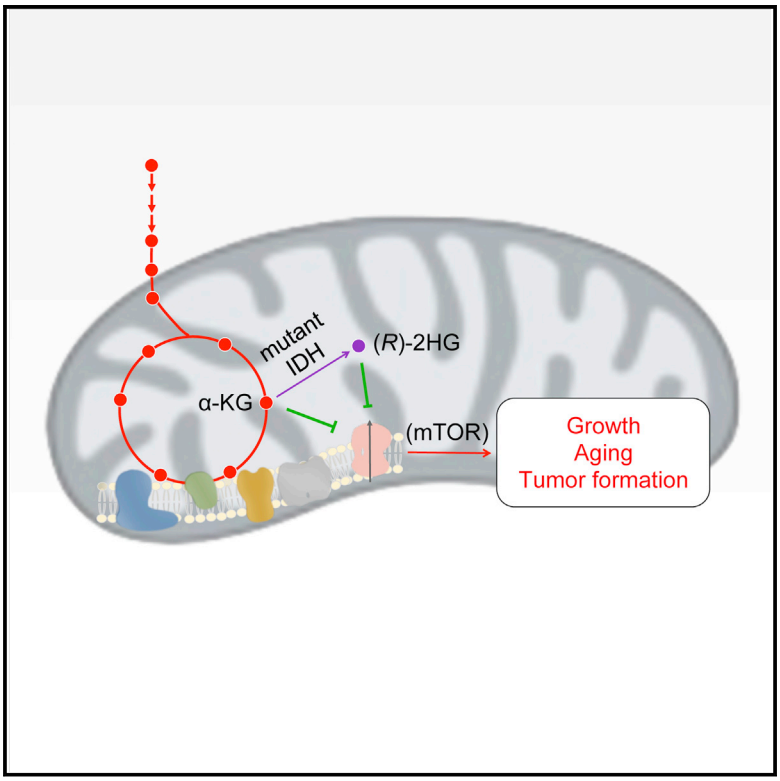
Short Article

Cell Metabolism

2-Hydroxyglutarate Inhibits ATP Synthase and mTOR Signaling

Graphical Abstract



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In Brief

Aberrant isocitrate dehydrogenase enzymes encoded by cancer-associated IDH1 and IDH2 gene mutations produce an oncometabolite, (*R*)-2HG. Fu et al. discover a growth-suppressive function of (*R*)-2-HG mediated by its binding and inhibition of ATP synthase. The resulting OXPHOS perturbation imparts extra vulnerability to glucose limitation in IDH mutant glioblastoma cells.

Highlights

- 2-HG, like α-KG, inhibits ATP synthase and extends the lifespan of *C. elegans*
- IDH1(R132H) mutant cells have reduced ATP content, respiration, and mTOR signaling
- IDH1(R132H) mutant cells exhibit an intrinsic vulnerability to glucose limitation
- ATP synthase is a target of 2-HG's growth-suppressive activity in IDH mutant cells

Fu et al., 2015, Cell Metabolism *22*, 508–515
September 1, 2015
http://dx.doi.org/10.1016/j.cmet.2015.06.009

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2-Hydroxyglutarate Inhibits ATP Synthase and mTOR Signaling

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http://dx.doi.org/10.1016/j.cmet.2015.06.009

SUMMARY

We discovered recently that the central metabolite α-ketoglutarate (α-KG) extends the lifespan of *C. elegans* through inhibition of ATP synthase and TOR signaling. Here we find, unexpectedly, that (*R*)-2-hydroxyglutarate ((*R*)-2HG), an oncometabolite that interferes with various α-KG-mediated processes, similarly extends worm lifespan. (*R*)-2HG accumulates in human cancers carrying neomorphic mutations in the isocitrate dehydrogenase (IDH) 1 and 2 genes. We show that, like α-KG, both (*R*)-2HG and (*S*)-2HG bind and inhibit ATP synthase and inhibit mTOR signaling. These effects are mirrored in IDH1 mutant cells, suggesting a growth-suppressive function of (*R*)-2HG. Consistently, inhibition of ATP synthase by 2-HG or α-KG in glioblastoma cells is sufficient for growth arrest and tumor cell killing under conditions of glucose limitation, e.g., when ketone bodies (instead of glucose) are supplied for energy. These findings inform therapeutic strategies and open avenues for investigating the roles of 2-HG and metabolites in biology and disease.

INTRODUCTION

Aberrant metabolism, long symbolic of inherited metabolic diseases, is now recognized as a hallmark of many other pathogenic conditions, including cancer (Warburg, 1956; Vander Heiden et al., 2009). Recently, we discovered that the common metabolite α-ketoglutarate (α-KG) increases the lifespan of adult *C. elegans* by inhibiting the highly conserved ATP synthase and the TOR pathway, mimicking dietary restriction in longevity (Chin et al., 2014). Furthermore, the observation that α-KG inhibits mTOR function in normal human cells implies a role for α-KG as an endogenous tumor suppressor metabolite (Chin et al., 2014). Known for its role in central carbon metabolism as a tricarboxylic acid (TCA) cycle intermediate, α-KG is universal to all cellular life. α-KG also serves as a co-substrate for a large family of dioxygenases with functions in cellular processes such as hypoxic response and epigenetic regulation. The identification of α-KG as a regulator of ATP synthase reveals a new mechanism for longevity regulation through metabolite signaling and suggests that there likely exist other metabolites that play signaling roles in aging. Particularly, metabolites that are similar in structure to α-KG may also modify lifespan through interactions with ATP synthase, and the lifespan effects of metabolites may correlate with their involvement in human disease.

