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ORIGINAL ARTICLE

# Rapamycin increases oxidative metabolism and enhances metabolic flexibility in human cardiac fibroblasts

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**Abstract** Inhibition of mTOR signaling using rapamycin has been shown to increase lifespan and healthspan in multiple model organisms; however, the precise mechanisms for the beneficial effects of rapamycin remain uncertain. We have previously reported that rapamycin delays senescence in human cells and that enhanced mitochondrial biogenesis and protection from mitochondrial stress is one component of the benefit provided by rapamycin treatment. Here, using two models of senescence, replicative senescence and senescence induced by the presence of the Hutchinson-Gilford progeria lamin A mutation, we report that senescence is accompanied by elevated glycolysis and increased oxidative phosphorylation, which are both reduced by rapamycin. Measurements of mitochondrial function indicate that direct mitochondria targets of rapamycin are succinate dehydrogenase and matrix alanine aminotransferase. Elevated activity of these enzymes could be part of complex mechanisms that enable mitochondria to resume their optimal oxidative phosphorylation and resist senescence. This interpretation is supported by the fact that rapamycin-treated cultures do not undergo a premature senescence in response to the replacement of glucose with galactose in the culture medium, which forces a greater reliance on oxidative phosphorylation. Additionally, long-term treatment with rapamycin increases expression of the mitochondrial carrier protein UCP2, which facilitates the movement of metabolic intermediates across the mitochondrial membrane. The results suggest that rapamycin impacts mitochondrial function both through direct interaction with the mitochondria and through altered gene expression of mitochondrial carrier proteins.

**Keywords** Senescence · Cardiac fibroblasts · Oxidative phosphorylation · Alanine aminotransferase · Rapamycin · Aging

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## Introduction

Cardiac fibroblasts represent the largest population of cells in the human heart. As with most fibroblast connective tissue cells, cardiac fibroblasts synthesize and degrade the extracellular matrix that provide the structural support for the heart. By sensing changes in their extracellular milieu, the cardiac fibroblasts are able to regulate the cell-derived extracellular matrix scaffolding and secrete growth factors and cytokines facilitating

adaption of the heart to normal and pathological stimuli (Souders et al. 2009). Cardiac fibroblasts also have roles in angiogenesis, neoplasia, and even electrical signaling and there is an evidence that senescent cells accumulate with age in the heart (Chimenti et al. 2003), although the impact of senescence specifically within the cardiac fibroblast population during aging requires further investigation.

Cellular senescence was initially defined by Hayflick and Moorhead as a cellular aging process that limits the number of cell divisions that somatic cells can undergo in culture (Hayflick and Moorhead 1961). However, it is now clear that senescence is also a stress response pathway parallel to apoptosis that can be activated by multiple stressors including oxidative stress, genotoxic stress, telomere attrition, and oncogene conversion (Campisi 2013). More recently, evidence has emerged that mitochondrial stress induces senescence, even in the absence of overt DNA (Nacarelli et al. 2016; Wiley et al. 2016). In terms of the relevance of senescence to aging of the organism, targeting senescent cells has been reported to provide protection in both progeroid models (Baker et al. 2011) and during normal aging (Baker et al. 2016), while the identification of senescent cells in multiple tissues as a function of age further demonstrates the importance of senescence in the aging process (Herbig et al. 2006; Jeyapalan and Sedivy 2008).

One of the key players in establishing replicative, genotoxic stress and oncogene-induced senescence is an increase in reactive oxygen species (ROS) (Colavitti and Finkel 2005; Lu and Finkel 2008; Nair et al. 2015). The generation of intracellular ROS appears to be a much earlier event than the onset of other senescent phenotypes as shown in a time-progression analysis of replicative senescence in human fibroblast (Kim et al. 2013). Mitochondria are a major source of intracellular ROS production and a significant increase in mitochondrial superoxide anion has been reported with increasing population doublings and replicative senescence in human fibroblasts, mesenchymal stem cells, and vascular smooth muscle cells (Bielak-Zmijewska et al. 2014; Estrada et al. 2013; Lerner et al. 2013; Nacarelli et al. 2016; Nacarelli et al. 2015; Passos et al. 2013; Passos et al. 2007; Wiley et al. 2016). Elevated non-phosphorylating mitochondrial respiration has been demonstrated in replicative, oxidative stress and oncogene-mediated senescence (Hutter et al. 2004; Kaplon et al. 2013; Nacarelli et al. 2015; Quijano et al. 2012). Not only are mitochondria potential sources of ROS, but also potential targets of ROS. During replicative senescence of human fibroblasts, evidence of ROS-induced oxidative damage was detected within the mitochondria (Ahmed et al. 2010), suggesting that the mitochondria are both a ROS source and a target during the senescence transition. Yet, aging-related decline in organismal metabolic rate refers not only to generation of abnormalities but also to "normal" mitochondrial changes which are adaptive to the aging processes. These changes include shifts in oxidative metabolites, rewiring in routes of carbon in the TCA cycle, and activity of mitochondrial enzymes and transporters. An increase in mitochondrial pyruvate oxidation, TCA cycle, and respiration has been observed in senescence induced through oncogene activation (Kaplon et al. 2013; Quijano et al. 2012), genotoxic stress (Wang et al. 2016), mitochondrial stress (Nacarelli et al. 2016), and in replicative senescence (Nacarelli et al. 2016; Takebayashi et al. 2015). Importantly, it has been demonstrated that senescence-mediated acceleration of mitochondria oxidative metabolism is accompanied by elevated glycolysis. Seminal studies on the metabolism of cellular senescence show that both glucose consumption and lactate production are elevated during replicative aging and senescence (Bittles and Harper 1984; Goldstein et al. 1982; Moiseeva et al. 2009; Dorr et al. 2013; Liao et al. 2014; Wang et al. 2016; James et al. 2015; Takebayashi et al. 2015). Thus, although in an essentially irreversible growth arrest, senescent cells exhibit a highly active metabolism that is a key characteristic of the senescent phenotype, although the precise mechanisms of balancing of different energetic pathways, including glycolysis and oxidative phosphorylation, are not fully understood.

