Received: 20 November 2022 | Revised: 25 December 2022 | Accepted: 27 December 2022

DOI: 10.1111/cas.15713

Cancer Science WILEY

ORIGINAL ARTICLE

RNAi screening reveals a synthetic chemical–genetic interaction between ATP synthase and PFK1 in cancer cells

Hiroki Kobayashi[1,2] | Shohei Takase[3] | Haruna Nishimura[1] | Ken Matsumoto[1,3] | Hironori Harada[2] | Minoru Yoshida[1,3,4,5]

[1]Seed Compounds Exploratory Unit for Drug Discovery Platform, RIKEN Center for Sustainable Resource Science, Saitama, Japan

[2]Laboratory of Oncology, School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

[3]Chemical Genomics Research Group, RIKEN Center for Sustainable Resource Science, Saitama, Japan

[4]Department of Biotechnology, Graduate School of Agricultural Life Sciences, The University of Tokyo, Tokyo, Japan

[5]Collaborative Research Institute for Innovative Microbiology, The University of Tokyo, Tokyo, Japan

Correspondence
Hiroki Kobayashi, Laboratory of Oncology, School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan.
Email: hkbys@toyaku.ac.jp

Minoru Yoshida, Chemical Genomics Research Group, RIKEN Center for Sustainable Resource Science, Wako, Saitama 351-0198, Japan.
Email: yoshidam@riken.jp

Funding information
Japan Society for the Promotion of Science, Grant/Award Number: JP19H05640 and JP19K16728; The SGH Foundation

Abstract

To meet cellular bioenergetic and biosynthetic demands, cancer cells remodel their metabolism to increase glycolytic flux, a phenomenon known as the Warburg effect and believed to contribute to cancer malignancy. Among glycolytic enzymes, phosphofructokinase-1 (PFK1) has been shown to act as a rate-limiting enzyme and to facilitate the Warburg effect in cancer cells. In this study, however, we found that decreased PFK1 activity did not affect cell survival or proliferation in cancer cells. This raised a question regarding the importance of PFK1 in malignancy. To gain insights into the role of PFK1 in cancer metabolism and the possibility of adopting it as a novel anticancer therapeutic target, we screened for genes that caused lethality when they were knocked down in the presence of tryptolinamide (TLAM), a PFK1 inhibitor. The screen revealed a synthetic chemical–genetic interaction between genes encoding subunits of ATP synthase (complex V) and TLAM. Indeed, after TLAM treatment, the sensitivity of HeLa cells to oligomycin A (OMA), an ATP synthase inhibitor, was 13,000 times higher than that of untreated cells. Furthermore, this sensitivity potentiation by TLAM treatment was recapitulated by genetic mutations of PFK1. By contrast, TLAM did not potentiate the sensitivity of normal fibroblast cell lines to OMA, possibly due to their reduced energy demands compared to cancer cells. We also showed that the PFK1-mediated glycolytic pathway can act as an energy reservoir. Selective potentiation of the efficacy of ATP synthase inhibitors by PFK1 inhibition may serve as a foundation for novel anticancer therapeutic strategies.

KEYWORDS
ATP synthase, glycolysis, PFK1, shRNA screening, Warburg effect

1 | INTRODUCTION

Cancer cells adopt aerobic glycolysis in a metabolic remodeling process called the Warburg effect. This provides an advantage to rapidly proliferating tumor cells by satisfying cellular bioenergetic and biosynthetic demands.[1,2] In addition, lactate, the final product of glycolysis, facilitates several characteristics of malignancies, including angiogenesis, immune escape, metastasis, and self-sufficient metabolism.[3] The metabolic remodeling of the Warburg effect is probably attained by the orchestration of multiple components such as oncogene activation, tumor suppressor mutations, epigenetics, and the extracellular environment, but the mechanism of its coordinated regulation remains

elusive. Glycolytic flux is mainly controlled by four steps: glucose import, glucose phosphorylation, fructose 6-phosphate phosphorylation, and lactate export.[4] Indeed, phosphofructokinase-1 (PFK1), which phosphorylates fructose 6-phosphate, can promote cell proliferation and tumor growth by increasing glycolytic flux in several cancers, including glioblastoma, colon cancer,[5,6] and cultured cancer cells.[7,8]