In the TCA cycle, α-KG is produced from isocitrate by isocitrate dehydrogenase (IDH). Catalytic arginine mutations in the *IDH1* and *IDH2* genes found in gliomas and acute myeloid leukemia (AML) result in neomorphic enzymes that, instead, convert α-KG to the structurally similar (*R*)-2-hydroxyglutarate ((*R*)-2HG), which accumulates to exceedingly high levels in these patients (Dang et al., 2009; Gross et al., 2010; Ward et al., 2010; Xu et al., 2011). (*R*)-2HG is now considered an oncometabolite, impairing epigenetic and hypoxic regulation through its binding to α-KG-dependent dioxygenases (Lu

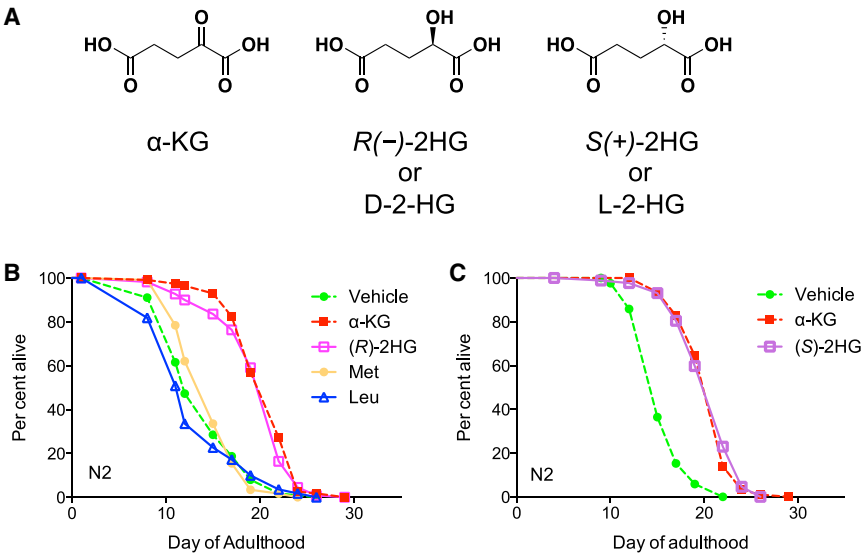

Figure 1. 2-HG Extends the Lifespan of Adult *C. elegans*

(A) Chemical structures of 2-hydroxyglutaric acid and α-ketoglutaric acid.

(B) (*R*)-2HG-supplemented worms. The mean lifespan (days of adulthood) with vehicle treatment (m_{veh}) = 14.0 (n = 112 animals tested). $m_{\alpha\text{-KG}}$ = 20.7 (n = 114), p < 0.0001 (log-rank test); $m_{(R)\text{-2HG}}$ = 20.0 (n = 110), p < 0.0001 (log-rank test); m_{Met} = 14.7 (n = 116), p = 0.4305 (log-rank test); mL_{eu} = 13.2 (n = 110), p = 0.3307 (log-rank test).

(C) (*S*)-2HG-supplemented worms. m_{veh} = 15.7 (n = 85); $m_{\alpha\text{-KG}}$ = 21.5 (n = 99), p < 0.0001 (log-rank test); $m_{(S)\text{-2HG}}$ = 20.7 (n = 87), p < 0.0001 (log-rank test).

All metabolites were given at a concentration of 8 mM. Two independent experiments were performed.

et al., 2012; Koivunen et al., 2012). The development of inhibitors of mutant IDH that normalize (*R*)-2HG levels is an attractive cancer therapeutic strategy (Wang et al., 2013; Rohle et al., 2013). Paradoxically, however, brain cancer patients with IDH mutations have a longer median overall survival than patients without mutations (Parsons et al., 2008; Yan et al., 2009; van den Bent et al., 2010), hinting at additional complexity in the biology of these cancers. (*R*)-2HG and (*S*)-2-hydroxyglutarate ((*S*)-2HG) have also been found to accumulate in tissues of individuals with germline mutations in genes encoding the corresponding 2-HG dehydrogenases (Kranendijk et al., 2012; Steenweg et al., 2010). The resulting 2-HG aciduria is associated with neurological manifestations whose molecular mechanisms are unknown (Kranendijk et al., 2010). We set out to identify additional targets of 2-HG to elucidate the mechanisms underlying the seemingly disparate 2-HG-related phenotypes.

RESULTS AND DISCUSSION

2-HG Extends the Lifespan of Adult *C. elegans*

We have demonstrated that α-KG promotes longevity through inhibition of ATP synthase (Chin et al., 2014). Given the structural similarity between α-KG and 2-HG (Figure 1A) and the association of 2-HG with cancer and neurological dysfunction, we asked whether 2-HG influences longevity. Surprisingly, both (*R*)-2HG and (*S*)-2HG increase the lifespan of *C. elegans* (Figures 1B and 1C). Notably, (*R*)-2HG, (*S*)-2HG, and α-KG interact distinctly with the α-KG-dependent dioxygenases (Koivunen et al., 2012; Tarhonskaya et al., 2014). Therefore, the similar effect of α-KG and (*R*)- and (*S*)-2-HG on lifespan points to a common mechanism that is independent of dioxygenases or any enantiomer-specific 2-HG effects (da Silva et al., 2002; Latini et al., 2005; Wajne et al., 2002; Chan et al., 2015). Because we identified the ATP synthase β subunit (ATP5B) as a target of α-KG (Chin et al., 2014), we asked whether 2-HG acts by a similar mechanism.

