Inhibiting mitochondrial respiration prevents cancer in a mouse model of Li-Fraumeni syndrome

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Li-Fraumeni syndrome (LFS) is a cancer predisposition disorder caused by germline mutations in *TP53* that can lead to increased mitochondrial metabolism in patients. However, the implications of altered mitochondrial function for tumorigenesis in LFS are unclear. Here, we have reported that genetic or pharmacologic disruption of mitochondrial respiration improves cancer-free survival in a mouse model of LFS that expresses mutant p53. Mechanistically, inhibition of mitochondrial function increased autophagy and decreased the aberrant proliferation signaling caused by mutant p53. In a pilot study, LFS patients treated with metformin exhibited decreases in mitochondrial activity concomitant with activation of antiproliferation signaling, thus reproducing the effects of disrupting mitochondrial function observed in LFS mice. These observations indicate that a commonly prescribed diabetic medicine can restrain mitochondrial metabolism and tumorigenesis in an LFS model, supporting its further consideration for cancer prevention in LFS patients.

Introduction

Growing evidence suggests that the mitochondrion plays a critical role in cancer development through various processes including metabolism, ROS generation, and cell signaling (1, 2). Increased oxidative phosphorylation and fatty acid oxidation have been observed in diffuse large B cell lymphoma as well as chronic lymphocytic leukemia, and the master regulator of mitochondrial biogenesis PGC-1α promotes cancer metastasis (3, 4). Furthermore, mitochondrial DNA–depleted (mtDNA-depleted) cancer cells cannot metastasize until they acquire host mtDNA (5). Mitochondria appear to be essential for cancer progression; however, their role in de novo tumor formation remains unclear.

Li-Fraumeni syndrome (LFS) is an autosomal dominant cancer predisposition syndrome caused predominately by germline mutations of *TP53*, encoding the tumor suppressor protein p53 (6). We previously reported that LFS family members who carried *TP53* mutations have increased oxidative metabolism (7). These findings were confirmed in a mouse model of LFS that demonstrated the distinction between p53 null and p53 mutant states in regulating the mitochondria (8, 9). Although the role of mitochondria in tumorigenesis in LFS remains unclear, their function is tightly integrated with cell survival and proliferation, suggesting their involvement. Therefore, we hypothesized that disrupting mitochondrial function in an LFS model would affect de novo tumorigenesis. Furthermore, we explored the feasibility of atten-

uating mitochondrial activity in LFS patients utilizing metformin, a well-tolerated antidiabetic medication that is known to inhibit respiration, but has not been demonstrated as doing so in humans.