Rapamycin, an inhibitor of mTOR metabolic and growth control pathway used as part of immunosuppressive therapy (Kennedy and Lamming 2016), has been intensively employed both as an investigational tool and a medicinal agent. It has been shown that rapamycin extends lifespan in multiple organisms including yeast, *C. elegans*, Drosophila, and mice, and improves late life cardiac function in mice (Dai et al. 2014); Miller et al. 2011). Rapamycin also provides benefit in settings of mitochondrial dysfunction such as mutations in the NDUFS4 mitochondrial subunit which models Leigh syndrome (Johnson et al. 2013), and in a lamin A/B null model (Ramos et al. 2012). We and other laboratories have reported that rapamycin delays replicative senescence in both normal human cells and cells from

Hutchinson-Gilford progeria syndrome (HGPS) patients as well as reduces the production of the senescence-associated secretory profile, known as the SASP (Cao et al. 2011b; Laberge et al. 2015; Lerner et al. 2013; Wiley et al. 2016). These studies demonstrate that rapamycin can delay or prevent senescence; however, a greater understanding of the metabolic reprogramming of the senescent cells and the potential impact of interventions to delay senescence, such as rapamycin, are needed to facilitate the development of targeted interventions that will modify metabolism and provide benefits associated with a reduced burden of senescent cells. In the present study, we have examined the mitochondrial oxidative phosphorylation processes both on intact cells and isolated organelles and the impact of rapamycin treatment in two settings of senescence, human cardiac fibroblasts grown to replicative senescence, and senescence induced by a mutant lamin A protein which underlies Hutchinson-Gilford progeria syndrome. This multidimensional approach enabled us to explore the potential mitochondrial targets of rapamycin which could be a part of mechanisms involved in senescence-mediated metabolic adaptation.

## Materials and methods

### Cell culture and cell culture reagents

Human cardiac fibroblast cells (ScienCell Inc. Carlsbad, CA) and HGPS patient-derived fibroblasts (Coriell Institute, Camden, NJ) were cultivated in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% MEM vitamins, and 1% MEM non-essential amino acids according to standard culture protocol for lifespan analysis of human diploid fibroblasts (Cristofalo and Charpentier 1980), with or without the addition of 1 nM rapamycin. Rapamycin was present in the cultures continuously. The HGPS fibroblasts, HGFDN0178 and AG01972, were obtained from the Hutchinson-Gilford Foundation and Coriell Research Institute respectively while the unaffected adult skin fibroblasts, HGFDFN168 and HGMDFN090, were obtained from the Hutchinson-Gilford Foundation. Progerin- and lamin-expressing cells were generated through retroviral introduction of cDNA constructs producing GFP-fusion proteins generously deposited in the Addgene site by Dr. Thomas Misteli (Scaffidi and Misteli 2008). Atypical nuclei were scored as any nuclei which exhibited abnormal morphology, including folding of the nuclear lamina and invaginations in the nuclear membrane. For galactose studies, cultures were transferred to Dulbecco's modified eagle medium (DMEM) containing either 10 or 20 mM galactose and followed for lifespan according to standard protocols for lifespan analysis as above.

### Cellular respiration, extracellular acidification, and glucose uptake measurements

Oxygen consumption and extracellular acidification rates were measured on a Seahorse XF24 Bioanalyzer (Seahorse Bioscience, North Billerica, MA), using the XF Cell Mito Stress Test and XF Mito Fuel Flex Test kits. Cells were seeded at a density of 15,000 to 25,000 cells per well in an XF24 microplate. Plates were placed in the bioanalyzer pre-loaded with the sensor cartridge containing oligomycin, carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP), and rotenone/antimycin A for the Mito Stress Test kit or UK5099(8 μM), BPTES (10 μM), and etomoxir (15 μM) for the Mito Fuel Flex Test kit. Respiration and acidification rates were assessed at least in triplicates as outlined in published methods (Hill et al. 2012; Dranka et al. 2011). In the inhibitory analysis, the rates of oxidation were calculated based on the percentage of oxygen consumption upon treatment with UK5099, an inhibitor of pyruvate oxidation, and with sequential addition of BPTES/etomoxir to interfere the glutamine/palmitate oxidation. The values were normalized to the number of cells harvested in each well at the completion of assay and counted using a Guava EasyCyte Mini (Guava Technologies, Hayward, CA). Glucose uptake was determined by measuring of glucose level in the media using an ACCU-CHEK glucometer (Roche, Basel, Switzerland). The difference in glucose concentration in the stock culture media and cultured cell media was normalized to cell number using a Guava EasyCyte Mini for cell counts.

### Isolation of mitochondria from cell cultures

Mitochondria were isolated by differential centrifugation. The cell pellet rinsed with ice-cold PBS (without $Ca^{+2}/Mg^{+2}$) was re-suspended in hypotonic solution (100 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EGTA) to let cells swell for 10 min on ice according to Panov (Panov et al. 2005). The suspension was

homogenized in a Teflon-glass homogenizer by gentle circular strokes following immediate dilution with hypertonic solution (1.25 M sucrose, 10 mM MOPS, pH 7.2), 1 ml per 10 ml cell suspension, to restore the buffer isotonicity. The mix was diluted with three volumes of isolation buffer (75 mM mannitol, 225 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EGTA, 0.1% fatty acid-free BSA). Cellular debris were sedimented at 980*g*, 4 °C for 5 min. Supernatant containing mitochondria was further centrifuged at 10,300*g*, 4 °C, for 20 min. The resulting crude mitochondria pellet was rinsed by centrifugation at the same parameters in 10 ml of MiR05 respiration buffer (110 mM sucrose, 60 mM K-lactobionate, 20 mM HEPES, pH 7.2, 1 mM $KH_2PO_4$, 3 mM $MgCl_2x6H_2O$, 0.5 mM EGTA, 20 mM taurine, 0.1% fatty acid-free BSA) (Gnaiger et al. 2000). The final pellet was re-suspended in 100 μl MiR05 by vortexing and stored on ice in a refrigerator. The mitochondria protein was determined by the Bradford protein assay.

### Measurement of oxidative phosphorylation of isolated mitochondria

The isolated mitochondria respiration was analyzed by high-resolution respirometry at 37 °C in a two-chamber respirometer OROBOROS Oxygraph-2K (Innsbruck, Austria) (Gnaiger 2008; Gnaiger et al. 1995; Pesta and Gnaiger 2012). The OROBOROS DatLab software was used for data acquisition and analysis. Each 2-ml chamber was loaded with 0.2 mg mitochondria protein. To assess different respirometric complexes, we applied sequential stimulation of mitochondria with 15 μM ADP (to assess state 4 respiration) following 2 mM ADP (to assess state 3 respiration) in the presence of metabolic substrates, namely 10 mM glutamate, 10 mM pyruvate, 10 mM succinate, and 15 μM palmitoyl-L-carnitine all in the presence of 2 mM malate along with inhibitors of NADH dehydrogenase (1 μg/ml rotenone) and adenine nucleotide transporter (5 μM carboxyatractilozide).

