And we examine how the interactions and protein networks change in different conditions, like in the presence of different drugs.

Just like genetics, proteomics alone has limited value. We put these pieces together. We combine genetics, proteomics, structural biology, and chemical biology to understand the functional relevance of our data.

How long does it take to make a PPI map?

It took many, many years to get to this point. I developed PPI maps in graduate school in budding yeast. We did that for a long time, but we pivoted when COVID-19 came along. We thought that if we knew what COVID-19 was doing, we could better predict future viral mutations and develop better vaccines and drugs.

We mapped the interactions between human and viral proteins for COVID-19 and other coronaviruses (5). We gathered a wealth of information in a matter of months, and at the time, I wondered how the cancer project took so many years. Out of fear during the pandemic, we quickly forced these proteomic and analytic technologies together and realized that the data available to develop PPI maps could be integrated more than we previously thought.

Forcing things to fit together can be incredibly powerful, both experimentally and computationally. That effort informed our work with cancer. We developed a disease agnostic pipeline that we can point at anything if we have a cell to look at and a list of potential disease-related genes.

CREDIT: NEVAN KROGAN

Nevan Krogan is a quantitative biologist from the University of California, San Francisco and the co-founder of the Cancer Cell Map Initiative.

When did you start your work developing PPI maps for cancer?

Trey Ideker, a biochemist from the University of California, San Diego and co-senior author on our recent set of *Science* papers, and I founded the Cancer Cell Map Initiative in 2014. We realized that sequencing data provided virtually unlimited information about cancer, but the functional units of a cell are proteins, not genes. Sequencing defined the sets of genes linked to cancer, but it was time for the next step. We wanted to look at the genomic data in a new way by developing these PPI maps. The goal of the initiative is to generate functional protein networks dysregulated in cancer and develop new therapeutic strategies for patients with cancer.

What was the most exciting finding in your recent set of studies?

We mainly focused on breast cancer and HNSCC in these studies. We looked at the genes most often mutated in these two cancers and subjected them to PPI mapping. In HNSCC, we looked at three different mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), a gene commonly mutated in cancer. The proteins interacting with PIK3CA were very different across mutations, but there was a set of point mutations that was prevalent in 5% of all cancers that resulted in a tighter connection with a protein called receptor tyrosine-protein kinase erbB-3 (HER3), which functions in the same pathway as PIK3CA. There is an antibody against HER3, so we wanted to know if HER3 inhibition could effectively treat cancer cells with mutations in PIK3CA. Cancer biologists told us that this inhibition would not work. This was true in most cases, but HER3 inhibition in mice with HNSCC tumors with PIK3CA mutations that caused a stronger interaction between PIK3CA and HER3 had a beautiful response. This has huge implications across many cancers.

Are you working on maps for other diseases?

We're developing maps for heart disease, neurodegenerative disease, pathogens, and other types of cancer. The next big set of papers we plan to publish will focus on neuropsychiatric disorders such as autism.

Our discovery platform can be applied to many different diseases. There's overlap between seemingly unrelated disorders at the biological level. The same genes mutated in breast cancer are being hijacked by COVID-19. The genes hijacked by Zika virus are mutated in Alzheimer's disease. These are things we don't see when looking at the individual gene level. Looking at complexes and pathways is where we will find the big discoveries. It will bring scientists from different disease areas together because a treatment for one disease could work for another. We're not just making connections between genes and proteins. We're making connections between scientists. That's the most exciting aspect of all of this. ■

This interview has been edited and condensed for clarity.

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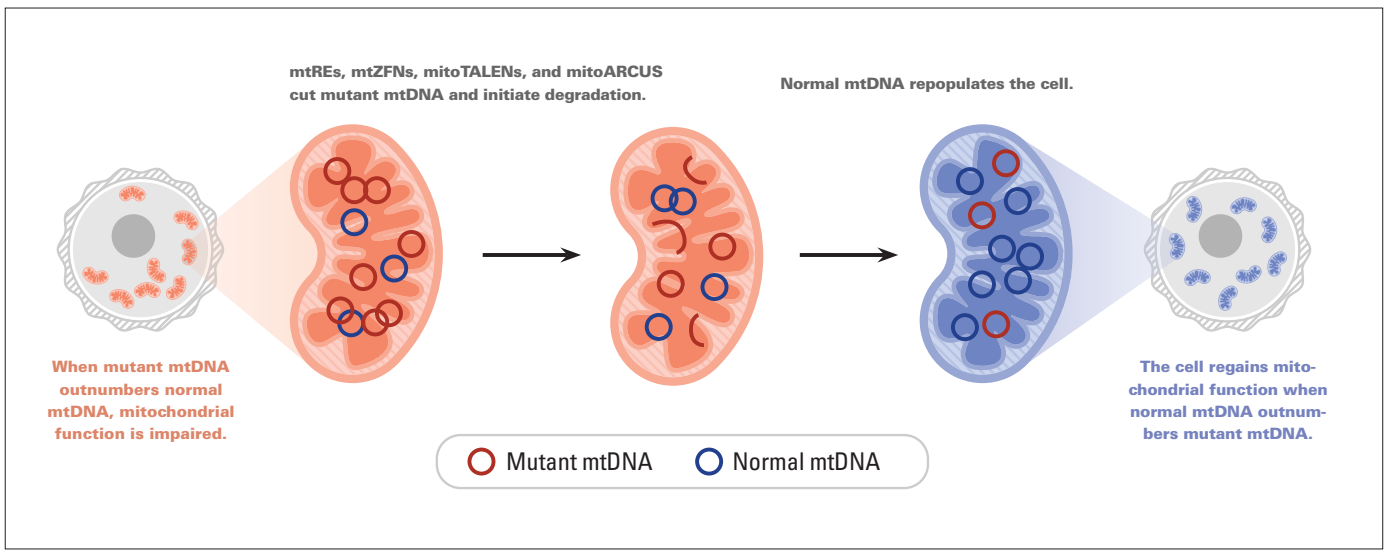
On the Road to Treating Mitochondrial Disease