Results and Discussion

Genetic disruption of mitochondria delays de novo tumorigenesis. Mitochondrial function in an established mouse model of LFS (*p53^{172H/H}*), created by knockin of the mouse equivalent of human p53 R175H hotspot mutation (10), was directly disrupted by introducing the DNA polymerase γ D257A mutant (*Polg^{mut}*) to generate frequent mtDNA mutations (11). Because the majority of *p53^{172H/H}* mice develop thymic lymphomas, we measured mitochondrial respiration of thymus tissue, which revealed decreased oxygen consumption rate (OCR) in a *Polg^{mut}* allele dose-dependent manner (Figure 1A). This decrease in oxidative metabolism was associated with increased blood lactate levels (Figure 1A). Similarly, decreased mitochondrial complex I, but increased glucose transporter 4 and hexokinase 2, which promote glycolysis, were observed in the skeletal muscle of *Polg* mutant mice (Supplemental Figure 1; supplemental material and unedited blots available online with this article; doi:10.1172/JCI88668DS1). This metabolic reprogramming markedly increased cancer-free survival of LFS mice. Compared with *p53^{172H/H}* mice, the median survival time of *Polg^{+/mut} p53^{172H/H}* and *Polg^{mut/mut} p53^{172H/H}* double-mutant mice was increased by 40% and 79%, respectively (Figure 1B). Further demonstrating the physiological effect of mitochondrial inhibition on *p53^{172H/H}* mouse survival, a positive correlation between blood lactate level and survival time was observed in the double-mutant mice (Figure 1B). In addition, the increased cancer-free survival time by *Polg* mutation in homozygous *p53^{172H/H}* mice was also reproduced in heterozygous *p53^{172R/H}* mice, which
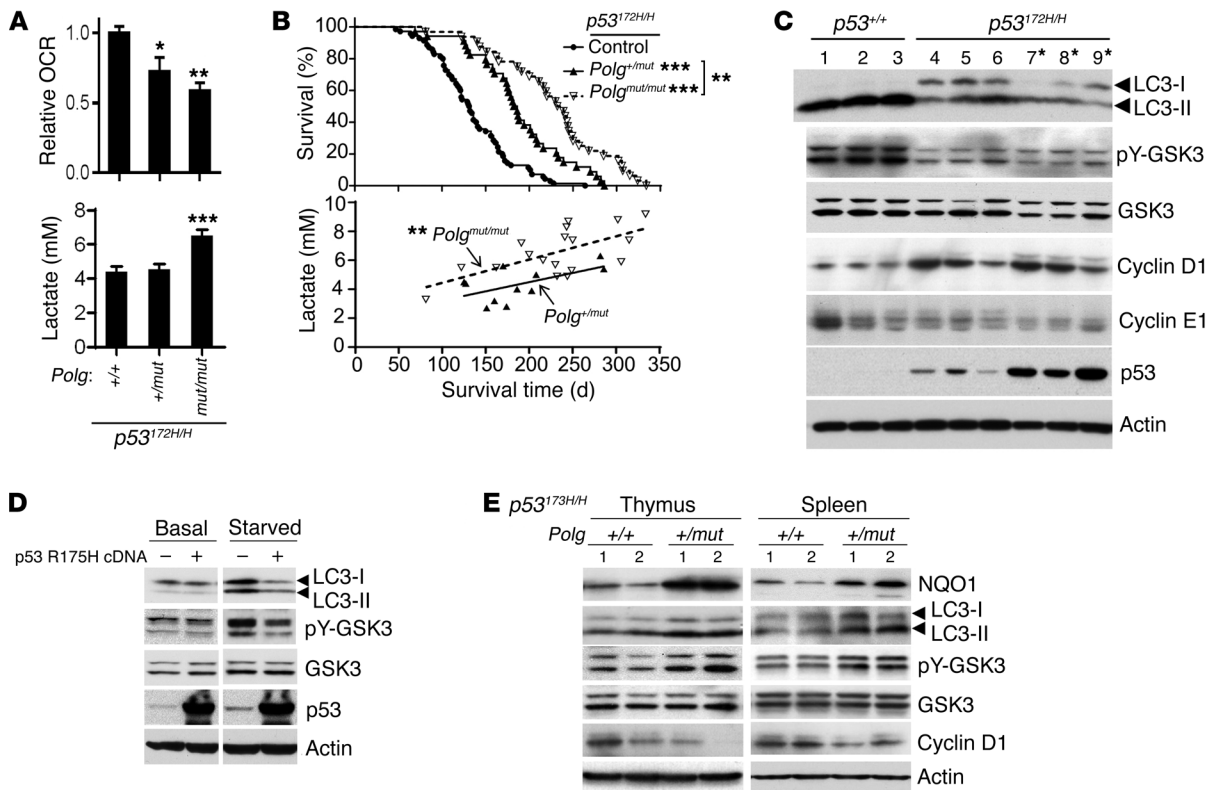
Figure 1. Genetic disruption of mitochondria reverses aberrant proliferation signaling and increases survival of _p53^172H/H_ mice. (**A**) OCRs of approximately 10-week-old mouse thymus cells (n = 5–7) and lactate in tail blood (average age approximately 12 weeks) (n = 14–19). (**B**) Upper panel shows Kaplan-Meier survival plot of _p53^172H/H_ control (n = 70), _Polg^+/mut_ _p53^172H/H_ (n = 34), and _Polg^mut/mut_ _p53^172H/H_ (n = 32) mice. Lower panel shows correlation between blood lactate level and survival time of _Polg^+/mut_ _p53^172H/H_ (r = 0.6, P = 0.057, n = 11) and _Polg^mut/mut_ _p53^172H/H_ (r = 0.7, P < 0.01, n = 18) mice. *P < 0.05; **P < 0.01; ***P < 0.001. (**C**) Immunoblots of approximately 10-week-old _p53^+/+_ and _p53^172H/H_ thymus tissues. Asterisks (lanes 7–9) indicate enlarged thymus samples with lymphoma. (**D**) Primary human myoblasts transduced with empty vector (–) or human p53 R175H cDNA (+) were serum starved for 3 hours prior to immunoblotting. (**E**) Immunoblots of thymus and spleen samples obtained from age-matched _Polg^+/+_ and _Polg^+/mut_ mice in _p53^172H/H_ background (lane 1, approximately 10 weeks old; lane 2, approximately 20 weeks old). Statistical differences by 1-way ANOVA (**A**), log-rank test (**B**, upper), and Pearson correlation coefficient test (**B**, lower).