### Transaminase assay

The activity of alanine aminotransferase (ALT) was measured in isolated mitochondria of the same preparation used for respirometric analysis using commercial assay kit according to the manufacturer protocol (Bio-Assay Systems, Hayward, CA). The 0.1 mg/ml mitochondria protein was used per sample, and both control and rapamycin treated went through one freezing-thawing cycle. Colorimetric detection of enzyme activity is based on quantification of pyruvate produced by ALT as a function of decrease of absorbance of associated NADH.

### Immunoblotting and immunoprecipitation

Cell protein extracts were prepared by extracting with RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich) and protein concentration was quantified using a bicinchoninic acid (BCA) assay (Pierce Biotechnology). Western blot analysis was performed using 15 to 30 μg of protein extracts run on SDS-PAGE and transferred onto nitrocellulose (Biorad) membranes. Blots were incubated with antibodies specific for beta-actin (A2066 Sigma-Aldrich) and progerin (EMD Millipore).

### Statistical analysis

Statistical analyses were performed with GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA). The statistical differences between control and rapamycin-treated cells and isolated mitochondria were estimated by paired, two-tailed Student's *t* test and were considered significant at $p < 0.05$.

## Results

### Cell respirometric and glycolytic activities

We examined the metabolic state of senescent (late passage greater than 90% lifespan completed) cardiac fibroblasts relative to early passage cardiac fibroblasts by monitoring oxygen consumption and extracellular acidification rates as a measure of activity of oxidative phosphorylation (OxPhos) and glycolysis respectively. The matched sets of cells had been cultured in the presence or absence of 1 nM rapamycin beginning at mid-lifespan (population doubling 27) and maintained until the control cultures began to approach senescence. Consistent with our previous observations, the senescent cells exhibited a higher basal oxygen consumption rate, i.e., OxPhos, up to 6 pmol/min/$10^5$cells vs 1 pmol/min/$10^5$cells in early passage cells, and increased glycolysis 5.5 vs 1 mg/dl/$10^5$cells, along with associated

medium acidification (Fig. 1). Long-term treatment of cells with rapamycin corrected all three processes back to the early passage cells.

## Cell oxidation of pyruvate and glutamine/palmitate

To explore the other energetic routes, we examined the ability of the late and early passage cells to maintain OxPhos via utilization of pyruvate and glutamine/palmitate. This was achieved through sequential addition of inhibitors of the corresponding mitochondrial pyruvate transporter (UK5099), glutaminase (BPTES), and palmitate transporter (etomoxir). About 40–50% inhibition of oxygen consumption by paired inhibitors indicates relative addiction of senescent cells to these three metabolites (Fig. 2a, b). There is a greater inhibition in early passage cells following the additional exposure to either BPTES/etomoxir in the presence of UK5099 (Fig. 2a) or UK5099 in the presence of BPTES/etomoxir (Fig. 2b). This suggests that the early passage cells are able to divert utilization when first challenged with the inhibitor. Interestingly, the late passage cells that went through the long-term exposure to 1 nM rapamycin exhibit an elevated oxidative capacity (Fig. 2c, d).

## Oxidative phosphorylation and transaminase activities of isolated mitochondria

To address the mechanisms of rapamycin-mediated metabolic reprogramming, we studied bioenergetic processes in isolated mitochondria of cardiac fibroblasts. To assess the direct mitochondria targets of rapamycin, we applied higher concentrations of rapamycin, 100 nM, 500 nM, and 5 μM, to the isolated mitochondria. The choice of these concentrations was made based on a large body of studies utilizing rapamycin for cell treatment (Kumar and Lombard 2016; Ramanathan and Schreiber 2009). We found that higher concentrations do not immediately alter respiration, but rather require at least 1 h of pre-incubation to develop detectable mitochondria effects. Figure 3 presents quantitative data of complex I and II and beta-oxidation respiration of mitochondria isolated from cardiac fibroblasts.

As seen from the data, rapamycin does not affect glutamate/malate oxidation in complex I (Fig. 3(a, b)). Similarly, pyruvate/malate oxidation is not affected (data not shown). Succinate oxidation is elevated in the presence of 100 nM rapamycin in both active state 3 to $44.9 \pm 2.2$ vs $35.4 \pm 0.8$ pmol/s/0.1 mg mitochondria protein and resting state 4 to $22.3 \pm 2.1$ vs $18.2 \pm 4.2$ pmol/s/0.1 mg mitochondria protein. To assess the complex II activity, rotenone was used prior to addition of succinate to demonstrate the direct targeting of succinate dehydrogenase by rapamycin. Complex I- and II-mediated changes of oxidation are not profound but statistically significant. Oxidation of palmitoyl-L-carnitine, at least in our experimental setting, was shown to be not affected, remaining in the presence of rapamycin at $15.3 \pm 1.8$ pmol/s/0.1 mg mitochondrial protein. To mimic in vivo heart physiological condition, we challenged the mitochondria with different substrates given over glutamate- and malate-stimulated respiration. In recent work on rat heart mitochondria, the importance of the use of tissue-specific substrate mixtures for physiological data has been described (Panov 2018). Addition of pyruvate over glutamate and malate increased the state 3 respiration of isolated mitochondria. The data presented in Fig. 4a, b demonstrates the original respirometric record of this protocol and the quantitative data of the effect of rapamycin on oxidative phosphorylation rates.

Interestingly, pretreatment with rapamycin caused dose-dependent elevation of pyruvate oxidation, while oxidation of glutamate and malate without pyruvate was not sensitive to rapamycin (Fig. 3(a, b)). Long-term treatment with 1 nM rapamycin for 14 days also elevates glutamate/malate/pyruvate oxidation, although shown to be statistically non-significant (Fig. 3b, black bar). These results led us to examine the activity of mitochondria alanine aminotransferase (mALT), the enzyme that promotes conversion of glutamate and pyruvate to α-ketoglutarate and alanine. Figure 4d shows the chain of reactions that involves transamination and further metabolizes α-ketoglutarate to succinate. Enzymatic activity of mALT was shown to be elevated also in a dose-dependent way in the presence of rapamycin (Fig. 4c).