ATP Synthase Is a Molecular Target of 2-HG

To determine whether 2-HG targets ATP5B, we first performed a drug affinity-responsive target stability (DARTS) analysis (Lomenick et al., 2009) using U87 human glioblastoma cells. We found that both (*R*)-2HG and (*S*)-2HG bind to ATP5B (Figure 2A; data not shown). Like α-KG, both 2-HG enantiomers inhibit ATP synthase (complex V) (Figures 2B and 2C; Figures S1A–S1C). This inhibition is specific because there is no inhibition by either enantiomer on other electron transport chain (ETC) complexes (Figures S1D–S1F) or ADP import into the mitochondria (Figure S1G). The inhibition of ATP synthase by 2-HG is also readily detected in live cells. Treatment of U87 cells (wild-type IDH1/2) with membrane-permeable octyl esters of 2-HG or α-KG results in decreased cellular ATP content (Figure S2A) and a decreased ATP/ADP ratio (Figure 2D; Figure S2B) under mitochondrially oxidative phosphorylation (OXPHOS) conditions, as with the ATP synthase inhibitor oligomycin (Figures S2A and S2B). As expected, both basal and ATP synthase-linked oxygen consumption rates (OCRs) are decreased in 2-HG-treated cells (Figure 2E; Figures S2C and S2D), and lifespan increase by 2-HG is dependent on ATP synthase (Figure S2E).

IDH1(R132H) Mutant Cells Have Decreased ATP Content and Mitochondrial Respiration

At normal cellular concentrations of ~200 μM (Gross et al., 2010), (*R*)-2HG is unlikely to cause significant inhibition of ATP synthase. However, in glioma patients with IDH mutations where (*R*)-2HG accumulates to 10–100 times of natural levels (Dang et al., 2009; Gross et al., 2010), inhibition of ATP synthase would be possible. To test this idea, we used U87 cells stably expressing IDH1(R132H), the most common IDH mutation in glioma (Yan et al., 2009). Similar to octyl (*R*)-2HG-treated cells, U87/IDH1(R132H) cells exhibit decreased ATP content and ATP/ADP ratio (Figure 3A) and OCR (Figure 3B) compared with isogenic IDH1(WT)-expressing U87 cells. Importantly, the decrease in respiration in IDH1(R132H) cells is attributable to ATP synthase (complex V) inhibition. Although there is a clear difference in basal respiration rates in U87/IDH1(WT)

