

# Mitochondria and the future of RASopathies: the emergence of bioenergetics

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**RASopathies are a family of rare autosomal dominant disorders that affect the canonical Ras/MAPK signaling pathway and manifest as neurodevelopmental systemic syndromes, including Costello syndrome (CS). In this issue of the *JCI*, Dard et al. describe the molecular determinants of CS using a myriad of genetically modified models, including mice expressing HRAS p.G12S, patient-derived skin fibroblasts, hiPSC-derived human cardiomyocytes, an HRAS p.G12V zebrafish model, and human lentivirally induced fibroblasts overexpressing HRAS p.G12S or HRAS p.G12A. Mitochondrial proteostasis and oxidative phosphorylation were altered in CS, and inhibition of the AMPK signaling pathway mediated bioenergetic changes. Importantly, the pharmacological induction of this pathway restored cardiac function and reduced the developmental defects associated with CS. These findings identify a role for altered bioenergetics and provide insights into more effective treatment strategies for patients with RASopathies.**

## RASopathies

RASopathies, a group of syndromic disorders caused by germline mutations in genes that affect the canonical Ras/MAPK signaling pathway, include Noonan syndrome (NS), NS with multiple lentiginos (NSML), Costello syndrome (CS), cardio-faciocutaneous syndrome (CFCS), neurofibromatosis type 1 (NF1), and other clinically related diseases (Figure 1A and ref. 1). Though individually rare, collectively, this family of disorders constitutes one of the world's largest groups of congenital diseases. Germline pathogenic variants result in similar yet distinct syndromes, the phenotypic characteristics of which can include facial abnormalities, short stature,

cardiac defects, hematopoietic defects, skeletal malformations, and certain types of cancer (1-3). These characteristics can be severe and life-threatening and may be present at birth or develop throughout one's lifetime. Unfortunately, effective targeted therapies for RASopathies remain elusive, with limited to no options available for most patients. Therefore, there is a critical need to identify effective treatments. Indeed, understanding the causal mechanisms associated with the development of each disease uniquely, through identification of the distinct point mutations within common genes, the panoply of signaling pathways affected by the genetic anomalies, and the potential molecular targets

associated with each, may help us find targeted and personalized approaches to treating patients (4).

The Ras/MAPK signaling pathway is critical for cellular homeostasis, cell differentiation, proliferation, and survival. Gain-of-function mutations and/or increased Ras and MAPK activities are associated with development of RASopathies and many other diseases, including cancer. However, because Ras/MAPK signaling is required for a multitude of cellular processes, identifying the appropriate inhibitors and their required level of inhibition to provide therapeutic efficacy without intolerable side effects, remains challenging.

A myriad of animal model systems developed to study RASopathies has helped identify many (or most) of the causal genes associated with this group of disorders. The pioneering studies also helped determine aberrant molecular functions of gene mutations, identifying possible therapeutic targets. More recently, tissue generated from human inducible pluripotent stem cells (iPSCs) has provided a powerful and versatile tool for studying human disease development in vitro as well as a system that allows investigation of disease pathophysiology and rapid high-throughput therapeutic testing. In the accompanying paper, Dard et al. utilized this iPSC model and a panoply of additional genetically modified models to understand the signaling pathways affected by mutations causal to CS (5).