We also examined a gene expression dataset of long-term rapamycin-treated cultures which we have generated to compare the impact of methionine restriction and rapamycin (Azar et al. 2018) for differential expression which could relate to the metabolic changes we observe here. This analysis revealed multiple changes in steady-state mRNA levels for transcripts related to mitochondrial function. Among these transcripts was the uncoupling protein 2 (UCP2) mRNA (adjusted $p < 0.001$). We verified the differential expression of UCP2 using PCR analysis of mRNA levels and by immunoblot for protein levels (Fig. 5S).

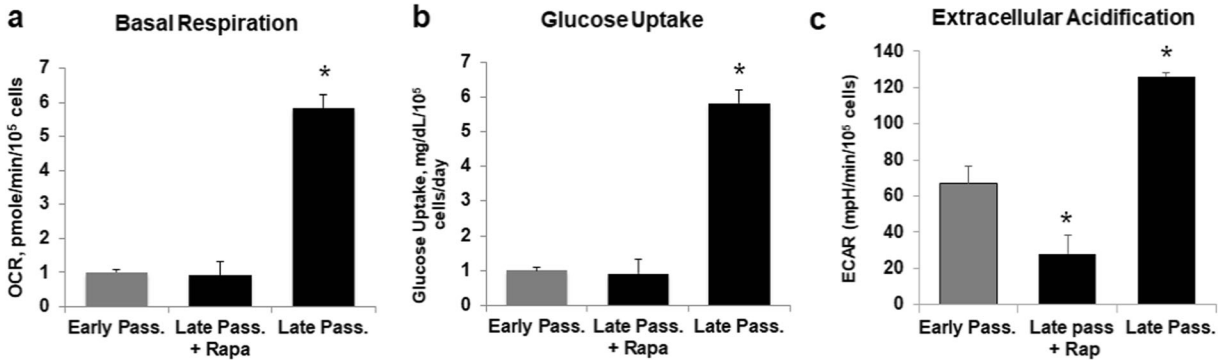


**Fig. 1** Late passage human fibroblasts are relatively glycolytic. Seahorse bioanalyzer measurements of basal respiration (**a**), glucose uptake (**b**), and extracellular acidification (**c**) in early and late passage (> 90% lifespan completed) human cardiac fibroblasts. The values were normalized to cell numbers in each well. Samples were measured in quintuplicate and results are representative of at least two independent experiments ± SD. The bars with an asterisk represent values that are significantly (*, $p < 0.05$) differ from the late passage ones

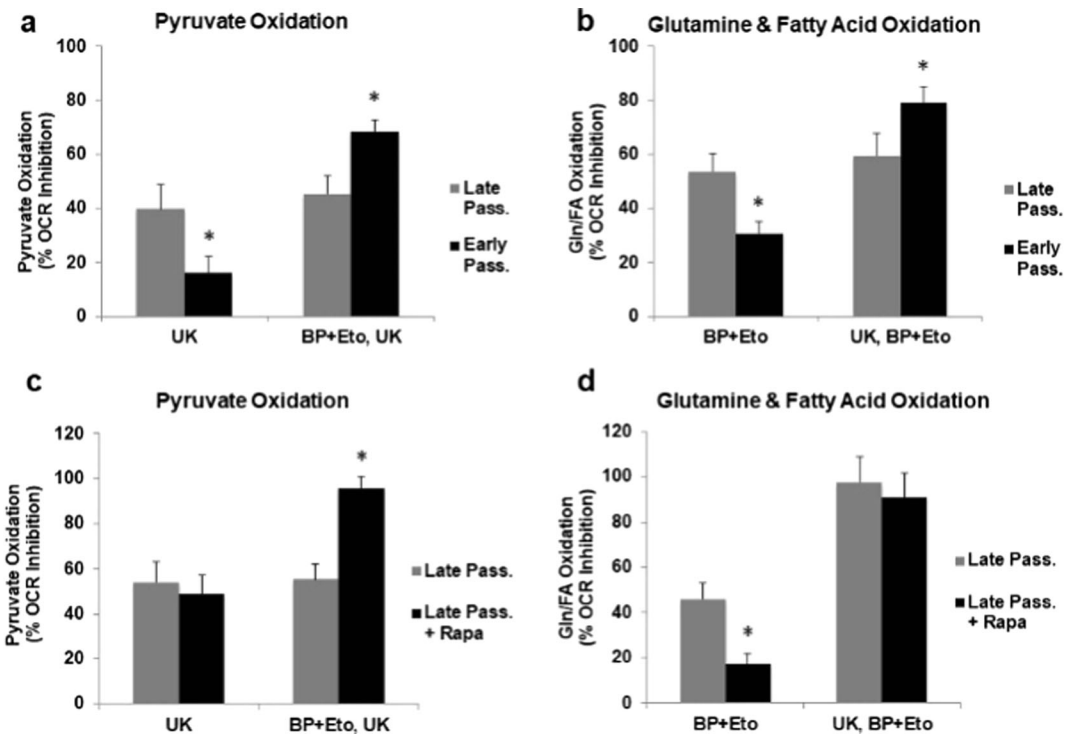


**Fig. 2** Distinct rates of pyruvate and glutamine/palmitate oxidation by early and late passage human fibroblasts and the effect of rapamycin. **a** The rates of pyruvate-mediated respiration were calculated as the percentage of inhibition of oxygen consumption by specific pyruvate transporter inhibitor UK5099. The rates of oxygen consumption obtained via sequential addition of first the glutamine and palmitate oxidation inhibitors, BPTES and etomoxir, and then UK5099 represent the capacity of pyruvate oxidation. **b** The rates of glutamine/palmitate oxidation were determined in the presence of corresponding inhibitors BPTES and etomoxir, while the capacity was determined following first the addition of pyruvate inhibitor UK5099 and then BPTES/etomoxir. **c**, **d** Effect of rapamycin on capacity of late passage cells to oxidize pyruvate and glutamine/palmitate in late passage fibroblasts maintained in the presence or absence of 1 nM rapamycin. Values are generated as in **b** through the addition of inhibitors and calculating the percent inhibition of oxygen consumption. In all cases, graphs represent the values of one of at least two independent experiments measured in triplicates and presented as mean ± SD; *$p < 0.05$ vs late passage