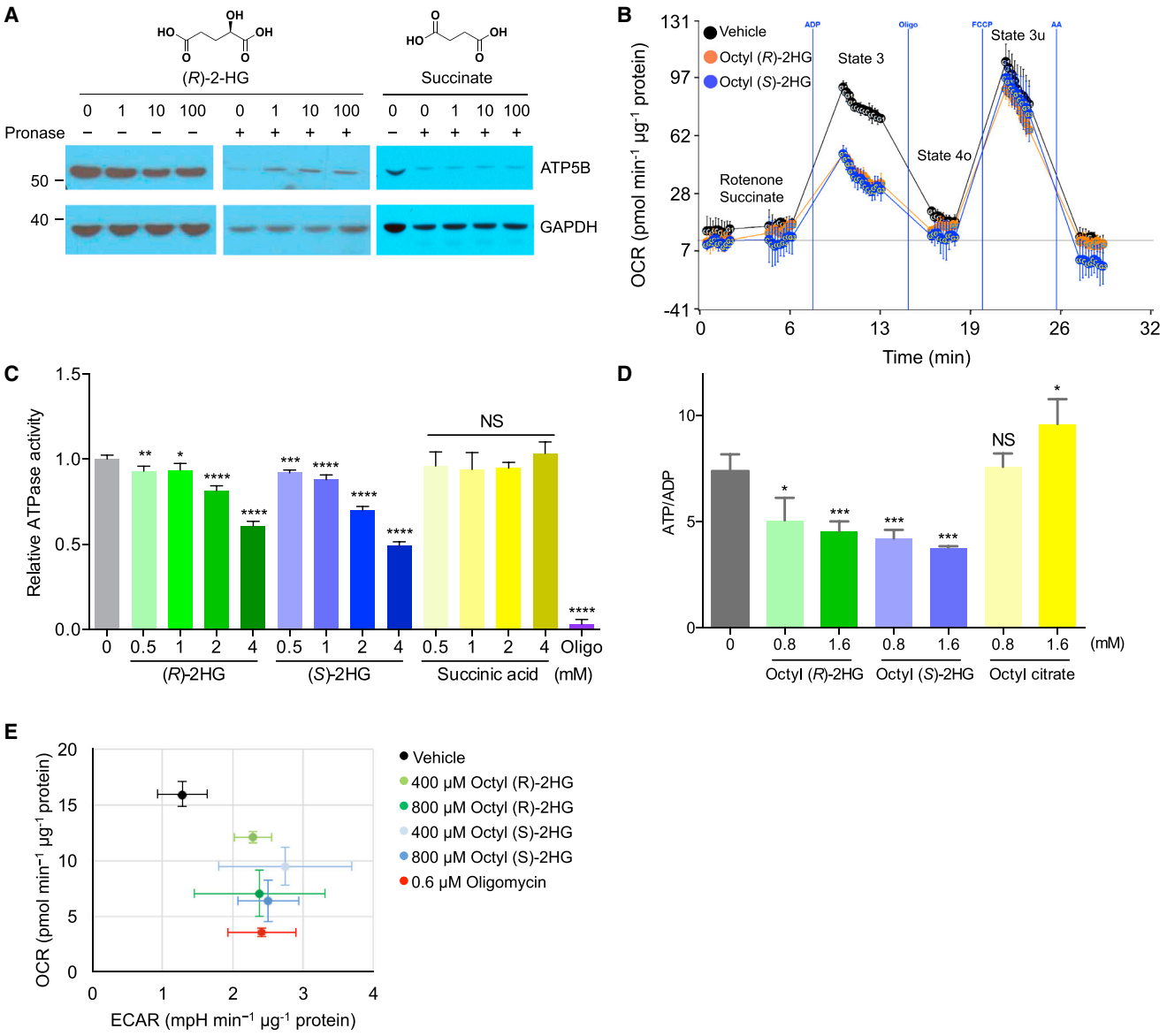

Figure 2. 2-HG Binds and Inhibits ATP Synthase

(A) DARTS identifies ATP5B as a 2-HG binding protein. U87 cell extracts were used. Succinate served as a negative control.

(B) Inhibition of ATP synthase by 2-HG. 2-HG, released from octyl 2-HG (600 µM), decreases ($p < 0.001$) state 3, but not state 4o or 3u, respiration in mitochondria isolated from mouse liver. Octanol was used as vehicle. Oligo, oligomycin; FCCP, carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone; AA, antimycin A.

(C) Inhibition of submitochondrial particle ATPase by 2-HG acid but not by succinic acid. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; NS, $p > 0.05$. Oligo, oligomycin (32 µM).

(D) Decreased ATP/ADP ratio in U87 cells treated with octyl 2-HG but not octyl citrate, indicating specificity and excluding any effect involving octanol. *$p < 0.05$, ***$p < 0.001$; NS, $p > 0.05$.

(E) Decreased respiration as indicated by OCR (**$p < 0.01$) in octyl 2-HG-treated U87 cells in glucose medium. Octanol shows no effect on OCR compared with DMSO.

For (A)–(E), results were replicated in at least two independent experiments. Unpaired t test, two-tailed, two-sample unequal variance was used for (B)–(E). Mean ± SD is plotted.

versus U87/IDH1(R132H) cells, oligomycin-insensitive respiration, which is independent of complex V, is not significantly different between IDH1(WT) and IDH1(R132H) cells (Figure 3C). Furthermore, complex V knockdown using ATP5B RNAi normalizes the respiration difference between IDH1(R132H) and IDH1(WT) cells (Figure 3D). Consistently, the difference in ATP content of U87/IDH1(WT) and U87/IDH1(R132H) cells is diminished upon treatment with octyl (*R*)-2HG (Figure 3E). Similar results were obtained in HCT 116 IDH1(R132H/+) cells (Figures S3A and S3B). In addition, the mitochondrial membrane potential in IDH1 mutant cells is higher than in IDH1 wild-type cells (Figure 3F; Figure S3C), consistent with the inhibition of complex V