have a much longer life span and more closely model the genotype that causes LFS in humans (Supplemental Figure 2A).

Inhibiting respiration reverses aberrant proliferation signaling in LFS thymus. More than 70% of _p53^172H/H_ mice developed lymphomas in the thymus (Supplemental Table 1); we therefore examined this tissue to identify biomarkers of mitochondrial regulated cell signaling. One mechanism of p53 tumor suppression is activation of autophagy, while mutant p53, including the human R175H mutation, can act in a protumorigenic manner by inhibition of autophagy (12, 13). Indeed, the thymic tissue of _p53^172H/H_ mice showed decreased levels of autophagosome membrane–associated LC3-II (lanes 4–9 versus 1–3, Figure 1C). Autophagy and associated endoplasmic reticulum stress can activate glycogen synthase kinase 3α/β by stimulating its autophosphorylation at tyrosine 276/216 (pY-GSK3) (14–16). Active GSK3 is necessary for downregulating cyclin D1 (17), a critical driver for cell proliferation that promotes tumorigenesis, including lymphoma formation (18, 19). Accordingly, increased cyclin D1 was inversely correlated with decreased pY-GSK3 and autophagy in _p53^172H/H_ mice along with reduced autophagy (lanes 4–9, Figure 1C). Cyclin E1, also, like cyclin D1, involved in G_1 cell cycle regulation, remained unchanged in _p53^172H/H_ mice, indicating a specific defect in the

cyclin D1 pathway (Figure 1C). To further demonstrate the effect of p53 R172H mutant on this signaling pathway, we examined the response of human primary myoblasts to serum deprivation. After 3 hours of serum starvation, control cells displayed increased autophagy and pY-GSK3, both of which were blocked by expressing human p53 R175H (homologous to mouse R172H) (Figure 1D). In vivo, LFS mouse thymus tissue also showed changes that were consistent with the inhibitory effects of mutant p53 on autophagy and GSK3 signaling (Supplemental Figure 3).

