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RESEARCH HIGHLIGHT
Krebs and an alternative TCA cycle!

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The tricarboxylic acid (TCA) cycle, first described by Krebs in the 1930s, is a center of activity for cellular metabolism, with respiratory organisms feeding specific nutrients into its cyclic sequence of catabolic biochemical reactions to produce energy and metabolites that underlie cell viability, proliferation, and function. Recently, a study by Arnold et al. in *Nature* identified an alternative, sub-compartmentalized version of the TCA cycle whose engagement is essential for changes in cell state.

Over the past century, our appreciation for and understanding of cellular metabolism and its regulation in cell physiology and disease has increased exponentially.[1] Starting in the early 1900s, biochemists began detailing the basic features of cellular metabolism. In 1937, Sir Hans Krebs (and William Johnson) introduced the citric acid cycle, also known as the tricarboxylic acid (TCA) cycle,[2] which is widely recognized as a center of activity for metabolism in respiring cells and organisms. Krebs's original description was of a cyclic sequence of eight biochemical reactions that catabolize nutrients to release intermediates and reducing equivalents as cellular currencies to meet metabolic demands for energy and biosynthesis. In mammalian cells, these reactions occur in the mitochondrial matrix with the currencies produced appearing identical for all cell types (i.e., acetyl-CoA, ATP, NAD^+, NADH), although the metabolic demands for each cell is context dependent and determined by the cellular microenvironment and tissue function.[1] The physiology of each cell requires different ratios of the metabolic currencies generated by distinct regulatory circuits, such as heterogenous TCA cycle substrate preferences and differential enzymatic activities,[3] to meet these requirements. How cells engage substrates and components of the TCA cycle preferentially for specific physiologies or pathologies remains incompletely understood.

Addressing this gap in knowledge resulted in a recent *Nature* study by Arnold et al.,[4] who queried whether core metabolism enzymes formed discrete functional modules based upon pairwise correlations of gene essentiality scores. Data for this computational assessment came from a Broad Institute resource, DepMap, which includes datasets from hundreds of cancer cells that describe gene co-dependencies using co-essentiality mapping from genome-wide CRISPR knockout screens. Unexpectedly, mining this co-essentiality mapping data revealed that TCA cycle-related genes clustered together but split into two distinct functional modules that depended on differences in the handling of citrate, a TCA cycle intermediate metabolite. One module

clustered genes associated with traditional TCA pathway cycling of citrate within mitochondria, as originally described by Krebs. In contrast, a second module clustered genes whose products represented an alternative, non-canonical TCA cycle-mapped biochemical pathway for the enzymatic conversion of citrate to malate. In this alternative pathway, citrate is exported out of mitochondria into the cytosol using a citrate/malate antiporter, SLC25A1. In the cytosol, ATP citrate lyase (ACL) cleaves citrate to produce acetyl-CoA and oxaloacetate, with malate dehydrogenase (MDH) converting oxaloacetate into malate. The subsequent import of malate through the SLC25A1 antiporter and mitochondrial matrix generation of oxaloacetate completes an alternative turn of the TCA cycle (Fig. 1).

An essential feature of this newly identified, bi-compartmentalized mammalian TCA cycle is the export of citrate from the mitochondria into the cytosol and concurrent import of malate from the cytosol into the mitochondria, thereby providing a coordinated mechanism for proliferating, respiratory cells to meet increasing energetic and biosynthetic demands simultaneously. In contrast to the traditional TCA cycle, use of this non-canonical citrate/malate shuttle blocks the loss of carbons as CO_2 in traditional cycling and instead produces the two-carbon carrier, acetyl-CoA, in the cytosol, which becomes available for acetylation reactions, de novo lipogenesis and sterol biosynthesis (Fig. 1). This TCA cycle alternative also regenerates cytosolic NAD^+ (Fig. 1) required to sustain continuous glucose oxidation to preserve proliferation. Rapidly proliferating cells, such as naïve or ground state mouse embryonic stem cells (mESCs) and cancer cells, were shown to engage the non-canonical TCA cycle, to support anabolic growth and build biomass for cell replication, rather than solely rely upon ATP-producing catabolic metabolism of the traditional TCA cycle.

Arnold et al. further showed that appropriate TCA cycle engagement is cell state dependent and a required component of cell state transitions. For example, during myogenic differentiation, mouse myoblasts activate the alternative TCA cycle pathway and then switch to the traditional TCA cycle during a transition to less proliferative mature myotubes, which aligns with Krebs's original findings on pigeon breast muscle.[5] A molecular mechanism for this cycle switch was not provided, although many TCA cycle genes are predicted targets of the myogenic transcription factor, MyoD. Similarly, during mESC differentiation, naïve mESCs undergo a metabolic shift upon exiting pluripotency, switching from canonical to alternative TCA cycle usage. Appropriate TCA