## CS

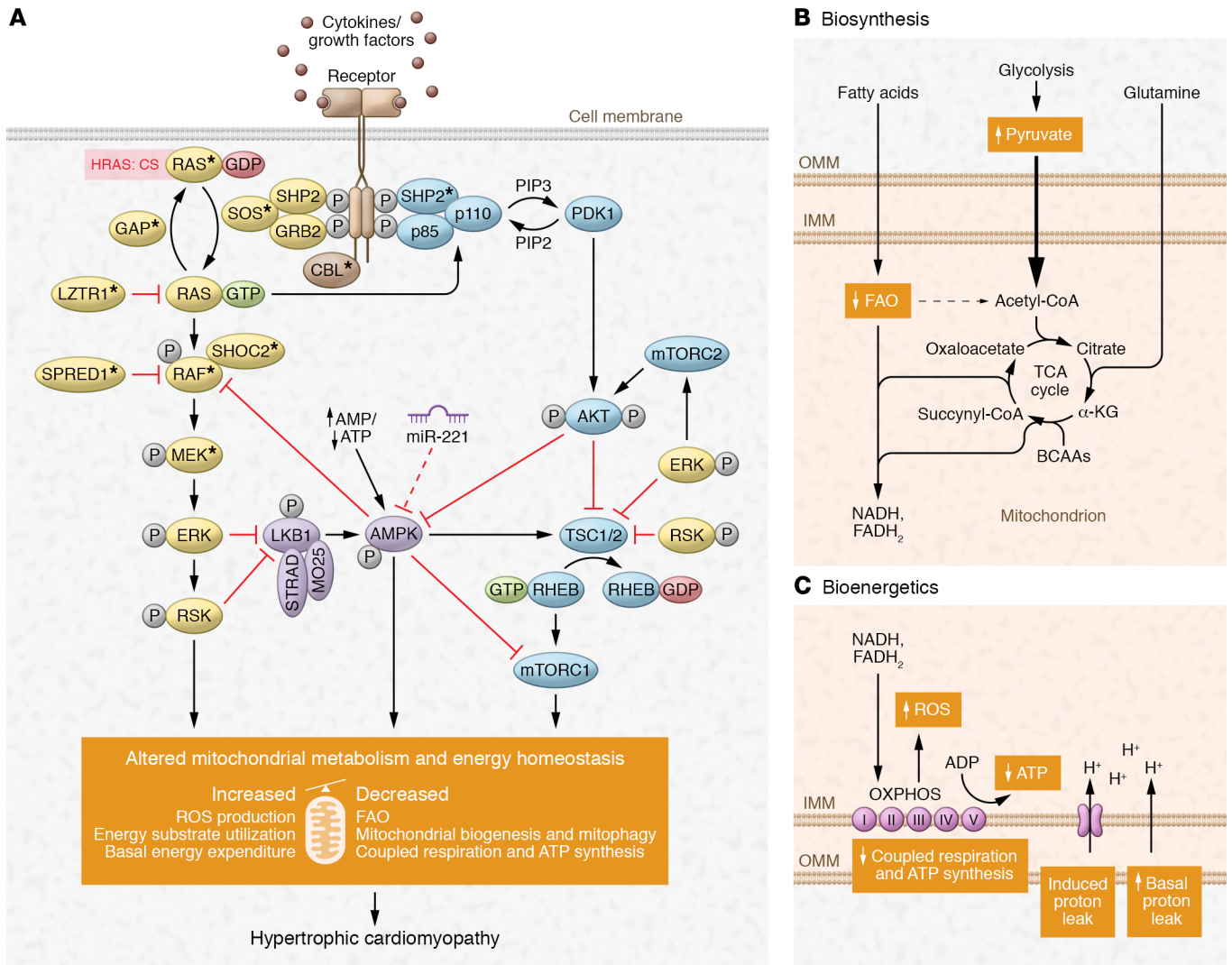
CS is a RASopathy caused by heterozygous gain-of-function germline mutations in HRAS. More than 80 percent of individuals with CS have mutations in the G12 position of HRAS (p.G12S variant). Importantly, the severity of the effects on downstream Ras and MAPK signaling depends largely on the specific mutations in HRAS directly affecting targeted tissues and cellular expression. Individuals with CS typically