We previously demonstrated that a small-molecule compound that we named tryptolinamide (TLAM) induces a metabolic switch from glycolysis to oxidative phosphorylation (OXPHOS) in HeLa cells, cybrids with mutated mitochondrial DNA, and fibroblasts from mitochondrial disease patient-derived induced pluripotent stem cells. To our surprise, we found that TLAM specifically inhibits PFK1.[9] PFK1 inhibition redirects the carbon flow from glycolysis toward the pentose phosphate pathway, which can reinforce antioxidative potential, thereby promoting tumor cell survival.[9–13] Collectively, PFK1 serves as a dual-function regulator that promotes and suppresses both cancer cell proliferation and survival in a context-dependent manner.

Synthetic lethality is a genetic interaction in which cell death is induced by the combination of mutations in two (or more) sensitive genes, while mutation of either one alone is insufficient to affect cell survival.[14,15] Synthetic lethality can also be achieved by chemical inhibition. Successful examples of synthetic lethality-based anticancer drugs are poly(ADP-ribose) polymerase (PARP) inhibitors for patients with BRCA-mutated ovarian, breast, or pancreatic cancer.[16,17] In cancer cells with loss-of-function mutations in either BRCA1 or BRCA2, which are involved in the homologous recombination repair pathway, PARP inhibitors are highly effective due to inhibition of alternative DNA repair pathways.[18,19] Thus, the discovery of synergistic genetic (or chemical–genetic) interactions can contribute to a deeper understanding of biological systems and to the development of novel anticancer therapeutics. Genome-wide screening techniques using RNAi or CRISPR/Cas9 are powerful approaches for identifying synthetic lethal interactions in mammalian cells.[20,21] Our group conducted large-scale RNAi screens to identify the determinants of sensitivity to each of the following: 2-deoxyglucose,[22] aurilide B, a natural cytotoxic marine product,[23] and prethioviridamide, a ribosomally synthesized and post-translationally modified anticancer peptide.[24]

In this paper, we performed large-scale RNAi screening to clarify the roles of PFK1 in cancer cell proliferation and survival, and identified a chemical–genetic interaction between genes coding subunits of ATP synthase (complex V) and PFK1. Importantly, no other respiratory chain complexes showed synthetic interaction with PFK1. This study advances our knowledge of how cancer cells cope with their higher energy demand and provides insights into anticancer therapeutics targeting cancer-specific metabolism.

2 | MATERIAL AND METHODS

2.1 | Pooled lentiviral shRNA screening

DECIPHER barcoded short hairpin RNA (shRNA) libraries (Human Modules 1, 2 and 3) were obtained from Cellecta. Each module contains 27,500 shRNAs covering approximately 5000 human genes, with five or six shRNAs per gene. The libraries share multiple shRNAs targeting 10 control genes, which have more statistical chances to be selected. Packaging into lentiviral particles using 293LTV cells was performed as described previously.[22] HeLa S3 cells were transduced with each of the lentiviral shRNA libraries at 50% efficiency in DMEM (Wako) containing 10% fetal calf serum (FCS; Nissui Pharmaceutical), 2 mM L-alanyl-L-glutamine (Nacalai Tesque), and 5 μg/ml Polybrene (Sigma). After 24 h, the viral supernatant was replaced with fresh medium. After an additional 24 h, the infected cells were selected with 2 μg/ml puromycin for 48 h. The cells were then split into two fractions, one treated with 50 μM TLAM (dissolved in media) and the other untreated. The cells were passaged three times to maintain exponential growth for 10 days. Cells from both fractions were collected and stored at −80°C for further manipulation. Preparation of genomic DNA from cell pellets, PCR amplification of barcodes, and barcode quantitation by next-generation sequencing were performed at Cellecta. Control genes were excluded from the candidates for causing synthetic lethality in conjunction with TLAM. The shRNAs which were absent in sequence reads in either fraction were excluded from analysis.