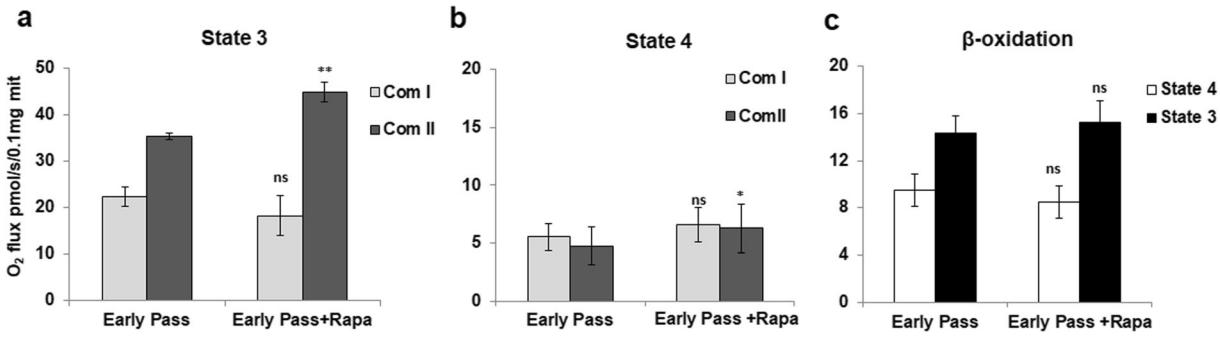


**Fig. 3** Oxidative phosphorylation activity of cardiac mitochondria. (a, b) Complex I-mediated oxidation of 10 mM glutamate and 2 mM malate does not change upon mitochondria incubation with 100 nM rapamycin. The complex II-mediated succinate oxidation significantly elevated in the presence of rapamycin in both active and resting states 3 and 4 correspondingly. (c) Oxidation of 15 μM palmitoyl-L-carnitine did not change noticeably, although the trend was observed in every experiment performed. Data are mean ± SD; $*p < 0.04$, $**p < 0.003$

## Rapamycin alters metabolism of lamin A mutant cells

In order to examine modulation of metabolism by rapamycin during senescence, we examined the effect of rapamycin on the metabolic state in cells expressing a mutant form of lamin A, which underlies Hutchinson-Gilford progeria syndrome (DeBusk 1972; Goldman et al. 2004). Rapamycin is known to extend the replicative lifespan of patient-derived HGPS fibroblasts potentially through an increase in autophagy (Cao et al. 2011a). Human fibroblasts expressing the mutant form of lamin A, progerin, displayed nuclear atypia consistent with the known impact of the lamin A mutation (Fig. 1S). We also verified that rapamycin rescues cells from premature senescence induced by progerin expression (Supplemental Materials, Fig. 2S). Human fibroblast cells expressing progerin exhibited an increase in basal oxygen consumption, glucose uptake, and extracellular acidification (Fig. 5a–c), while rapamycin treatment reduced all of these parameters in a manner similar to the effects in the late passage cells (black bars, Fig. 5a–c).

We next examined the impact of rapamycin treatment on the ability of progeria cells to maintain oxygen consumption rates following a block in the utilization of either pyruvate or glutamine and palmitate through the sequential addition of corresponding inhibitors UK5099, BPTES, and etomoxir. Although not as robust as the impact on normal fibroblasts, this analysis suggests that rapamycin-treated cultures had an enhanced capacity to utilize either pyruvate or glutamine/fatty acids (Fig. 6a, b).

In order to directly test whether rapamycin treatment allows human fibroblasts to resist senescence induced by a metabolic stress, we grew the normal cardiac fibroblasts in glucose-free culture media supplemented with either 10 or 20 mM galactose. Due to the additional phosphorylation events required to process galactose for use in glycolysis (Holden et al. 2003), cells are relatively more reliant on oxidative phosphorylation when grown in galactose-containing medium. We found that the normal human fibroblasts undergo a premature senescence when provided galactose instead of glucose as a source of energy (Fig. 7, gray lines compared to gray dashed lines). Consistent with our interpretation of an enhanced metabolic flexibility, rapamycin provided protection against galactose-induced premature senescence (rapamycin-treated cultures are indicated by black lines in Fig. 7).

## Discussion

We have employed oxygen consumption rates as an indirect measure to gain insight into the metabolic state of senescent cells and to examine the impact of rapamycin on metabolism as it relates to senescence. First, we found that senescent cells are relatively reliant on both glycolysis and OxPhos (Fig. 1a, b). The parallel activity of both energetic routs is not inherently controversial. To compensate for their mitochondrial dysregulation, senescence cells could employ mechanisms which have been observed in tissues with high energy demand. For example, muscle cells are capable of converting glycolytic lactate to pyruvate to be further oxidized by mitochondria (Elustondo et al. 2013). In our senescent cell measurements, the elevated lactate