One direct effect of inhibiting mitochondrial respiration is lower mitochondrial membrane potential, which can induce mitophagy/autophagy, potentially disrupting proliferation signaling in LFS mice. Because homozygous _Polg^mut/mut_ mice have accelerated involution of the thymus, we focused on the heterozygous _Polg^+/mut_, which causes no overt phenotype (11), but conferred a substantial increase in cancer-free survival of _p53^172H/H_ mice. Notably, the _Polg^+/mut_ state caused decreases in both mitochondrial complex I subunit and activity levels in mutant p53 R172H thymus and spleen (Supplemental Figure 4). This disruption of mitochondria induced autophagy and pY-GSK3 as well as NAD(P)H dehydrogenase quinone 1 (NQO1) levels, while cyclin D1 was downregulated despite the presence of mutant p53 (Figure 1E). _Polg_ mutation also activated the autophagy/
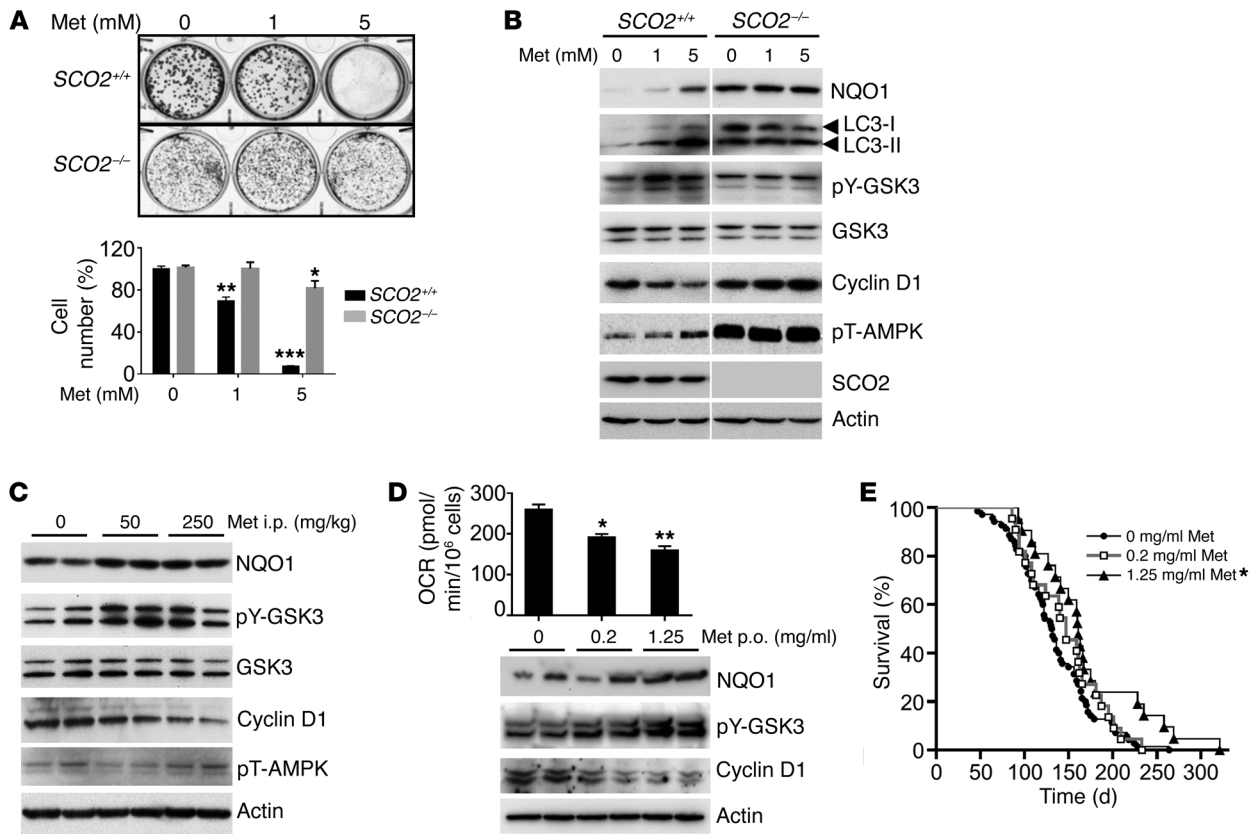
Figure 2. Metformin induces antiproliferation signaling by inhibiting respiration and prevents tumorigenesis. (**A**) Crystal violet staining and quantification of *SCO2*⁺/⁺ and *SCO2*⁻/⁻ HCT116 cells after metformin (Met) treatment for 10 days (*n* = 4–6). (**B**) HCT116 cells treated with metformin overnight prior to immunoblotting. pT-AMPK is from a separate gel using the same samples. Representative of 3 experiments. (**C**) *p53*[172H/H] mice (approximately 10 weeks old) were injected (i.p.) with metformin daily for 1 week prior to thymus immunoblotting. (**D**) OCR and immunoblots of thymus from approximately 7-week-old *p53*[172H/H] mice treated with metformin by mouth (p.o) in drinking water for 3 weeks (*n* = 6–7). (**E**) Kaplan-Meier survival plot of *p53*[172H/H] mouse treated with metformin in drinking water (*n* = 21–70). Statistical difference by 2-way ANOVA (**A**), 1-way ANOVA (**D**), and log-rank test (**E**). *$P < 0.01$; **$P < 0.001$; ***$P < 0.0001$.