2.2 | Enrichment analysis

Enrichment analysis was performed using Enrichr, a web-based tool.[25–27]

2.3 | Cell culture

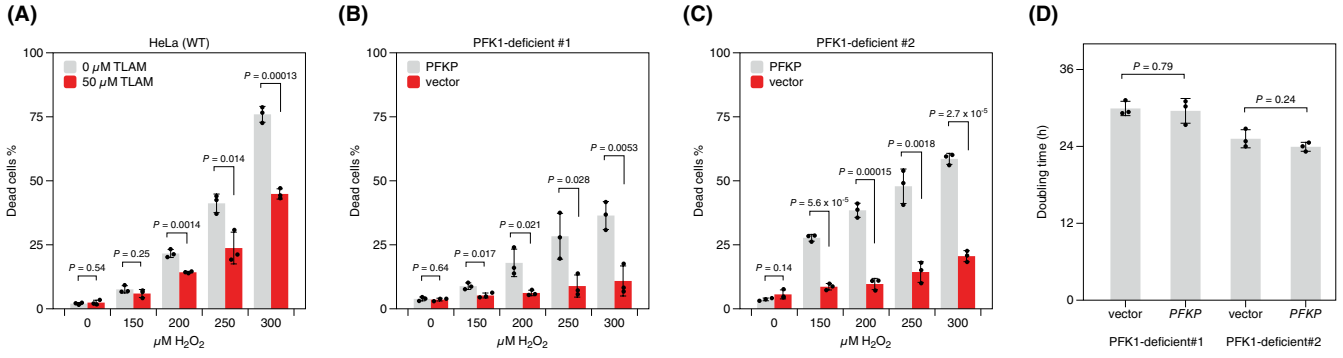
A431, H1299, RXF-631L, HT29, HeLa cells, and the HeLa-derived cell lines were maintained in RPMI1640 (Gibco) supplemented with 10% FCS and 100 units/ml penicillin–streptomycin (Gibco). HeLa S3, 293LTV, IMR90, and TIG3 cells were maintained in DMEM supplemented with 10% FCS and 100 units/ml penicillin–streptomycin.

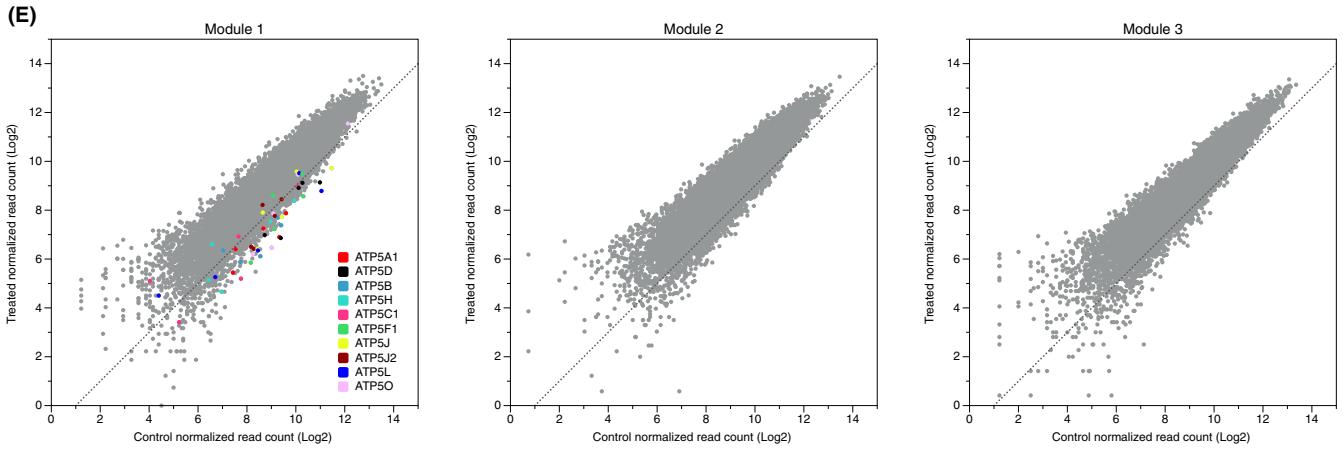
2.4 | Reagents

Tryptolinamide (TLAM) was purchased from Maybridge. Oligomycin A (OMA), rotenone (RTN), and antimycin A (AMA) were purchased from Sigma. Hydrogen peroxide was purchased from Wako. Venturicidin A (VCA) was purchased from Santa Cruz Biotechnology.

