mitochondrial proteins. We developed MitoPerturb-Seq, a high-throughput single-cell approach to interrogate these nuclear–mitochondrial interactions. This method revealed cellular responses to the depletion of mitochondrial DNA, indicating that it will enable the discovery of cell-type specific vulnerabilities to mitochondrial dysfunction.

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The problem

Mitochondria contain their own genome. This mitochondrial DNA (mtDNA) comprises 37 genes, which are required for oxidative phosphorylation (OXPHOS)[1]. Each cell contains hundreds to thousands of copies of mtDNA, with the exact copy number (CN) varying from cell-to-cell in a tissue-specific manner[1]. Mutations in mtDNA often only affect a proportion of molecules in each cell — a state known as heteroplasmy[1].

Changes in MtDNA CN and heteroplasmy, which are sensed and controlled by nuclear-encoded mitochondrially-targeted proteins, can affect OXPHOS capacity, underly inherited mitochondrial diseases and contribute to aging and neurodegenerative conditions[1]. However, current knowledge of the nuclear–mitochondrial interactions governing these mtDNA dynamics remains incomplete. A deeper understanding of the nuclear-encoded pathways that modulate, sense and respond to these key mitochondrial parameters therefore has the potential to inform therapies targeting mitochondrial dysfunction.

The solution

We developed MitoPerturb-Seq, a single-cell workflow that integrates various previously reported technologies. Two key advances enabling MitoPerturb-Seq were single-cell CRISPR screening through CRISPR droplet sequencing (CROP-seq)[2] and multi-modal profiling of mtDNA, chromatin accessibility and gene expression via whole-cell combined assay for transposase-accessible chromatin using sequencing (ATAC-seq) and RNA sequencing (RNA-seq)[3] **(Fig.1a)**.

Using this integrated approach, we screened a library of 13 nuclear candidate genes that were previously suggested to be implicated in the regulation of mtDNA CN or heteroplasmy levels. Following CRISPR editing of target genes, we were able to simultaneously measure mtDNA abundance, heteroplasmy level and nuclear chromatin accessibility from single-cell ATAC-seq, as well as gene expression and, thanks to the CROP-seq library design, the identity of the CRISPR guide RNA (gRNA) from RNA-seq. These measurements allowed us to link nuclear gene perturbations with changes in mtDNA CN and/or heteroplasmy and the transcriptional and epigenetic responses to these changes at single-cell resolution.

Our MitoPerturb-Seq screen identified three genes — Tfam, Opa1 and Polg — that, when individually perturbed, caused mtDNA CN depletion (**Fig.1b**) and increased cell-to-cell variance of heteroplasmy levels (**Fig. 1c**). Analysis of differential gene expression revealed a common transcriptional response to these changes, which partly centred on induction of the mitochondrial integrated stress response (mtISR) through the transcription factor Atf4, as confirmed by Atf4 DNA adenine methyltransferase identification sequencing (DamID-seq)[4]. However, there was a marked difference in the strength of the transcriptional response to gene perturbation, with Opa1 knockout most strongly upregulating Atf4 target gene expression despite causing less profound mtDNA depletion than Tfam or Polg knockout.

Combined RNA and mtDNA profiling next allowed us to correlate mtDNA CN with cell-cycle progression; a progressive increase in CN across cell-cycle stages was seen and validated *in vitro* using the fluorescent ubiquitination-based cell cycle indicator (FUCCI) system. This result indicates that mtDNA replication is independent of cell-cycle stage.

Future directions

A key feature of MitoPerturb-Seq is its ability to measure differences in mtDNA CN and heteroplasmy at the single-cell level. Virtually every cell has a different mtDNA CN and a unique heteroplasmy level. The energy demand of individual cell-types varies widely, even within tissues, leading to highly divergent, disease-relevant responses to mtDNA variation. Until recently, most studies relied on bulk analysis of homogenised tissue to probe the mechanisms underlying mitochondrial pathology, obscuring cell-to-cell heterogeneity and limiting sensitivity. MitoPerturb-Seq represents a notable step forward, allowing us to capture the mosaic nature of mtDNA dynamics in disease-relevant contexts.