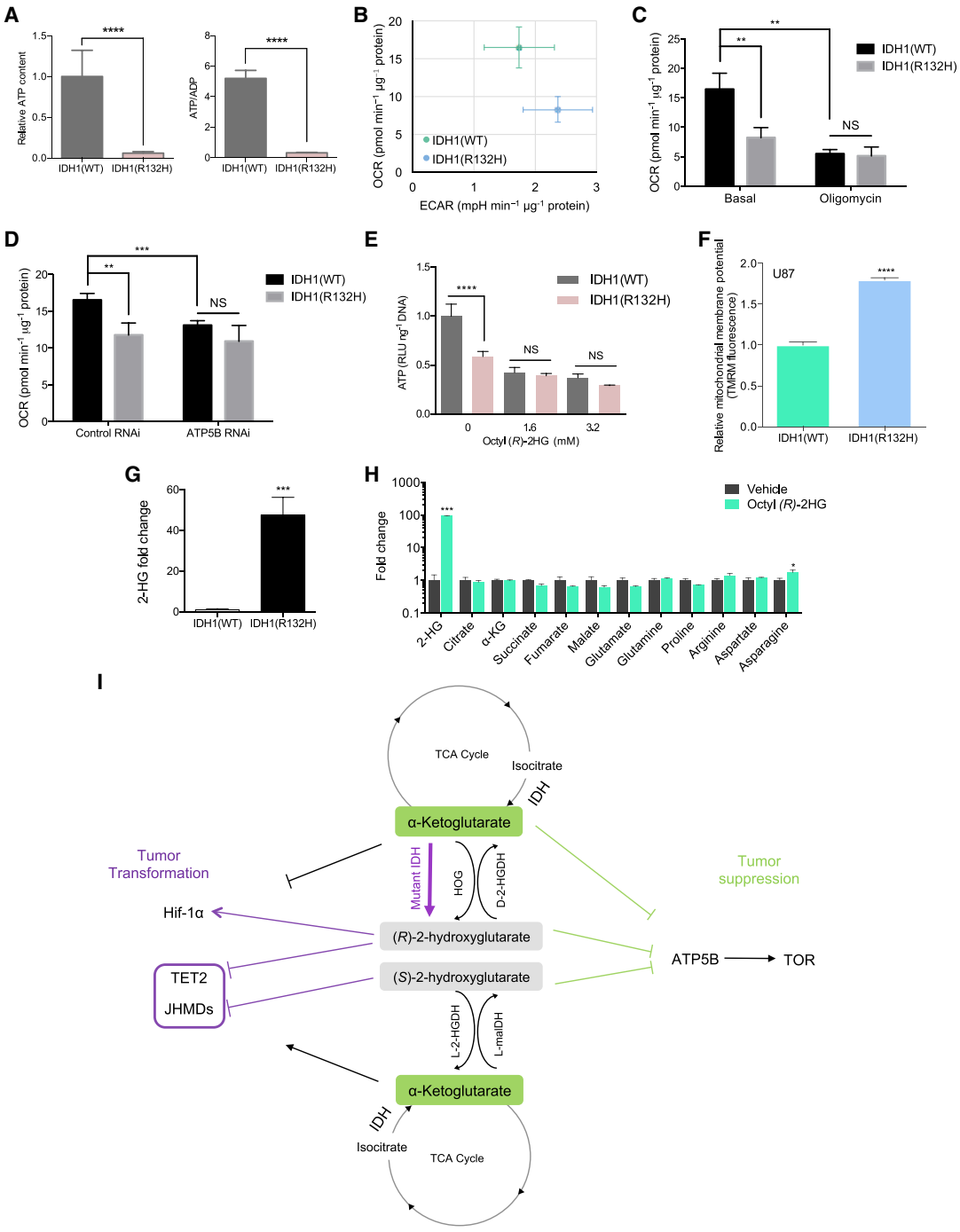

Figure 3. Inhibition of ATP Synthase in IDH1(R132H) Cells

(A) Decreased ATP levels and ATP/ADP ratio in U87/IDH1(R132H) cells (****$p < 0.0001$).

(B) Decreased respiration in U87/IDH1(R132H) cells (**$p = 0.0037$).

(C and D) Decreased respiration in U87/IDH1(R132H) cells is complex V-dependent (**$p < 0.01$, ***$p < 0.001$; NS, $p > 0.05$).

(E) Decreased ATP content in U87/IDH1(R132H) cells is attributable to (*R*)-2HG (****$p < 0.0001$; NS, $p > 0.05$).

(F) Increased mitochondrial membrane potential in U87/IDH1(R132H) cells normalized to cell number (****$p < 0.0001$).

(G) 2-HG accumulation in U87/IDH1(R132H) cells (***$p = 0.0003$).

(H) Metabolic profile of octyl *(R)*-2HG-treated U87 cells (***$p < 0.001$, *$p = 0.0435$). It is possible that the flux rate changed without affecting the absolute abundance of the intermediates.

(I) Model of metabolite signaling through ATP synthase inhibition.

Unpaired t test, two-tailed, two-sample unequal variance was used for (A)–(H). Mean ± SD is plotted. Results were replicated in at least two independent experiments.

(Johnson et al., 1981). In contrast, inhibition of ETC complex I, III, or IV causes dissipation of the mitochondrial membrane potential (Johnson et al., 1981).

The intracellular (*R*)-2HG levels are ~20- to 100-fold higher in U87 and HCT 116 cells expressing IDH1(R132H) than in control cells (Figure 3G; Figure S3D). The elevated (*R*)-2HG levels are comparable with those found in cells treated with octyl (*R*)-2HG (Figure 3H), and levels reported for IDH1 mutant tumor samples (Dang et al., 2009; Gross et al., 2010; Reitman et al., 2011). The detection of similar (*R*)-2HG levels in tumors as in octyl (*R*)-2HG-treated cells suggests that the tumor cells likely experience reduced ATP synthase and mitochondrial respiration, raising potential prognostic or therapeutic implications (see below).

2-HG Accumulation Does Not Alter the Levels of Common Metabolites

The metabolite 2-HG is linked to the TCA cycle and related amino acid metabolic pathways (Figure 3I). To explore potential metabolic changes upon octyl 2-HG treatment, we measured metabolite levels in octyl 2-HG-treated cells cultured in 1,2-^{13}C-glucose-containing medium by liquid chromatography/mass spectrometry (LC/MS). As expected, 2-HG accumulates 20- to 100-fold more after octyl 2-HG treatment (Figure 3H; Figure S3E). There is no dramatic change (<2-fold) in TCA cycle metabolites or related amino acids (Figure 3H; Figure S3E). As expected, the bulk of the increased 2-HG came from the hydrolysis of exogenously provided octyl 2-HG, as indicated by the unlabeled M+0 isotopomer (Figure S3F; data not shown for octyl (*S*)-2HG treatment). There is also no major change (<2-fold) in labeled TCA cycle intermediates and related amino acids (Figure S3G). Similarly, treatment with octyl α-KG causes an increase in α-KG levels without other substantial changes in the metabolic profile (Figure S3H). The steady-state metabolic profiles observed in 2-HG-treated (or α-KG-treated) cells support the notion that the bioenergetic shift results from the direct inhibition of ATP synthase by 2-HG (or α-KG) rather than secondary effects (Figure 3I).

IDH1(R132H) Mutant Cells Exhibit Intrinsic Vulnerability to Glucose Limitation

As the end component of the mitochondrial ETC, ATP synthase is a major source of cellular energy and the sole site for OXPHOS (Walker, 2013). When glycolysis is inhibited, for example, under conditions of glucose insufficiency, cells are forced to rely on mitochondrial respiration as a source of ATP. The inherent inhibition of ATP synthase and mitochondrial respiration in mutant IDH1 cancer cells therefore suggests a potential Achilles heel for these cancers. Supporting this idea, when cultured in glucose-free, galactose-containing medium to ensure that respiration is the primary source of energy, IDH1(R132H) cells exhibit drastically decreased cell viability (Figure 4A; Figure S4A). These results indicate a particular sensitivity of IDH1(R132H) mutant cells to the deprivation of glucose. The mutant cell line is not sensitive to fetal bovine serum (FBS) deprivation (data not shown), indicating that its increased vulnerability to glucose starvation is specific. This vulnerability to glucose starvation is also evident in U87 cells treated with octyl α-KG or octyl 2-HG (Figures 4B–4D; Figure S4B) and in ATP5B knockdown cells (Figure 4E). These findings raise the possibility that cancer cells with the IDH1(R132H) mutation (and the concomitant ATP synthase/mitochondrial respiration defect) may also be particularly sensitive to nutrient conditions analogous to glucose limitation.