Recent advancements in mitochondrial genome editing technologies take scientists one step closer to developing viable treatments for mitochondrial diseases, which affect 1 in 4300 adults.

BY DANIELLE GERHARD, PHD

MITOCHONDRIAL DISEASE, whether inherited or acquired over the lifespan, can result in devastating, multisystem symptoms and premature death. Although mitochondrial disease can result from mutations in the nuclear genome, a majority of adult-onset and a quarter of childhood-onset disease arises from mutations in mitochondrial DNA (mtDNA). Housed in the mitochondria, the powerhouses of cells, mtDNA exclusively encodes genetic information for mitochondrial function — namely energy production.

With no available treatments for mitochondrial disease, personalized symptom management is the primary therapy. However, recent technological advancements in mitochondrial genome editing give hope that therapies are within reach.



CREDIT: ASHLEIGH CAMPSALL

Most of the therapeutic strategies for mitochondrial diseases are designed to shift heteroplasmy, or the ratio of normal mitochondrial DNA (mtDNA) (blue circles) to mutant mtDNA (red circles) in cells. When mutant mtDNA outnumbers normal mtDNA, cells struggle to perform essential mitochondrial functions, and clinical symptoms emerge. Mitochondrially-targeted restriction endonucleases and programmable endonucleases and meganucleases (like mtZFNs, mitoTALENs, and mitoARCUS) produce double-stranded breaks in mutant mtDNA to initiate their degradation. As mutant mtDNA are eliminated, normal mtDNA repopulate the cell and restore mitochondrial function.

Shifting heteroplasmy

A single human cell has only one nucleus, but it can house hundreds of mitochondria. Each mitochondrion contains numerous copies of circular mtDNA that are continuously replicated. This adds up to nearly 1000 copies of mtDNA per cell. In a given cell, pathogenic mutant mtDNA often coexists with normal mtDNA in a state referred to as heteroplasmy. The presence of mutated mtDNA by itself doesn't cause disease, but disease results from unfavorable ratios of mutant to normal mtDNA. Once the number of mutant mtDNA copies surpasses a certain threshold and outnumbers the normal mtDNA, clinical symptoms emerge.

This sliding scale of heteroplasmy and pathogenicity raises an interesting clinical possibility when it comes to designing effective therapies for mitochondrial diseases. "You really don't have to eliminate mutant mitochondrial DNA completely," said Carlos Moraes, a mitochondrial biologist at the University of Miami Miller School of Medicine. "You can just reduce it by 20 or 30%, sometimes even less, and have a very dramatic phenotypic difference."

Reducing mutant mtDNA is no easy feat, but techniques are constantly improving. Despite the remarkable advancements seen in nuclear genome engineering using CRISPR-Cas9 technology, its use in mitochondria is controversial (1). This has led researchers to pursue alternative approaches.

Restriction endonucleases open the door to mtDNA editing

One reason for an overabundance of mtDNA in a cell is the absence of a good damage repair system. Unlike nuclear DNA, mtDNA lacks a reliable repair mechanism for double-strand breaks. "The general rule is that if there's a double-strand break, the cell prefers to degrade this molecule rather than trying to repair it," said Michal Minczuk, a mitochondrial biologist at the University of Cambridge.

In the early 2000s, with a genetic approach for targeting mtDNA unavailable, researchers focused their efforts towards shifting heteroplasmy using restriction endonucleases. Mitochondrially targeted restriction endonucleases (mtREs) recognize specific nucleotide sequences and induce double-strand breaks to linearize mtDNA and trigger its degradation.

In 2001, Moraes and his colleagues described their design for a plasmid containing an mtRE (2). They tested their system in cultured cells that had a mix of normal mtDNA and mutated mtDNA engineered to express the specific nucleotide sequence targeted by their mtRE plasmid. In this proof-of-concept study, they noticed an increase in normal mtDNA, suggesting that mtREs eliminated mutant mtDNA and shifted heteroplasmy. In a 2005 follow-up study, the same group demonstrated mtREs in a mouse model with two non-pathogenic mtDNA genotypes (3).