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**Figure 1. Crosstalk among RAS/MAPK, PI3K/AKT-mTOR, and AMPK pathways in RASopathy-induced mitochondrial biogenesis.** (A) RASopathies are a group of developmental disorders that include CS. Ligands binding to tyrosine kinase receptors recruit proteins, such as GRB2 and SHP2, to the membrane and aid in the transactivation of guanine nucleotide exchange factors, such as son of sevenless (SOS). SOS catalyzes RAS activation to initiate signaling events involving the kinases RAF, MEK, ERK, and RSK. GTPase activating proteins hydrolyze GTP to GDP, which inactivates RAS GTPases and terminates downstream signaling. Crosstalk between the RAS/MAPK and PI3K/AKT-mTOR pathways can occur in a cell-type and stimulation-dependent manner. AMP-activated protein kinase (AMPK) is a key regulator of cellular energy homeostasis. In healthy cells, the three pathways crosstalk to balance energy availability with cellular function. RASopathy mutations impair the energy balance, altering AMPK protein expression levels and inducing aberrant post-translational modifications. miR-221\* may inhibit AMPK expression and serve as a downstream effector of RAS/MAPK signaling. (B) Under physiological conditions, the mitochondrial TCA cycle uses intermediates from catabolism of energy substrates, such as fatty acids, glucose, glutamine, and branched chain amino acids (BCAAs), to produce energy-rich FADH<sub>2</sub> and NADH, components for biosynthesis and OXPHOS. In RASopathies, mitochondrial ATP production may favor pyruvate derived from glycolysis over substrate derived from fatty acid oxidation (FAO). OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane;  $\alpha$ -KG,  $\alpha$ -ketoglutarate. (C) Mitochondrial OXPHOS efficiently produces ATP. Electrons from electron donors FADH<sub>2</sub> and NADH flow through a series of protein complexes (I–IV) within the mitochondrial membrane and pump protons from the matrix and across the inner membrane. The electrochemical proton gradient generates free energy that is coupled with the synthesis of ATP from ADP and Pi by complex V (ATP synthase). Dysfunctional mitochondrial complexes in RASopathies lead to increased basal proton leaks, decreased ATP, and increased ROS production, resulting in HCM. Asterisks indicate RASopathy mutations.

present with increased birth weight, craniofacial abnormalities, and gastroesophageal reflux with oral aversion, which results in a failure to thrive. Individuals can also have skin anomalies, including excessive wrinkling, redundancy of skin over the hands and feet, deep plantar and palmar

creases, and increased risk of benign or malignant tumors. Musculoskeletal abnormalities, such as hypotonia, are also reported. Most importantly, like many of the other RASopathies, the most detrimental aspect of CS is the development of severe cardiac hypertrophy. However, the molec-

ular mechanisms linking HRAS activation with cardiac dysfunction remain unclear.

A recent translational study of 11 patients with CS showed bioenergetic anomalies, including a low fasting blood glucose, low levels of insulin-like growth factor-1, and elevated total cholesterol (6).

Moreover, patients had increased resting energy expenditure, indicating increased cellular basal metabolism (6). Similarly, HRAS p.G12S mice show mitochondrial fatty acid oxidation defects, hypoglycemia, poor weight gain, and cardiomyopathy (7).

In this issue of the *JCI*, Dard et al. suggest that bioenergetic alterations of the heart cause CS-associated hypertrophic cardiomyopathy (HCM) (5). Indeed, disruption of mitochondrial function is a recognized contributing factor in stress-induced heart disease and cardiomyopathy. The findings reveal disruption of mitochondrial proteostasis and defective oxidative phosphorylation in the hearts and skeletal muscle of CS mice (5). Importantly, the authors also uncovered a mechanism that implicates inhibition of AMP-activated protein kinase (AMPK) in CS. The observation that AMPK signaling is involved in mitochondrial turnover, proteostasis, and bioenergetics is not new; neonatal cardiomyocytes from AMPK $\alpha$ 2-knockout mice show repressed expression of energy metabolism-related genes (8). However, Dard and colleagues described the role of AMPK in RASopathies (5), and its effects on mediating aberrant mitochondrial energetics in these disorders may reveal targets for treatment of HCM in patients with CM. Analysis of the master regulators, such as AMPK, on mitochondrial fatty acid oxidation might help identify what role HRAS mutations have in the bioenergetic process.