2.5 | Cell number assay

Cells were exposed to test compounds in RPMI1640 supplemented with 10% FCS. After incubation for 4 days, the cells were fixed with 4% paraformaldehyde (PFA) for 15 min. After rinsing with phosphate-buffered saline (PBS), the cells were stained with 1 μg/ml Hoechst 33342 (Molecular Probes). The fixed and stained cells were rinsed once with PBS, then imaged and counted using the Celigo S imaging cytometer system (Nexcelom Bioscience).





(F)

Module1

gene	number
ATP5A1	5
ATP5D	5
ATP5B	4
ATP5H	4
ABCB7	3
ATP5C1	3
ATP5F1	3
ATP5J	3
ATP5J2	3
ATP5L	3
ATP5O	3
COX10	3
DNAJA3	3
ECSIT	3
EIF2S2	3
GNB2L1	3
NDUFS8	3
PSMB2	3
TCP1	3

Module2 and 3

gene	number
KIAA0664	4
C14orf156	3
CXorf15	3
FBXL5	3
FKRP	3
INCENP	3
KRT14	3
RPS4X	3
SLC25A39	3
UBE2S	3
CLPX	3
COPB1	3
COPS2	3
LSM6	3
MRPL12	3
MRPL48	3
NAPA	3
SON	3
U2AF2	3

(G)

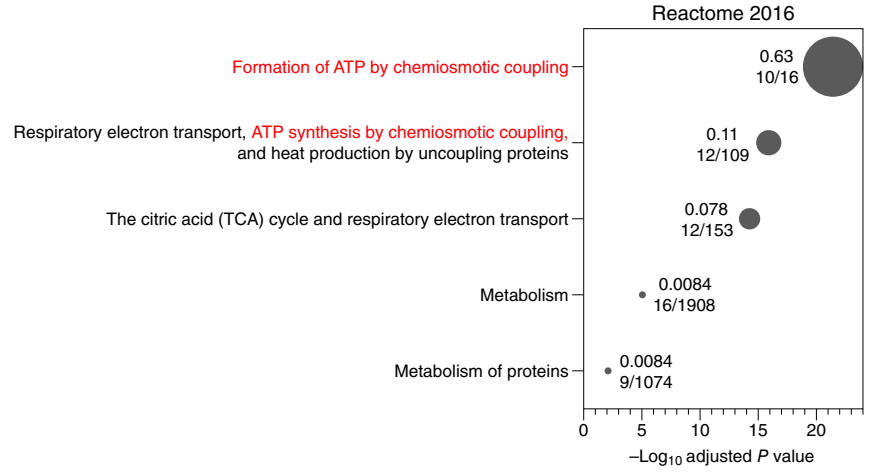


FIGURE 1 Identification of ATP synthase-coding genes that determine the sensitivity to tryptolinamide (TLAM), a phosphofructokinase-1 (PFK1) inhibitor. (A–C) Effects of pharmacological (A) or genetic perturbation (B, C) of PFK1 activity on cell survival under oxidative stress conditions. (D) Doubling time in PFK1-deficient #1/vector, PFK1-deficient #1/PFKP, PFK1-deficient #2/vector, and PFK1-deficient #2/PFKP under normal culture conditions. Throughout, data represent means ± SD of three independent experiments. *P* values were calculated using Student's two-tailed *t*-test ($n = 3$). (E) HeLa S3 cells were infected with gene Modules 1–3 and split into two subpopulations, one treated with TLAM and the other untreated. Relative read counts of each barcode were compared between treated and untreated cells (control). The area under the dashed line represents a barcode abundance ratio below 0.5. (F) Candidate genes that determine the sensitivity to TLAM. shRNAs against ATP synthase are highlighted. (G) Enrichment analysis with the genes listed in (F), performed using the Reactome 2016 database set. Numeric labels represent the gene ratio, defined as the ratio of genes within a given module that are found in any given gene set. The size of each dot represents the gene ratio. Normalized read count data are included in Appendix S1.