In complex organisms, glucose limitation can occur as a consequence of ketosis, wherein cells use ketone bodies (instead of glucose) for energy. Ketosis is naturally induced upon prolonged starvation (or fasting), during which cells derive energy from fat reservoirs while sparing protein in muscle and other tissues from catabolism. Ketosis can also be induced by feeding a low-carbohydrate, high-fat "ketogenic diet," which has shown benefits against cancer (Stafford et al., 2010). One reason for this may be that tumor cells largely depend on glucose for growth and survival. Because the metabolism of ketone bodies depends entirely on OXPHOS, one prediction is that inhibiting ATP synthase (or other ETC components) in cancer cells would confer a survival disadvantage if ketone bodies were the only source of energy. Because U87 cells are unable to utilize ketone bodies for energy, we determined the effect of ketogenic conditions using HCT 116 cells expressing mutant IDH1. When cultured in glucose-free medium containing the ketone body *(R)*-3-hydroxybutyrate, IDH1 mutant HCT 116 cells showed a profound decrease in viability compared with the parental cells (Figure 4F), confirming the suspected metabolic weakness of IDH mutant cells. These results further support our discovery that (*R*)-2HG accumulation in mutant IDH cancer cells results in ATP synthase inhibition and also suggest novel metabolic therapeutic strategies in cancer treatment.

Decreased mTOR Signaling and Cell Growth by 2-HG

Inhibition of ATP synthase leads to decreased TOR signaling in mammalian cells, worms, and flies (Chin et al., 2014; Sun et al., 2014). We found that ATP5B knockdown (Figure 4G), treatment with octyl esters of 2-HG (Figure 4H), and IDH1(R132H) mutation (Figure 4I) all decrease the phosphorylation of mTOR complex 1 substrates. This effect occurs initially (4 h) in an AMPK-independent manner, with 2-HG decreasing mTOR signaling without significantly altering AMPK activity. However, prolonged exposure to 2-HG (24 h) also activates AMPK (Figure S4C), consistent with the idea that mTOR itself may directly sense ATP (Dennis et al., 2001) in addition to responding to AMP levels through crosstalk with AMPK (Shaw, 2009; Inoki et al., 2012).

TOR is a major regulator of cell growth (Blagosklonny and Hall, 2009). Consistent with the decreased TOR signaling, we observed growth inhibition in ATP5B knockdown cells (Figure S4D), in cells treated with octyl α-KG or octyl 2-HG (Figures S4E–S4G), and in IDH1(R132H)-expressing cells (Figures S4H and S4I). ATP5B RNAi normalizes the growth difference between IDH1(R132H) and IDH1(WT) cells (Figure S4J). Growth inhibition by 2-HG (and by α-KG) is also observed in WI-38 normal human diploid fibroblasts, in immortalized non-malignant HEK293 cells, and in other cancer cell lines tested (Figures S4K–S4N). These results suggest that, when present in excess, 2-HG acts as a growth-inhibitory metabolite across cell types. Further work is warranted to test whether the growth-inhibitory effect of (*R*)-2HG underlies the longer median overall survival of glioma patients with IDH mutations.

SUMMARY

We demonstrate that, similar to α-KG, both enantiomers of 2-HG bind and inhibit ATP synthase and extend the lifespan of