GSK/cyclin D1 pathway in approximately 1-year-old heterozygous *p53*[172R/H] mice, indicating the preservation of this cell signaling in the genotype modeling LFS patients (Supplemental Figure 2B).

Metformin can reproduce the effects of genetically disrupting mitochondrial function in LFS mice. Based on its known inhibitory effect on mitochondria and excellent safety profile in humans, metformin represented an ideal therapeutic agent for a test of whether it can reproduce the antiproliferation signaling induced by *Polg* mutation. Because metformin has pleiotropic effects on cells, we utilized a pair of isogenic respiratory-proficient and -deficient cell lines to demonstrate that its antiproliferation effect acts via inhibition of respiration. Metformin inhibited the proliferation of wild-type human colon cancer HCT116 cells, but did not markedly affect nonrespiring cells with homozygous deletion of synthesis of cytochrome *c* oxidase 2 (*SCO2*⁻/⁻), an essential factor for the assembly of respiratory complex IV (Figure 2A) (20). In parallel, metformin reproduced the in vivo cell signaling changes caused by mitochondrial disruption only in respiring *SCO2*⁺/⁺ cells (Figure 2B). Despite the high concentration of metformin used in vitro, the autophagy/GSK3/cyclin D1 signaling components remained unchanged in *SCO2*⁻/⁻ cells with high basal levels of NQO1 and phosphorylated AMPK (pT-AMPK) (Figure 2B). It is notable that, although pT-AMPK is thought to be a major target of

metformin, pY-GSK3 appeared to be more sensitive to metformin than pT-AMPK in respiring cells, as seen by its strong induction at 1 mM compared with 5 mM for pT-AMPK (Figure 2B).

Questions have been raised about the clinical relevance of in vitro findings using millimolar concentrations of metformin versus the micromolar range plasma levels observed in patients on therapeutic doses of metformin (21). Thus, we examined whether treating LFS mice with metformin at human therapeutic levels would induce antiproliferation signaling (22). The i.p. injection of metformin at 250 mg/kg body weight reproduced the autophagy/ GSK3/cyclin D1 signaling cascade, as observed in cultured cells (Figure 2C). Notably, pY-GSK and cyclin D1 levels were again more sensitive to metformin than pT-AMPK (Figure 2C). This was consistent with the report that pT-AMPK induction by metformin cannot be detected in tissues in contrast with cultured cells (23).