2.6 | Imaging and estimation of doubling time

To estimate the doubling times shown in Figure 1D, cells were seeded into 96-well plates (Essen Biosciences), then imaged every 1 h using a time-lapse imaging system (IncuCyte Zoom, Essen Biosciences). Obtained images were analyzed for confluence using IncuCyte software. Doubling time was determined as the time to progress from 30% to 60% confluence. For Figure 4A,B, cells were seeded into 384-well plates (Corning). After 24 h of incubation, cells were exposed to compounds, then imaged every 1 h using a IncuCyte Zoom system.

2.7 | Cell-cycle analysis and detection of dead cells

For detection of dead cells in Figure 1A–C, cells were exposed to hydrogen peroxide for 30 h (for HeLa cells in the presence or absence of 50 μM TLAM) or 24 h (for HeLa-derived cells). Then, the cells were stained with LIVE/DEAD Fixable Violet dye (Thermo Fisher) and subjected to flow cytometry using a cell analyzer (Guava, Millipore). For cell-cycle analysis, cells were exposed to OMA and/or TLAM for 4 days, then fixed with ice-cold 70% ethanol over 2 h at 4°C. After washing with PBS, the cells were stained with 50 μg/ml propidium iodide and 10 μg/ml RNase for 30 min, and subjected to flow cytometry using a cell analyzer. Data were analyzed using Flow Jo (ver. 10.6, BD Biosciences).

2.8 | Glycolytic capacity assay

An XFe96 extracellular flux analyzer (Agilent) was used to measure the extracellular acidification rate and analyze glycolytic capacity. HeLa or HeLa-derived cells were seeded at a density of 10,000 cells per well of an XFe96 cell culture microplate and incubated for 24 h. Before the assay, cells were equilibrated for 1 h in a non-CO_2 incubator with RPMI1640 (R1383; Sigma) containing 1% FCS and 60 μM TLAM. Injector ports were used to deliver reagents including glucose (10 mM), OMA (1 μg/ml), and 2-deoxyglucose (50 mM; Sigma). After measuring the extracellular acidification rate, the cells were fixed with 4% PFA, stained with Cytotox Green reagent (Essen Biosciences), and imaged using a IncuCyte Zoom system. The glycolytic capacity values were normalized to the cell area calculated from the obtained images using IncuCyte software.

2.9 | Statistical information

Unless indicated otherwise, data are presented as means ± SD. The number of biological replicates per experiment, statistical tests, and significance are specified in every figure legend.