Our screen was performed in mouse embryonic fibroblasts carrying a specific heteroplasmic mtDNA variant (m.5024C>T in the $tRNA^{Ala}$ gene) and using a relatively small pooled CRISPR library. So, whilst the identification of Tfam, Opa1 and Polg as regulators of mtDNA CN provides a robust proof-of-principle for our workflow, we have not yet tapped into the potential of MitoPerturb-Seq to discover novel modulators of mtDNA across multiple cell-types.

Our future work will focus on increasing the throughput of MitoPerturb-Seq and incorporating additional mitochondrial and cellular phenotyping modalities[5], so that we can screen larger libraries across different heteroplasmic mtDNA variants and cell-types, including *in vivo*; ultimately, we hope to translate MitoPerturb-Seq into a clinical benefit for patients with mitochondrial disease.

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EXPERT OPINION

"This study introduces an integrative strategy that combines multiple concepts of single-cell genomic techniques to investigate mitochondrial genome regulation at the level of individual cells. The resulting approach has broad applicability to mitochondrial genetics and offers new opportunities to explore mtDNA–nuclear cross-talk across diverse cell types, organ systems, and disease contexts." **Leif S. Ludwig, Berlin Institute of Health, Berlin, Germany**

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FIGURE

Fig.1 | MitoPerturb-Seq allows the perturbation of mtDNA maintenance in single-cells. a. Schematic of the MitoPerturb-Seq workflow: transduction of a pooled CRISPR gRNA library is followed by cell fixation and permeabilization to allow combined RNA-seq and ATAC-seq from single whole cells that retain their mitochondria. RNA-seq identifies gRNAs and the single-cell transcriptome; ATAC-seq provides nuclear chromatin accessibility and assesses mtDNA coverage and heteroplasmy. **b, c**. Representative results from MitoPerturb-Seq in MEFs carrying a heteroplasmic $tRNA^{Ala}$ mutation. Single-cell mtDNA coverage (**b**) and heteroplasmy levels (**c**) for each of the genetic perturbations are shown, illustrating mtDNA depletion (pairwise t-tests with Bonferroni multiple testing correction) in cells with Tfam, Polg and Opa1 perturbation. © 202x, XXX

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BEHIND THE PAPER

We initially attempted to link mtDNA CN and heteroplasmy to transcriptional responses at the single-cell level using plate-based methods, such as genomic & transcriptomic sequencing (G&T-seq). However, this approach lacked the throughput required to profile multiple cell types or to conduct forward genetic screening. The breakthrough that made MitoPerturb-Seq possible was the ability to directly sequence mtDNA in single cells via whole-cell ATAC-seq[3].

When designing our experiments, we hoped to discover new modulators of mtDNA heteroplasmy, and conducted our pilot screens in heteroplasmic mouse cells. Even if we did not see shifts in heteroplasmy, we were really excited when the first data showed severe mtDNA depletion in cells transduced with certain gRNAs. This finding meant that we could now identify perturbations and study mtDNA within the same single cell to identify meaningful mtDNA-related phenotypes. MitoPerturb-Seq is already creating numerous opportunities to study previously inaccessible questions. **J.A. & S.B.**

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The figure for this Briefing is composed of **3** panels.

- Panel a = Fig **1a** from the original paper
- Panel b = Fig **2J** from the original paper
- Panel c = Fig **2K** from the original paper

Icons in Fig. 1a have been created with Bio-Render under license.

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References only above this box

FROM THE EDITOR

"Understanding how mutations on mitochondrial DNA give rise to disease is challenging due to heteroplasmy. By developing and implementing MitoPertub-Seq the authors overcome this limitation and unveil specific responses that regulate the response to challenges to mtDNA integrity." **Dimitris Typas, Senior Editor, *Nature Structural &***