Based on these findings, we tested the feasibility of a cancer-free survival study in *p53*[172H/H] mice treated with metformin in drinking water. A dose of 1.25 mg/ml metformin in drinking water was previously shown to result in plasma concentrations within the therapeutic range observed in humans and to inhibit xenograft growth in mice (22, 24). The thymus of mice fed metformin showed a dose-dependent decrease in oxygen consumption and the induction of antiproliferation signaling cascade (Figure 2D).
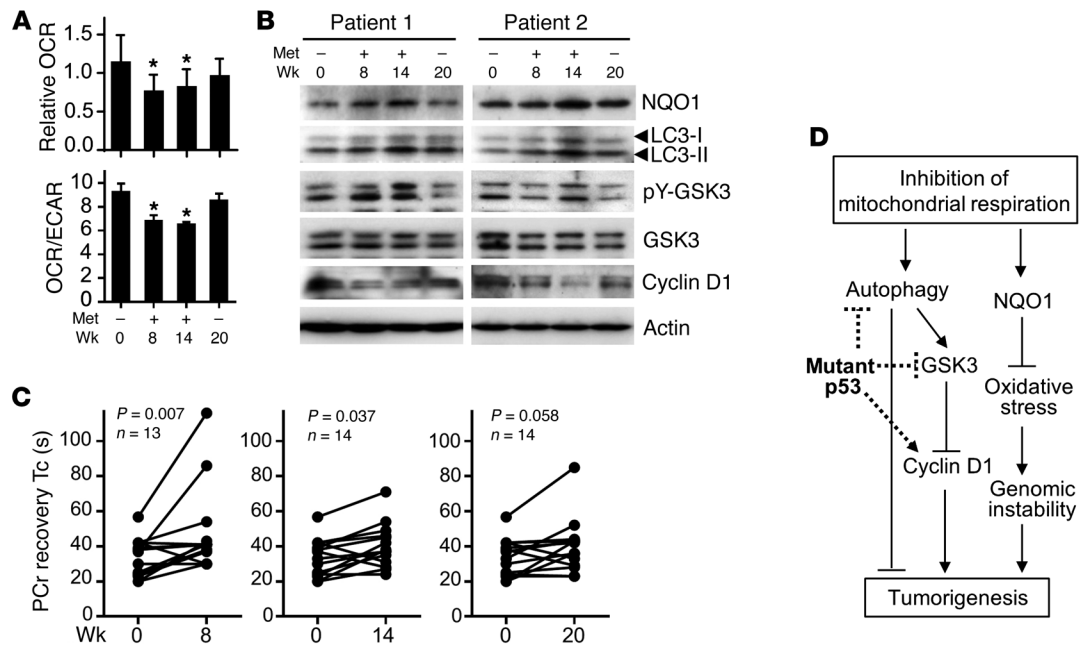
Figure 3. Metformin inhibits mitochondria and activates antiproliferation signaling in LFS patients. (**A**) Effect of metformin on OCR and OCR/ECAR ratio of LFS patient blood MNCs (n = 13–14). (**B**) Representative immunoblots of MNCs from 2 out of 6 examined patients. (**C**) Effect of metformin treatment on PCr recovery time constant (Tc) after exercise in patient skeletal muscle. (**D**) Mitochondrial inhibition counteracts tumor-promoting activities of mutant p53 (dotted lines). Statistical differences by 1-way ANOVA (**A**) and Wilcoxon matched-pairs signed rank test (**C**). *P < 0.01.

Initiating treatment with 1.25 mg/ml metformin after the mice were weaned (~4 weeks old) increased its median and mean cancer-free survival times by 22% and 27%, respectively, while 0.2 mg/ml metformin treatment showed only a trend of increased survival time (Figure 2E). Although less effective in prolonging cancer-free survival than the genetic approach, the improvement in survival by metformin treatment was nonetheless remarkable, as lymphomas can develop early in *p53$^{172H/H}$* mice.

Metformin inhibits mitochondrial function and activates antiproliferation signaling in LFS patients. We next examined whether metformin could elicit similar effects in LFS patients. As part of a clinical protocol examining the safety and tolerability of metformin, healthy LFS patients carrying germline *TP53* mutations were examined for mitochondrial function and antiproliferation biomarkers before (week 0), during (weeks 8 and 14), and after (week 20) metformin treatment (Supplemental Table 2 and Supplemental Figure 5A) (25). Blood samples were obtained at the indicated time points, and mononuclear cells (MNCs) were isolated for simultaneous measurements of OCR and extracellular acidification rate (ECAR) as markers of oxidative and glycolytic metabolism, respectively. Metformin treatment at the maximum dose of 2,000 mg daily at weeks 8 and 14 substantially reduced OCR as well as the OCR to ECAR ratio as an index of oxidative metabolism, while 6 weeks of medication washout at week 20 resulted in recovery of respiration (Figure 3A and Supplemental Figure 5B). As a control, we confirmed that the acute removal of metformin in preparation for the assay does not affect the inhibition (Supplemental Figure 6). The metformin treatment reproduced the pattern of increased NQO1, LC3-II, and pY-GSK3 activation and decreased cyclin D1 in MNCs (Figure 3B). Finally, we measured skeletal muscle phosphocreatine (PCr) recovery kinetics after exercise in vivo as a marker of