Since then, mtREs have provided valuable insights into how to shift heteroplasmy and how mtDNA degrades after treatment (4), but the approach relies on naturally-occurring restriction endonucleases that target specific sites in mtDNA; the chance that mtREs target a unique pathogenic mutation are slim. "We knew that the mtRE approach was very limited, so we were waiting over the years until DNA editing enzymes started to appear," said Moares.

Programmable nucleases provide selective targeting

To target disease causing mtDNA mutations, researchers turned to artificial means. In 2008, while he was a postdoctoral fellow at the University of Cambridge, Minczuk and his colleagues tested an artificial restriction enzyme known as a zinc finger nuclease (ZFN), which can be customized to target specific DNA sequences.

They focused on correcting a mutation in a mitochondrial gene that shows up in two maternally-inherited mitochondrial diseases that can't be targeted by any known mtREs. Minczuk's team first worked out how to transport their ZFN into mitochondria to create a mitochondrially-targeted zinc finger nuclease (mtZFN) (5).

Then they tested the new tool in cultured cybrid cells, which are cells created by fusing nuclear genes from one cell line with mitochondrial genes of another cell line, allowing them to parse out the contributions of mtDNA in disease (6). When Minczuk and his team applied their mtZFN to the cybrid cells, the mutated mtDNA degraded, providing valuable evidence that programmable endonucleases held promise for targeting unique pathogenic mutations in mtDNA.

Minczuk continued down this road, making additional modifications to his mtZFN system to improve binding specificity and efficiently target point mutations and deletions in mtDNA (7).

Around the same time, Moraes and his colleagues released another programmable endonuclease, mitochondrially-targeted TAL-effector Nucleases (mitoTALENs). They demonstrated their mitoTALENs in 2013 by permanently shifting heteroplasmy in patient-derived cybrid cells with point mutations or deletions (8).

> "You really don't have to eliminate mutant mitochondrial DNA completely. You can just reduce it by 20 or 30 percent, sometimes even less, and have a very dramatic phenotypic difference."
>
> *- Carlos Moraes, University of Miami Miller School of Medicine*

Carlos Moraes, a mitochondrial biologist at the University of Miami Miller School of Medicine, pioneered restriction endonucleases for degrading mutant mitochondrial DNA. Now, his research focuses on improving programmable endonuclease and meganuclease systems like mitoTALENs and mitoARCUS.

CREDIT: CARLOS MORAES

Up until 2016, the only method available for testing these tools against pathogenic mtDNA mutants were heteroplasmic cultured cybrid cells. But this changed when scientists introduced the first mouse model for a heteroplasmic pathogenic mtDNA mutation (9). Using this new mouse model, both Minczuk and Moraes validated their mtZFN and mitoTALEN systems *in vivo* (10,11).

Meganucleases minimize drawbacks

mtZFNs and mitoTALENs successfully shift heteroplasmy *in vitro* and *in vivo*, but there are still roadblocks for advancing these treatments to the clinic.

"MitoTALENs are great, and they're very specific, but they have the problem that they're very large proteins," said Moraes. This makes packing them into small adeno-associated viruses (AAV), commonly used to deliver gene therapies to humans, extremely difficult. To introduce the mitoTALEN to mice, Moraes needed to inject two viruses simultaneously. "If you think of translating it to the clinic, it's cumbersome and expensive already to do with one virus, so you can imagine with two. Over the years, we have been trying to find simpler platforms that retain that kind of specificity."

Michal Minczuk, a mitochondrial biologist at the University of Cambridge, modified zinc finger nuclease technology to target specific sequences in mitochondrial DNA.

CREDIT: MICHAL MINCZUK

Earlier this year, Moraes and his team introduced a new mitochondrially-targeted meganuclease (12). Compared to other nucleases, meganucleases contain a larger recognition site, but are smaller in size, lending to a better fit inside a viral delivery system.

In collaboration with scientists at Precision Biosciences, Moraes developed a new gene editing platform that uses a mitochondrially-targeted I-CreI meganuclease (mitoARCUS) designed against a unique mitochondrial mutation. When the team delivered mitoARCUS into mice with mutated mtDNA, it eliminated mutant mtDNA in the liver. With its compact size and enhanced specificity, the mitoARCUS system is a promising tool for removing mutant mtDNA.

Breakthroughs with base editors

Base editing tools like the popular CRISPR-Cas9 system are attractive to scientists working with mtDNA, but that technology requires customized guide RNAs to direct the enzymes where to cut the DNA. Mitochondria seem to lack the machinery needed to import the single-stranded guide RNA.

In 2020, a groundbreaking study in *Nature* introduced a novel, CRISPR-free approach for base editing mtDNA (13). A research group at Harvard University discovered a bacterial toxin they named DddA that can base edit double stranded DNA and enter mitochondria. The researchers used this system to edit human mtDNA genes, successfully converting cytosine to thymine in different locations throughout the genome, showing for the first time that base editing is possible in mitochondria.

Currently, DddA only introduces cytosine to thymine edits, but this new technology holds exciting possibilities for mitochondrial gene correction (14). "This tool will also be very useful in the development of novel mouse models where we will be able to engineer a certain type of mutation by embryo or somatic delivery of base editors," said Minczuk. ■

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