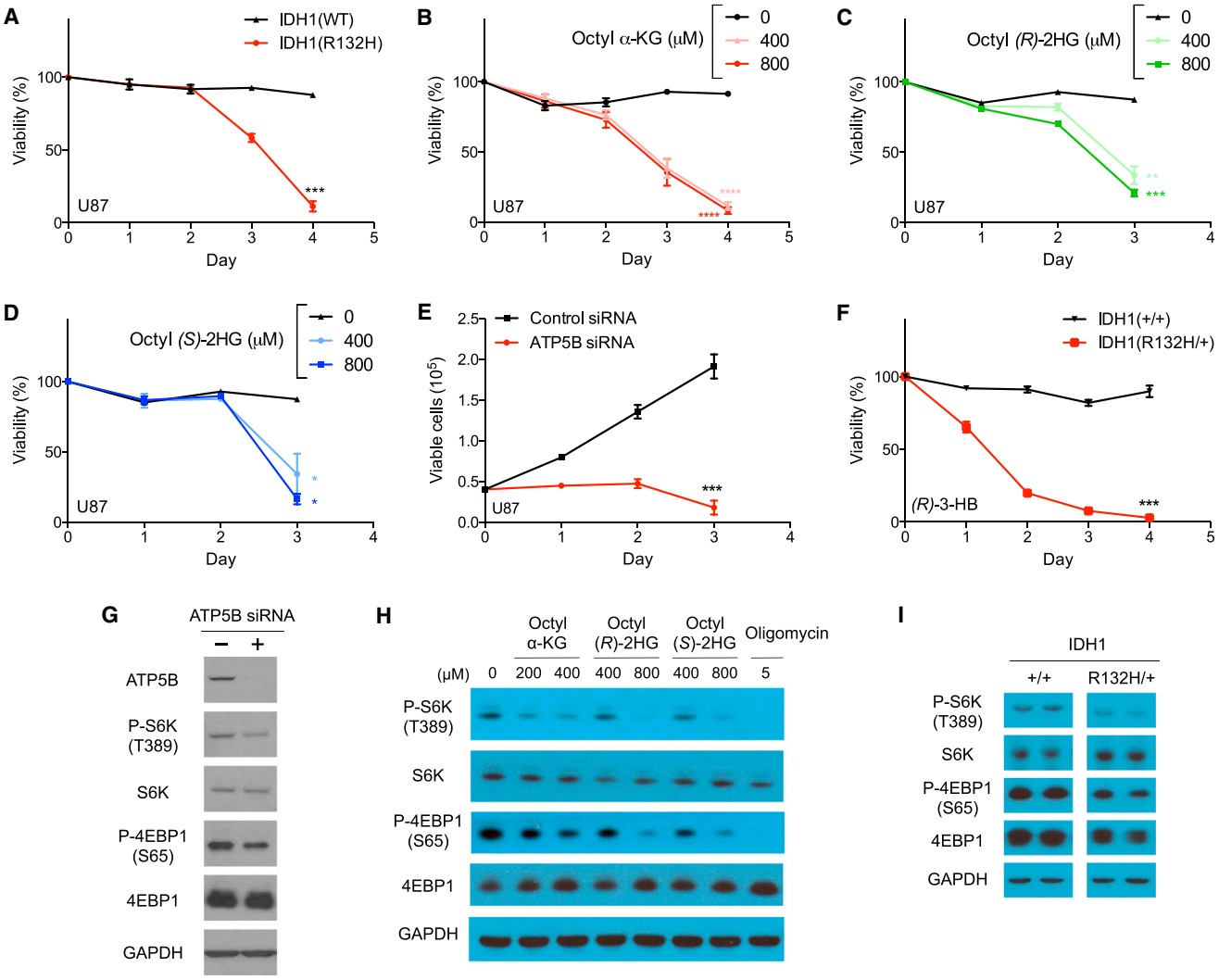

Figure 4. Inherent Vulnerability, or the Loss of Cell Viability, Characteristic of Cells with ATP5B Knockdown, 2-HG Accumulation, or IDH Mutations

(A) U87/IDH1(R132H) cells have increased vulnerability to glucose starvation (***$p < 0.001$).

(B–D) Octyl α-KG- or octyl 2-HG-treated U87 cells exhibit decreased viability upon glucose starvation (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

(E) ATP5B knockdown inhibits U87 cell growth (***$p = 0.0004$).

(F) HCT 116 IDH1(R132H/+) cells exhibit increased vulnerability to glucose-free medium supplemented with *(R)*-3-hydroxybutyrate (***$p < 0.001$).

(G–I) U87 cells with ATP5B knockdown or octyl esters of α-KG or 2-HG treatment and HCT 116 IDH1(R132H/+) cells exhibit decreased mTOR complex 1 activity in glucose-free, galactose-containing medium.

For (A)–(E), cells were cultured in galactose medium. All lanes in (I) are on the same blot. Spaces indicate the positions of unnecessary lanes that were removed digitally. Octanol has no effect on mTOR activity. Unpaired t test, two-tailed, two-sample unequal variance was used for (A)–(F). Mean ± SD is plotted. Results in (A)–(I) were replicated in at least two independent experiments.

C. elegans. Inhibition of ATP synthase by these related metabolites decreases mitochondrial respiration and mTOR signaling. Both 2-HG and α-KG exhibit broad growth-inhibitory effects and reduce cancer cell viability under glucose-restricted conditions. It is now recognized that (*R*)-2HG, which accumulates in IDH mutant cancers, facilitates oncogenic transformation. Little is known, however, about how (*R*)-2HG modifies the phenotype of IDH mutant gliomas when the tumors are formed. Our findings suggest that, in addition to interfering with various α-KG binding factors with importance in cancer, (*R*)-2HG also acts, through inhibition of ATP synthase and mTOR signaling downstream, to decrease tumor cell growth and viability. The latter property may contribute to the improved prognosis of IDH mutant glioma patients. This idea is consistent with emerging findings that the inhibition of ETC complex I in cancer could be an effective therapeutic strategy (Wheaton et al., 2014; Birsoy et al., 2014). Together, these findings highlight a hopeful approach to cancer prevention and treatment by targeting certain aging pathways through metabolic modulation.

The effects of excess 2-HG are likely to be context-dependent. Although its growth-inhibitory effects may be beneficial in cancer, in 2-HG aciduria, the inhibition of ATP synthase and

the resulting impaired mitochondrial function could contribute to neurological dysfunction (da Silva et al., 2002; Wajne et al., 2002; Kölker et al., 2002; Latini et al., 2005). Therefore, the identification of ATP synthase as a target of both 2-HG enantiomers provides a congruent molecular basis for 2-HG-associated cancer and neurological disorders. We postulate that altered mitochondrial energy metabolism may contribute to the inverse susceptibility to cancer and neurodegenerative diseases (*e.g.*, Parkinson's disease). Finally, our findings raise the possibility that nutrient and/or metabolic intervention per se, such as diets that lower the reliance on glucose, and/or approaches that perturb cellular energy metabolism (e.g., by targeting OXPHOS), may benefit glioma patients. Such approaches may be particularly valuable for improving the survival of glioma patients without IDH mutations, who otherwise have no means to inherently curb mitochondrial respiration, and for cancer prevention and treatment in general.