## Bioenergetics

Cells metabolize glucose to carbon dioxide through the mitochondrial TCA cycle and through oxidative phosphorylation (OXPHOS), which is the most effective way to generate ATP (refs. 9, 10 and Figure 1, B and C). Relative amounts of ATP, ADP, AMP, and phosphocreatine/creatinine modulate intracellular energy, while levels of glucose, fatty acids, and amino acids regulate nutrient status (9). In addition, calcium and ROS influence cellular energy metabolism (ref. 10 and Figure 1, B and C). Importantly, transcriptional regulation and activation of downstream RAS signaling pathways control metabolic sensors (10). One example involves the activation of the AMPKs. Once activated, AMPKs induce catabolic pathways needed to produce ATP, increase glycolysis,

and enhance oxidation phosphorylation, through mitochondrial biogenesis, while simultaneously switching off the anabolic pathways that consume ATP, such as protein, fatty acid, and cholesterol synthesis (9). Consequently, AMPKs serve as critical modulators of bioenergetics, targeting multiple genes involved in lipid, glucose, and protein metabolism, including peroxisome proliferator-activated receptor  $\gamma$  coactivator 1- $\alpha$  (PGC1 $\alpha$ ) as well as genes involved in cell growth, autophagy, polarity, transcription, and ion transport (11).

## Bioenergetic dysfunction in Ras/MAPK signaling

Many symptoms associated with mitochondrial diseases are also present in RASopathies, especially those RASopathies that develop cardiomyopathies. Like the mitochondrial mutations that cause mitochondrial disorders, increased Ras/MAPK signaling can promote anaerobic glycolysis, glucose uptake, autophagy, lipid synthesis, and nucleotide synthesis through the pentose phosphate pathway (9). Increased pathway activation can also modulate mitochondrial respiration, although the mechanisms remain unclear. Therefore, it seems probable that gain-of-function mutations associated with increased Ras/MAPK signaling in RASopathies also alter bioenergetics, leading to increased mitochondrial dysfunction and worsened pathological outcomes. Dard et al. identified molecular abnormalities that affect mitochondrial homeostasis in CS (5). Previous studies in HRAS<sup>G12S/+</sup>-transgenic mice also identified effects in energy homeostasis in CS, with decreased fatty acid oxidation, altered metabolism of glucose, and impaired energy homeostasis (7).

Bioenergetic effects of Ras/MAPK activation have been extensively studied in cancer, where pathway activation is linked to increased turnover of mitochondria through enhanced mitophagy, modulating mitochondrial fission and dynamics (9). However, mitophagy alone cannot explain the mechanisms linking Ras and MAPK activation with OXPHOS; a concomitant increase in mitochondrial biogenesis is also likely required. ROS produced mitochondrially may play a role in cancer cell transformation by RAS or may mediate oncogene-induced cellular senescence (8). Moreover, HRAS p.G12V

mutations can stimulate mitochondrial metabolism and increase the rate of oxygen consumption (12, 13). Finally, AMPK signaling has been associated with mitochondrial proteostasis (11, 14); its activation induces mitophagy and the selective degradation of mitochondria by autophagy and stimulates mitochondrial biogenesis through PGC1 $\alpha$  (9). However, how any or all these processes directly tie into Ras/MAPK signaling and how they specifically affect RASopathies remains unclear (15).

AMPK dysfunction and mitochondrial respiratory chain defects are frequent causes of HCM and cardiomegaly (8, 16–19). Dard et al. (5) showed that both mRNA and protein expression levels of AMPK were strongly inhibited in CS. Likely, the combination of aberrant signaling upstream of AMPK, disrupted localization of signaling effectors, and/or changes in cellular distribution contributed to the decreased AMPK activity observed in CS. The authors also suggested another explanation: a dysregulation in modulating microRNAs (miRs). Specifically, they identified an induction in miR-221\*, which they suggest modulates and reduced AMPK expression and activity in CS cell models. Previous work established a potential role for miR-221\* in Ras activation (20). Dard and authors also established an association between miR-221\* and AMPK $\alpha$ 2 expression; inhibiting miR-221\* in CS models rescued cellular energy homeostasis (5). However, given that miRs often have multiple targets with differential expression between mice and humans, as well as other limitations, additional studies are required to fully understand how miR-221\*, or other miRs, affects RASopathies and if the inhibition of miRs can reverse or treat patient phenotypes, including HCM, short stature, and/or the craniofacial anomalies found in RASopathies.