3 | RESULTS

3.1 | Genome-wide synthetic lethality screening with the PFK1 inhibitor TLAM

Phosphofructokinase-1 has three isoforms: platelet isoform (PFKP), muscle isoform (PFKM), and liver isoform (PFKL). Using CRISPR/Cas9 technology, we previously established PFK1-deficient HeLa cell lines in which PFKP and PFKL were completely knocked out but one or more WT PFKM alleles still existed. Our previous study demonstrated that these PFK1-deficient cells were viable even in the presence of TLAM, which abolished all remaining PFK1 activity.[9] Using two independently established PFK1-deficient cell lines and modified versions of these cell lines into which PFKP was reintroduced, we confirmed that pharmacological or genetic perturbation of PFK1 activity provided a survival advantage under oxidative stress induced by H_2O_2 (Figure 1A–C) due to the promotion of the pentose phosphate pathway, as evidenced in previous studies.[10–13] These results suggested that PFK1 activity is not required for cell survival. Next, we investigated the impact of PFK1 on cell proliferation rates using two different cell lines. Although PFK1-deficient cells into which PFKP was reintroduced were previously shown to have high glycolytic activity,[9] there was no difference in doubling times between PFK1-deficient and PFKP-restored cells, suggesting that inhibition of PFK1 activity does not affect cell proliferation under normal culture conditions (Figure 1D). Given the functional importance of PFK1 in glycolysis, its apparent dispensability for HeLa cell survival and proliferation suggests the existence of an unknown pathway that supports cell proliferation in the absence of PFK1. To address this issue, we screened for synthetic-lethal genes using systematic gene knockdown in the presence of the specific PFK1 inhibitor TLAM. To do this, we used pooled shRNA libraries targeting ~15,000 human genes consisting of three modules (Human Modules 1, 2, and 3). HeLa S3 cells were transduced with each of the libraries, then half of the cells were treated with 50 μM TLAM for 10 days while the other half remained untreated. After the 10-day period, the cells were harvested and the constructs integrated in each fraction were quantitated by next-generation sequencing of associated molecular barcodes. Cells harboring shRNAs against candidate synthetic-lethal genes were expected to be depleted in the TLAM-treated fraction but not in the untreated fraction. We then calculated the ratio of abundances of each shRNA sequence between the two fractions (Figure 1E and Appendix S1). We considered a gene to be a candidate for causing synthetic lethality in conjunction with TLAM if its abundance ratio (treated:untreated) was lower than 0.5 for at least three of the corresponding shRNA sequences except for control genes. Ten of the 39 genes that met the criteria were found to encode subunits of mitochondrial respiratory chain complex V, which is responsible for ATP synthesis. Statistical analysis revealed surprisingly high enrichment (Figure 1E–G), suggesting that inhibition of ATP synthase and PFK1 exhibits a strong synthetic genetic interaction.

3.2 | Validation of synthetic chemical–genetic interaction using ATP synthesis inhibitors

To validate the interaction between PFK1 and ATP synthase, we used ATP synthase inhibitors in place of gene silencing. Oligomycin A has been widely used as a potent inhibitor of the mitochondrial ATP synthase since 1958.[28,29] As seen in previous reports,[30,31] OMA showed biphasic cytotoxic effects on HeLa cells, with a moderate reduction of cell numbers in a wide range of low concentrations (10^{-3}–10^{0} μg/ml) (first inflection phase) and eradication of cells at 10 μg/ml (second inflection phase) (Figure 2A). Tryptolinamide potentiated the cytotoxicity of OMA by more than 13,000 times, as shown by a comparison of IC_{50} values following the administration of OMA alone (IC_{50} = 3500 ng/ml) versus OMA together with TLAM (IC_{50} = 0.26 ng/ml). This potentiation was observed in a wide range of OMA concentrations, corresponding to the first inflection phase. The potentiation by TLAM also occurred when venturicidin A (VCA), another ATP synthase inhibitor, was used in place of OMA (Figure 2B). Like the shRNA screening, these results indicate that simultaneous inhibition of ATP synthase and PFK1 results in synthetic lethal interaction.

In addition to mitochondrial ATP synthase (mitochondrial respiratory chain complex V), other mitochondrial respiratory complexes are involved in fully functional ATP synthesis. Therefore, we examined whether TLAM potentiated the cytotoxic effects of mitochondrial respiratory complex inhibitors. We used RTN for complex I inhibition and AMA for complex III inhibition. While RTN exhibited marked cytotoxicity at 1 μM in HeLa cells, TLAM potentiated this toxicity by four times based on a comparison of IC_{50} values (without TLAM: 0.28 μM; with TLAM: 0.073 μM) (Figure 2C). TLAM potentiated the toxicity of 0.01 μM AMA but not that of higher concentrations (Figure 2C). Obviously, the stimulatory effect of the combination of TLAM and OMA or VA was much greater and was observable in a wider range of OMA or VCA than that of TLAM with RTN or AMA. This is consistent with the result of shRNA screening; the screen highly enriched the genes encoding subunits of complex V, but no other mitochondrial respiratory complexes were identified as having a clear chemical–genetic interaction with TLAM.

3.3 | Validation of the synthetic chemical–genetic interaction using PFK1 mutants

To verify the chemical–genetic and chemical–chemical interactions, we tested the effects of TLAM in combination with OMA on PFK1-deficient cells. These cells were highly sensitive to OMA, consistent with the effect of TLAM on OMA-treated wild-type cells (Figure 3A). Furthermore, reintroduction of PFKP into PFK1-deficient cells reversed their susceptibility to OMA, and they became as sensitive as wild-type cells (Figure 3B). These results confirmed that simultaneous inhibition of ATP synthase and PFK1 demonstrates a synthetic chemical–genetic interaction.