EXPERIMENTAL PROCEDURES

Lifespan Analysis

Lifespan experiments were performed as described previously (Chin et al., 2014). Lifespan assays were conducted at 20°C on solid nematode growth medium (NGM). L4 or young adult animals were placed onto NGM assay plates containing D-2-HG (Sigma, catalog no. H8378), L-2-HG (Sigma, catalog no. 90790), α-KG (Sigma, catalog no. K1128), or vehicle control (H_2O). Assay plates were seeded with OP50. For RNAi experiments, NGM assay plates also contained 1 mM isopropyl-β-D-thiogalactoside (IPTG) and 50 μg/ml ampicillin and were seeded with the appropriate RNAi feeding clone (Thermo Scientific/OpenBiosystems). The *C. elegans* TOR (*let-363*) RNAi clone was obtained from Joseph Avruch (Massachusetts General Hospital/Harvard). To assess the survival of the worms, the animals were prodded with a platinum wire every 2–3 days, and those that failed to respond were scored as dead. Worms that ruptured, bagged, or crawled off the plates were censored. Lifespan data were analyzed using GraphPad Prism. *p* Values were calculated using the log-rank (Mantel-Cox) test unless stated otherwise.

Target Identification Using DARTS

DARTS was performed as described previously (Lomenick et al., 2009).

Measurement of Mitochondrial Respiration

Mitochondrial respiration was analyzed using isolated mitochondria (Brand and Nicholls, 2011). Animal studies were performed under approved University of California Los Angeles (UCLA) animal research protocols.

Cell Growth and Viability Assays

Cells were seeded in 12-well plates and, after overnight incubation, treated with the indicated concentrations of each compound. After harvesting, cells were stained with acridine orange (AO) and 4′,6-diamidino-2-phenylindole (DAPI). Cell number and viability were measured based on AO and DAPI fluorescence as measured by NC3000 (ChemoMetec) following the manufacturer's instructions.

Metabolic Profile Analysis

Cells were cultured for 24 hr and rinsed with PBS, and medium containing 1,2-^{13}C-glucose (1 g/l) was added. After 24-hr culture, cells were rinsed with ice-cold 150 mM NH_4AcO (pH 7.3), followed by addition of 400 μl cold methanol and 400 μl cold water. Cells were scraped off and transferred to an Eppendorf tube, and 10 nmol norvaline as well as 400 μl chloroform were added to each sample. For the metabolite extraction, samples were vortexed for 5 min on ice and spun down, and the aqueous layer was transferred into a glass vial and dried. Metabolites were resuspended in 70% ACN, and a 5-μl sample was loaded onto a Phenomenex Luna 3u NH2 100A (150 × 2.0 mm) column. The chromatographic separation was performed on an UltiMate 3000RSLC (Thermo Scientific) with mobile phases A (5 mM NH_4AcO [pH 9.9]) and B (ACN) and a flow rate of 300 μl/ min. The gradient ran from 15% A to 95% A over 18 min, 9 min isocratic at 95% A, and re-equilibration for 7 min. Metabolite detection was achieved with a Thermo Scientific Q Exactive mass spectrometer run in polarity switching mode (+3.0 kV / −2.25 kV). TraceFinder 3.1 (Thermo Scientific) was used to quantify metabolites as the area under the curve using retention time and accurate mass measurements (≤3 ppm). Relative amounts of metabolites were calculated by summing up all isotopomers of a given metabolite and normalized to the internal standard and cell number. Natural occurring ^{13}C was accounted for as described by Yuan et al. (2008).

Statistical Analyses

All experiments were repeated at least two times with identical or similar results. Data represent biological replicates. Appropriate statistical tests were used for every figure. Mean ± SD is plotted in all figures. See the Supplemental Experimental Procedures for details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.06.009.

AUTHOR CONTRIBUTIONS

The majority of experiments were designed and performed by X.F.; lifespan assays by R.M.C.; DARTS by H.H.; mitochondrial respiration study design and analyses by L.V. and K.R.; enzyme inhibition assays by R.M.C.; compound syntheses by G.D., Y.X., and M.E.J.; and metabolomic profiling and analysis by X.F. and D.B. S.L., L.T., D.A.N., M.Y.P., F.F., C.C., R.M.P., M.A.T., A.L., K.F.F., M.J., S.G.C., T.F.C., T.G.G., D.B., H.R.C., M.E.J., L.V., and K.R. provided guidance, specialized reagents, and expertise. X.F., K.R., and J.H. wrote the paper. X.F., R.M.C., and J.H. analyzed data. All authors discussed the results and contributed to aspects of preparing the manuscript.

ACKNOWLEDGMENTS

We thank Chris Walsh for insightful suggestions and advice. This work was supported by the Oppenheimer Program, NIH grants R01 AT006889 and P01 HL028481, and UCLA Jonsson Cancer Center Foundation and National Center for Advancing Translational Sciences UCLA Clinical and Translational Science Institute (CTSI) Grant UL1TR000124. X.F. is a recipient of a China Scholarship Council scholarship. R.M.C. is a postdoctoral fellow supported by the UCLA Tumor Immunology Training Program (NIH T32 CA009120). M.Y.P. was a trainee of the UCLA C&MB training grant (NIH T32 GM007185). Support for L.V. and K.R. was from the Leducq Foundation. This paper is dedicated to Dr. Judith Gasson in honor of her 20 years as Director of the Jonsson Comprehensive Cancer Center.

Received: October 16, 2014
Revised: February 27, 2015
Accepted: June 10, 2015
Published: July 16, 2015

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