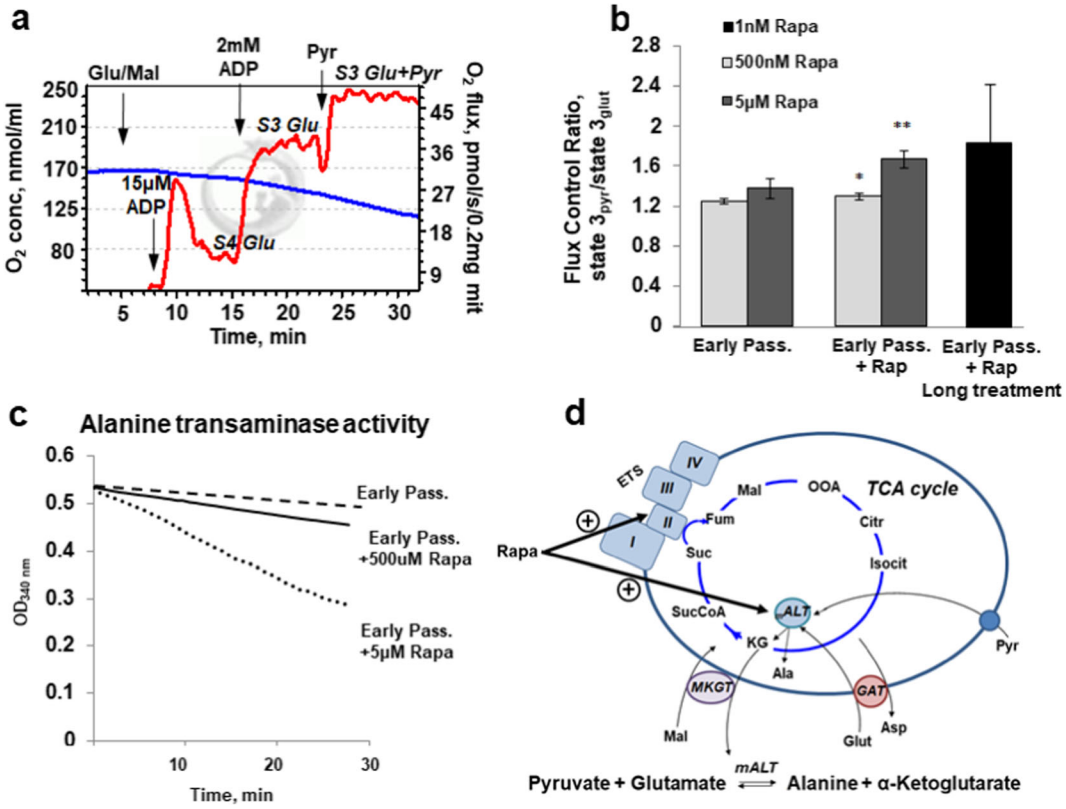


**Fig. 4** Combinatory oxidation of glutamate, malate, and pyruvate by isolated cardiac mitochondria. **a** The original oxygraphic protocol applied to study of oxidation of the substrate combination. Addition of 15 μM of ADP enabled to evaluate state 4 and addition of 2 mM ADP to read out the maximal state 3 respiration rates. Sequential addition of 10 mM glutamate and 2 mM malate and then 10 mM pyruvate gave higher rates of oxygen consumption than the glutamate and pyruvate alone. **b** Quantitative flux control ratios were obtained as state $3_{pyr}$/state $3_{glut}$. Pre-incubation of mitochondria with rapamycin for 1 h dose-dependently elevated glutamate/malate/pyruvate oxidation. Elevation of pyruvate oxidation over glutamate/malate was also observed in mitochondria isolated from cells long treated with rapamycin for 14 days. Data are mean ± SD; $*p < 0.044$, $**p < 0.0021$. **c** Alanine aminotransferase activity also dose-dependently elevates in the presence of rapamycin. **d** The schematics demonstrates transamination of pyruvate and glutamate by mitochondria matrix alanine aminotransferase and the formed α-ketoglutarate further converts in TCA cycle to succinate and activates succinate dehydrogenase-dependent respiration. *Abbreviations:* ETS, electron transport system; mALT, mitochondria alanine aminotransferase; MKGT, malate-α-ketoglutarate transporter; GAT, glutamate-aspartate transporter

production (Fig. 1c) is in support of this possibility. Second, we found that when challenged by inducing a block on specific metabolic pathways, senescent cells are less able to shift between carbon sources to maintain oxygen consumption, compared to early passage cells (Fig. 2). Whether the change in metabolism is a cause or consequence of senescence is not clear. However, it appears that rapamycin both reduces the dependence on glycolysis and enhances the ability to utilize other respiratory metabolites. It appears that the metabolic rejuvenation of senescence cells through long-term treatment with a low (nM) dose of rapamycin may occur via reprogramming of pyruvate and glutamate oxidation, and possibly fatty acids (Fig. 2). To better understand this metabolic reprogramming and to explore the mitochondria targets of rapamycin, we examined the direct effects of rapamycin on mitochondria isolated from cardiac fibroblasts. These oxygraph studies demonstrated that short-term treatment with rapamycin (1 h) causes elevation of complex II but not complex I activity both in active and resting states of respiration (Fig. 3(a, b)). Interestingly, similar effects of rapamycin on succinate dehydrogenase activity have been reported in studies on drosophila (Villa-Cuesta et al. 2014). The rates of beta-oxidation of cardiac fibroblasts mitochondria had a tendency to increase in the presence of rapamycin, although not statistically significant (Fig. 3(c)). Palmitoyl-L-carnitine was added

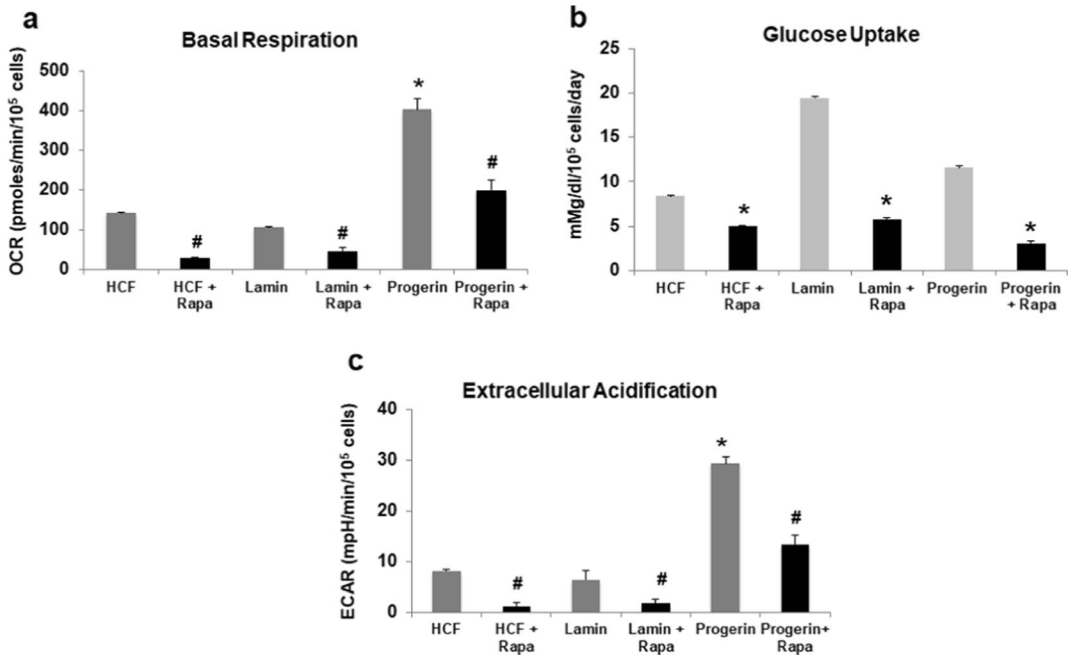


**Fig. 5** Progerin expression induces a glycolytic phenotype which is reversed by rapamycin. Measurements of basal respiration (**a**), glucose uptake (**b**), and extracellular acidification (ECAR) (**c**) in human cardiac fibroblasts (HCFs) expressing lamin A or progerin maintained with or without 1 nM rapamycin. Oxygen consumption rates were determined using a Seahorse bioanalyzer. Oxygen consumption and extracellular acidification rates were normalized to cell number. Data presented as mean ± SD ($n = 2$–3, in triplicates). The differences are shown to be statistically significant with $p < 0.05$ in comparison to the late passage + rapamycin values