## Bioenergetic dysfunction in other RASopathies

Mutations in mitochondrial genes in RASopathies suggest an overlap in mitochondrial biogenesis. Though not specifically evaluated by Dard et al. (5), mitochondrial dysfunction in RAS-related genetic disease manifests in observable mitochondrial defects, decreased adipogenesis, fat malabsorption, lymphatic

disorders, increased energy expenditure, and enhanced insulin signaling (9). In NS, mutations in *PTPN11*, the gene encoding the protein tyrosine phosphatase SHP2, lower mitochondrial membrane potential and ATP cellular content and increase ROS levels (21). Increased SHP2 activity has also been associated with increased complex IV activity and decreased ATP synthesis, altering cellular energy homeostasis (22, 23). Conversely, a BRAF-associated NSML mutation had decreased complex I activity (22). In NF1, data are more controversial; whereas decreased mitochondrial respiration, mediated by inhibition of respiratory chain complex II was noted in cells lacking NF1, increased mitochondrial respiration was observed in *Drosophila* lacking NF1 (24). Previous studies in CFC Syndrome also indicate mitochondrial dysfunction, with multiple enzyme deficiencies in OXPHOS, reduced ATP production, and a deficiency in muscular coenzyme Q, an enzyme required for transport of electrons from complexes I and II to complex III (22).

## Factors contributing to mitochondrial dysfunction in RASopathies

Germline or somatic HRASG12S/A mutations can alter mitochondrial proteome homeostasis and OXPHOS in a tissue-specific manner (5). Indeed, at the level of the respiratory chain, tissues exhibit large differences in how they compose OXPHOS machinery (10). Heterogeneity of mitochondrial function within different cells means changes in cellular and mitochondrial microenvironments may regulate energy production. In CS, these differences in tissue expression can affect craniofacial development, heart structure and function, skin, and predisposition to cancer (25). Differences in the way RASopathy mutations interact with downstream effectors of the Ras/MAPK pathway also affect tissue function to mediate disease severity. However, how tissue specificity, localization, crosstalk, and/or degree of expressivity in various cell types and tissues contribute to mitochondrial dysfunction and bioenergetics in RASopathies remains unknown. Dard et al. suggest that AMPK inhibition alone cannot sufficiently induce heart dysfunction; additional stressors, such as dysregulated signaling

through the HRAS/MAPK pathway or another challenge in mitochondrial bioenergetics, must occur (5).

## Potential therapeutic approaches and targeted treatments

The pathophysiology of most RASopathies includes systemic alterations of energy metabolism, but the molecular mechanisms associated with this dysfunction were previously unknown. Therefore, the therapeutic implications of this study are great, in that they broaden the scope of the therapeutic potential for treating patients with RASopathies. First, metabolic remodeling and hormonal control of glucose and lipid catabolism can modulate bioenergetic regulation (26). Therefore, drugs that drive energy metabolism (e.g., AICAR, bezafibrate, resveratrol, cannabinoids, vitamins, etc.) could be considered as modulators of bioenergetics in RASopathies (27). Here, too, Dard et al. identified a benefit in treating CS with a downstream effector of AMPK, bezafibrate, an agonist of PPAR $\alpha$  (ref. 28). The authors rescued or prevented defective mitochondrial dysfunction, improved heart function, and increased survival in various CS models (5). Moreover, they went on to show that a combination of bezafibrate with urolithin A, a natural compound produced by gut bacteria (29, 30), provided a synergistic effect, rescuing the genetic developmental defects in the CS zebrafish model and preventing the HCM phenotype in the CS mouse (5).

Clinical trials for use of Ras and MAPK inhibitors in RASopathies are ongoing, but the results appear promising (31). Combining these inhibitors, perhaps at lower concentrations to prevent side effects, with bioenergetics compounds could prove fruitful. The findings by Dard et al. (5) provide optimism that targeted and effective treatments for RASopathies are on the horizon.

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