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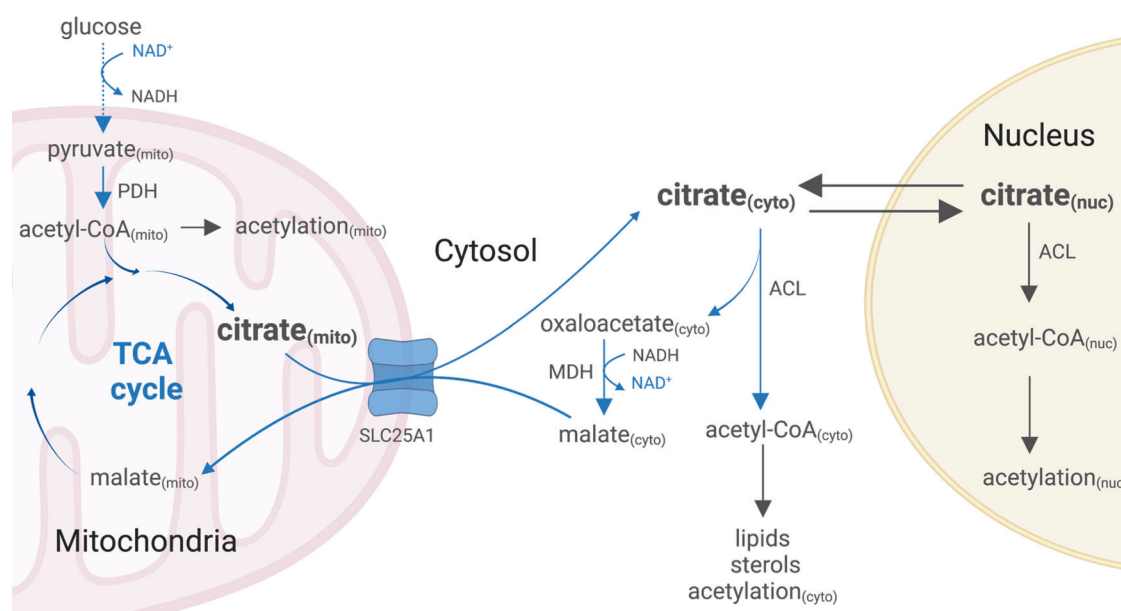
Fig. 1 A non-canonical TCA cycle. Arnold, Jackson and colleagues propose an alternative TCA cycle wherein citrate is exported out of the mitochondria (mito) to the cytosol (cyto) by citrate/malate antiporter SLC25A1 and then cleaved by ACL into (1) cytosolic acetyl-CoA for biosynthetic and acetylation reactions, and (2) oxaloacetate to regenerate NAD^+ and produce malate, which is then imported into mitochondria to complete one turn of the TCA cycle without the loss of CO_2. Cytosolic citrate could also contribute to nuclear (nuc) citrate concentrations, wherein ACL also converts citrate into acetyl-CoA to power acetylation reactions. Created with BioRender.com.

cycle engagement was indispensable for this fate transition because ACL inhibition impaired the exit of naive mESCs from pluripotency. It remains open whether this TCA cycle switch is a driver or an essential but passive condition for mESC exit from pluripotency or other cell state transitions. Nevertheless, these combined results reveal yet another component of dynamic TCA cycle behavior in different mammalian cell types and states, with engagement of the correct TCA cycle activity required for cell fate transitions, further illustrating an active, or at least permissive, role that cellular metabolism has in cell identity.[6]

The requirement of differential TCA cycle engagement could also link cycle activity to histone acetylation and gene expression. The epigenome is sensitive to metabolite pools that can serve as substrates and co-factors for chromatin-modifying enzymes. Global histone modifications can be dynamically regulated by changes in acetyl-CoA pools,[7] and, in particular, switching from the traditional to alternative TCA cycle activity could increase histone acetylation through ACL-dependent production of additional cytosolic acetyl-CoA[8] (Fig. 1). ACL is localized in both the cytosol and nucleus and contributes to acetyl-CoA production in both compartments. Considering how the role of metabolites varies within different subcellular compartments, such as in the mitochondria, cytosol, or nucleus,[9] the site(s) of acetyl-CoA generation and trafficking may influence cell fate specificity. A distinction between mitochondrial and nuclear-cytoplasmic acetyl-CoA pools is well-established, and recent studies have also shown that the nuclear pool is distinct from the cytosolic pool.[7,10] Although Arnold et al. used clever ^{13}C-nutrient isotope tracing to

quantify compartment-specific metabolism, and [2]H-glucose tracing to indirectly dissect NAD^+/NADH pools in the mitochondria and cytosol, it could be interesting to also examine the impact of this new pathway on the nucleus. Differences in subcellular crosstalk from traditional versus alternative TCA cycle pathways could regulate cellular identify and potentially offer new strategies to enrich for cells at different developmental or functional states, for fundamental studies or applications in biomedicine. Lastly, this new pathway is termed the non-canonical or alternative TCA cycle. It probably deserves a better name!

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