alone and in combination with glutamate, malate, and pyruvate. The importance of application of hybrid substrates has been demonstrated on rat heart mitochondria (Panov 2018). Following that experimental approach, we added pyruvate to mitochondria-oxidizing glutamate and malate. The moderate rates of glutamate/malate-stimulated oxygen consumption elevated upon addition of pyruvate. Interestingly, rapamycin was able to further increase the state 3 respiration higher than in control mitochondria. The increase of oxygen consumption when both glutamate and pyruvate are present could be due to active transamination supported by mitochondria alanine aminotransferase (ALT). The enzymatic analysis confirmed the ability of rapamycin to activate

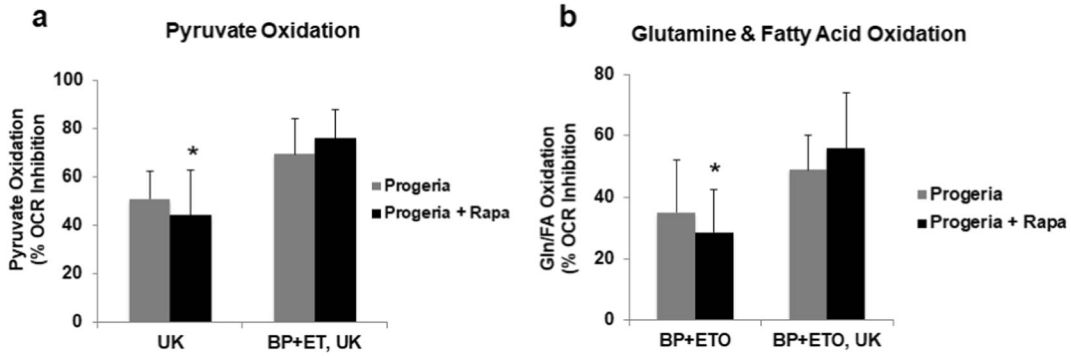


**Fig. 6** Rapamycin improves metabolic flexibility in human fibroblast cells expressing progerin. Measurements of the capacity for oxidizing pyruvate (**a**) or glutamine/palmitate (**b**) in late passage fibroblasts maintained in the presence or absence of 1 nM rapamycin. Measurements were made as in Fig. 2. The data were normalized to cell number in each well. Data presented as mean ± SD ($n = 2$, in triplicates). The differences are shown to be statistically significant with $p < 0.05$ in comparison to the late passage values

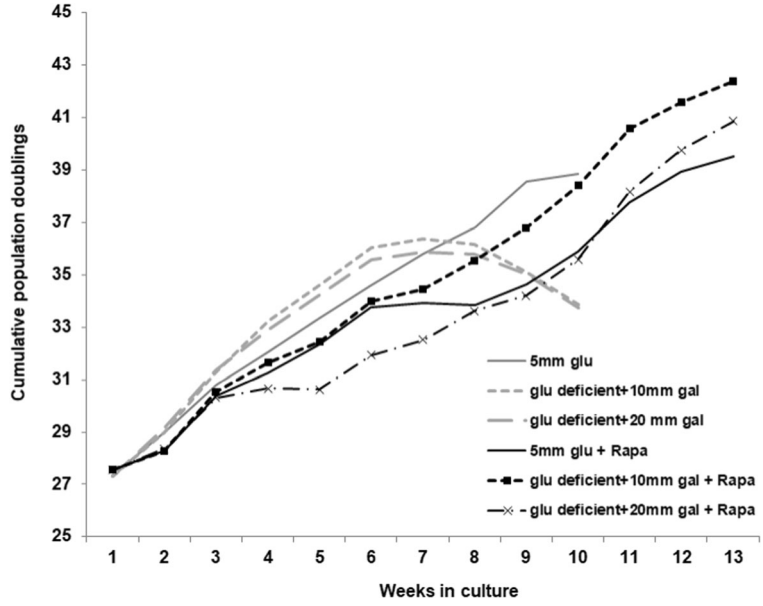


**Fig. 7** Rapamycin prevents premature senescence induced by galactose. Human cardiac fibroblasts maintained with or without 1 nM rapamycin were transferred to glucose-free medium supplemented with either 10 or 20 mM galactose at a population doubling of 27.5. Cumulative population doublings were calculated as described in "Materials and methods" section

ALT (Fig. 4c). As a result of transamination, pyruvate and glutamate metabolize to alanine and α-ketoglutarate with further conversion of latter to succinate. The operation of α-ketoglutarate dehydrogenase in TCA cycle that converts α-ketoglutarate to succinyl-CoA and then to succinate could be a rescue mechanism that