oxidative phosphorylation capacity using phosphorus-31 magnetic resonance spectroscopy (^{31}P-MRS) (7). Treatment with metformin (weeks 8 and 14) increased the PCr recovery time constant, providing in vivo evidence of decreased mitochondrial activity that is necessary for regenerating PCr in muscle (Figure 3C).

In the current study, we have demonstrated that the genetic perturbation of mitochondrial respiration in a mouse model of LFS can markedly improve cancer-free survival in association with inhibition of the aberrant proliferation signaling caused by p53 mutation (Figure 3D). Pharmacologically inhibiting mitochondrial respiration using metformin also improved cancer-free survival and reproduced the cell-signaling cascade observed in the genetic model. Importantly, treatment of LFS patients with metformin inhibited respiration in blood MNCs and evoked similar anti–cell proliferation signaling that could serve as treatment biomarkers. Furthermore, we have provided in vivo evidence that metformin can decrease mitochondrial activity in the tissue of LFS patients, which is known to give rise to rhabdomyosarcoma, a cancer associated with the syndrome.

One possible mechanism by which mitochondrial disruption prevented tumorigenesis is through increased oxidative stress. However, the overall decrease in cellular ROS levels associated with decreased nuclear NRF2 suggested that increased oxidative stress may not be the major mechanism underlying our observations (Supplemental Figure 7). The marked improvement in survival of *p53$^{172H/H}$* mice by the homozygous mutation of *Polg* could be explained by increased apoptosis, but this was not detected in the heterozygous *Polg* mutant state, as previously reported (Supplemental Figure 8) (11, 26). Although our study was not designed to address whether inhibiting respiration prevents cancer specifically in LFS, our findings are in agreement with the growing evidence

indicating the importance of mitochondria in tumorigenesis. In this regard, inhibiting the expression of citrate transport protein (CTP), a gene that is induced by a gain-of-function p53 mutant and increases mitochondrial respiration, blunts the accelerated xenograft growth of both p53 mutant and p53 null cancer cells (27). Such observations point to the essential role of the mitochondrion in tumorigenesis regardless of p53 status and intrinsic mitochondrial function.

In summary, our study provides evidence that disrupting mitochondrial respiration can delay cancer development in LFS, a cancer predisposition syndrome for which there is currently no therapeutic option. Despite epidemiologic evidence of decreased cancer incidence associated with metformin treatment in diabetic patients, biomarkers correlating its treatment effect to tumor suppression have not been identified. By using a LFS mouse model with concurrent translation of the findings to patients, our study provides in vivo evidence of the inhibitory effects of metformin on mitochondria and provides a mechanistic rationale for investigating the chemopreventive efficacy of metformin in populations at high risk of cancer.

Methods

Statistics. GraphPad Prism software (version 6.02) was used for 1-way ANOVA test for multiple comparisons, the log-rank test for animal survival, the Pearson correlation coefficient test, and the Wilcoxon matched-pairs signed rank test (2-tailed) for before and after metformin treatment in patients. Values are shown as mean ± SEM. A *P* value of less than 0.05 was considered significant.

Study approval. All animal procedures were approved by the Animal Care and Use Committee of the NHLBI-NIH. All human subjects provided written informed consent. The IRB of the NCI and NHLBI approved the protocols (ClinicalTrials.gov NCT01981525 and NCT00406445).

See Supplemental Methods for additional information.

Author contributions

PYW, JL, JGK, MFS, SLT, JZ, JHP, and PMH designed and performed the experimental studies. FLW, PLM, MP, CMA, SAS, ATF, and PMH designed and performed the clinical studies. RDH and CMB managed patients. PYW, JL, and PMH wrote the manuscript.

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