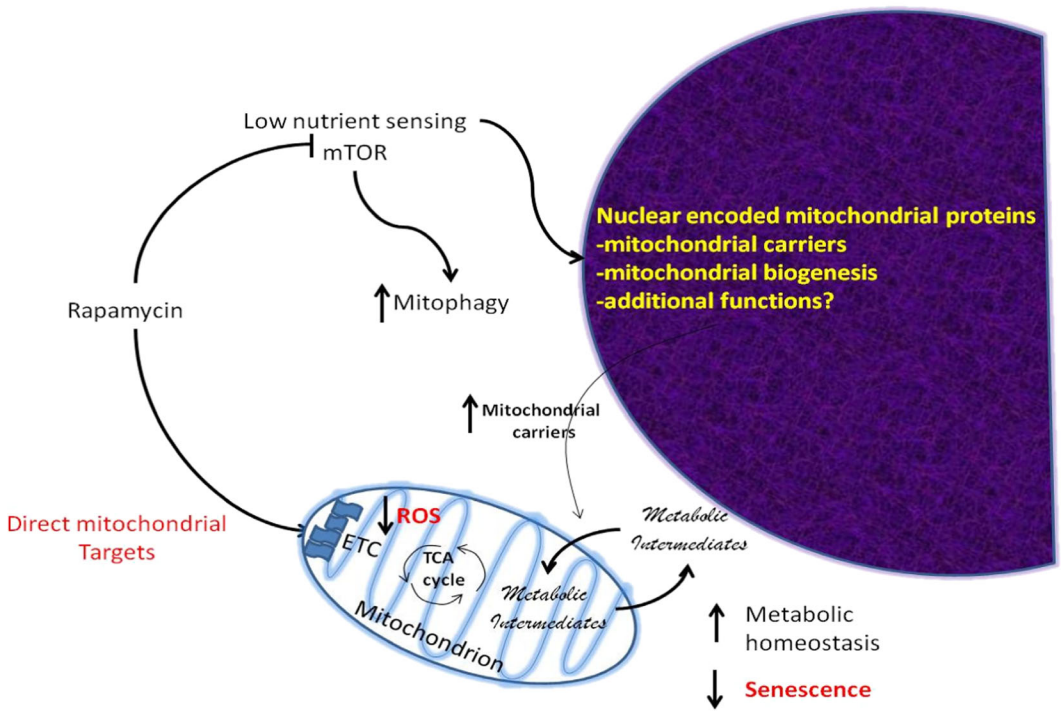


**Fig. 8** Model of metabolic responses to rapamycin improving metabolic homeostasis. Rapamycin interacts directly with the mitochondria to provide enhanced complex II function and inhibits the mTOR complex which impacts nuclear-encoded mitochondrial genes including mitochondrial carrier proteins which shuttle metabolic intermediates across the mitochondrial membrane providing greater metabolic flexibility to meet the demand for ATP and metabolic precursors and reduced ROS production. This increased flexibility prevents metabolic catastrophe, which can result in senescence (Nacarelli and Sell 2017)

enables mitochondria functionality based on substrate-level phosphorylation (Chinopoulos et al. 2010). The data on ALT activity demonstrating elevated production of α-ketoglutarate by rapamycin which further proceeds to generation of succinate in TCA is in agreement with the increased activity of succinate dehydrogenase promoted by rapamycin (Fig. 3(a, b)). Thus, as shown on the schematics on Fig. 4d, we revealed at least two potential direct mitochondria targets of rapamycin, succinate dehydrogenase and alanine aminotransferase. In addition, our long-term treatment of cells with rapamycin reveals potentially indirect mitochondrial targets of rapamycin. For example, we have performed gene expression profiling of long-term rapamycin-treated cultures (Azar et al. 2018) and find several changes related to mitochondrial metabolism. Among the induced set of genes is the uncoupling protein 2, which displays elevated transcript and protein levels following prolonged treatment with rapamycin (Supplementary Materials, Fig. 5S). Biochemical analysis has demonstrated that UCP2 acts as a transporter of 4 carbon, Krebs cycle intermediates and participates in the regulation of substrate phosphorylation (Vozza et al. 2014). The intact cell data corroborated by those obtained with isolated mitochondria enabled us to explore mitochondria constituents that could be involved in aging-mediated dysfunctions and be corrected by rapamycin. The observed changes in UCP2 are consistent with this data and indicate that altered gene expression of proteins related to mitochondrial function is impacted by rapamycin. These results extend our previous observations on the impact of rapamycin on NFE2L2 (NRF2) signaling (Lerner et al. 2013) to enhance mitochondrial biogenesis as well as clearance.

In addition to cardiac fibroblasts, we also examined the metabolic state of HGPS patient-derived skin fibroblasts relative to skin fibroblasts from unaffected individuals. HPGS fibroblasts exhibited a glycolytic phenotype similar to the progerin-expressing HCFs (Supplementary Materials, Fig. 3S) and rapamycin provided a similar shift towards oxidative metabolism (Supplementary Materials, Fig. 4S).

Interestingly, when cells are forced to rely upon oxidative phosphorylation by replacing glucose with galactose in the culture medium rapamycin treatment allows cells to maintain proliferative potential which suggests protection against metabolic stress. Galactose requires conversion to glucose-1-phosphate via the Leloir pathway to enter metabolism (Holden et al. 2003), which places an extra metabolic demand on the cells and a relative reliance on oxidative phosphorylation for ATP production. In terms of the relationship to senescence, replacement of glucose with micromolar concentrations of galactose has been shown to induce senescence in human fibroblasts, while cells harboring a $Val^{32}Met$ mutation in galactokinase display accelerated senescence (Elzi et al. 2016). Thus, these results and our observations support the concept that metabolic imbalance can induce senescence and that rapamycin improves metabolic homeostasis to prevent senescence caused by metabolic catastrophe (Fig. 8).

In the literature, there are numerous links between mitochondrial function and senescence. For example, complete disruption of mitochondrial function induces senescence (Wiley et al. 2016) through an AMPK-dependent manner, while low levels of mitochondrial stress induce senescence in an mTOR-dependent manner (Nacarelli et al. 2016) and depletion of mitochondria in senescent cells leads to a reduction in SASP production (Correia-Melo et al. 2016). Proteomic analysis suggests that HGPS also involves mitochondrial dysfunction (Peinado et al. 2011; Rivera-Torres et al. 2013). In this regard, it is interesting to note that an improved metabolism has recently been reported in LMNA $^{-/-}$ mice treated with rapamycin (Liao et al. 2016). In this case, the authors find an improved balance between lipolysis and glucose utilization which allows the LMNA $^{-/-}$ mice to survive in the face of profound metabolic dysfunction. These results are consistent with previous reports suggesting a mitochondrial defect in Hutchinson-Gilford progeria and our observations regarding the impact of rapamycin on metabolic flexibility. It is interesting to note that both rapamycin treatment and low IGF-1 levels also provide enhanced ability to maintain metabolic balance when challenged by a high fat diet (Chang et al. 2009; Lorenzini et al. 2014, Salmon et al. 2015).

Based on our studies, we believe that rapamycin increases metabolic flexibility defined as the ability to utilize multiple carbon sources to satisfy metabolic demands, and that this flexibility averts a metabolic stress contributing to the lifespan extension that is provided by rapamycin treatment, both in cell culture and in the organism. This effect appears to be mediated both by direct interaction of rapamycin with the mitochondria and through increased expression of mitochondrial transporters such as UCP2.

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