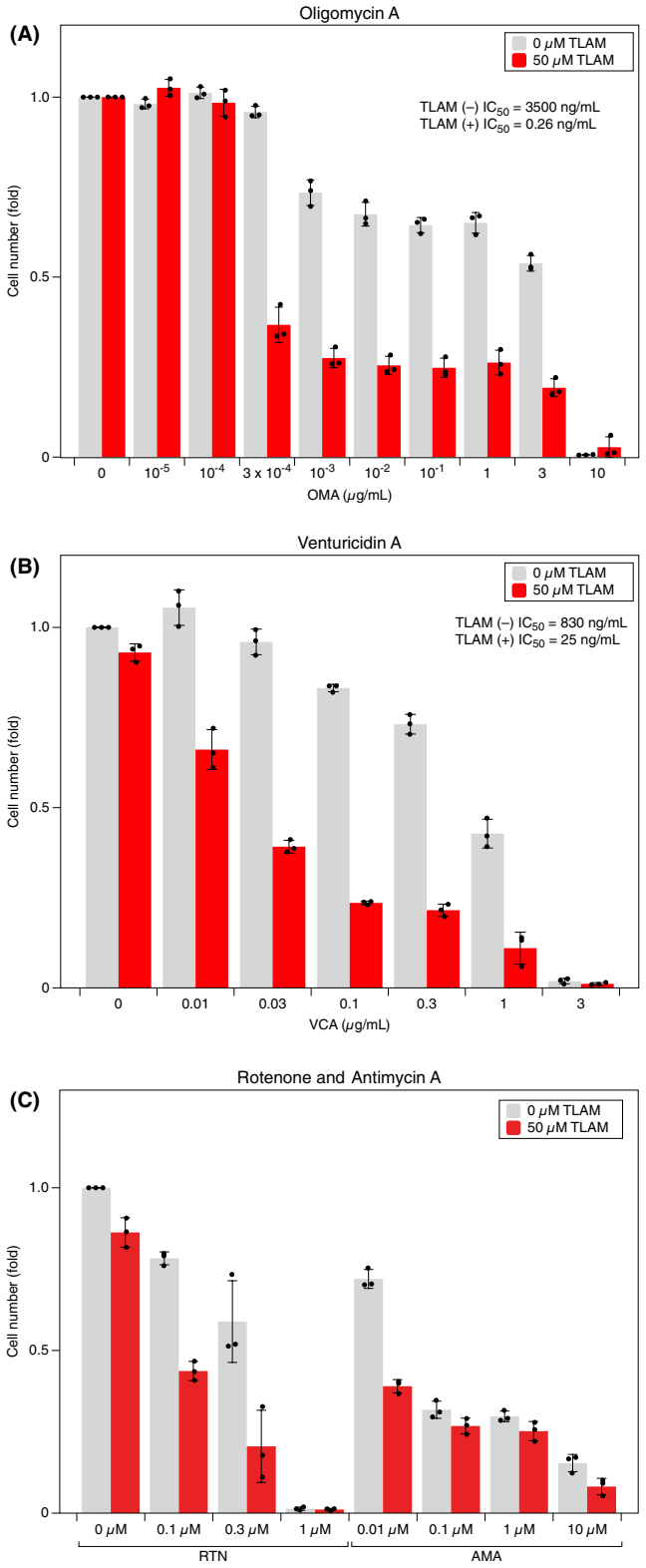


FIGURE 2 Validation of the synergistic interaction between phosphofructokinase-1 and ATP synthase using ATP synthesis inhibitors. (A–C) Effects of mitochondrial respiratory complex inhibitors [(A) oligomycin A (OMA), (B) venturicidin A (VCA), (C) rotenone (RTN) and antimycin A (AMA)] on HeLa cell number in the presence or absence of 50 μM tryptolinamide (TLAM). Throughout, data represent means ± SD of three independent experiments.

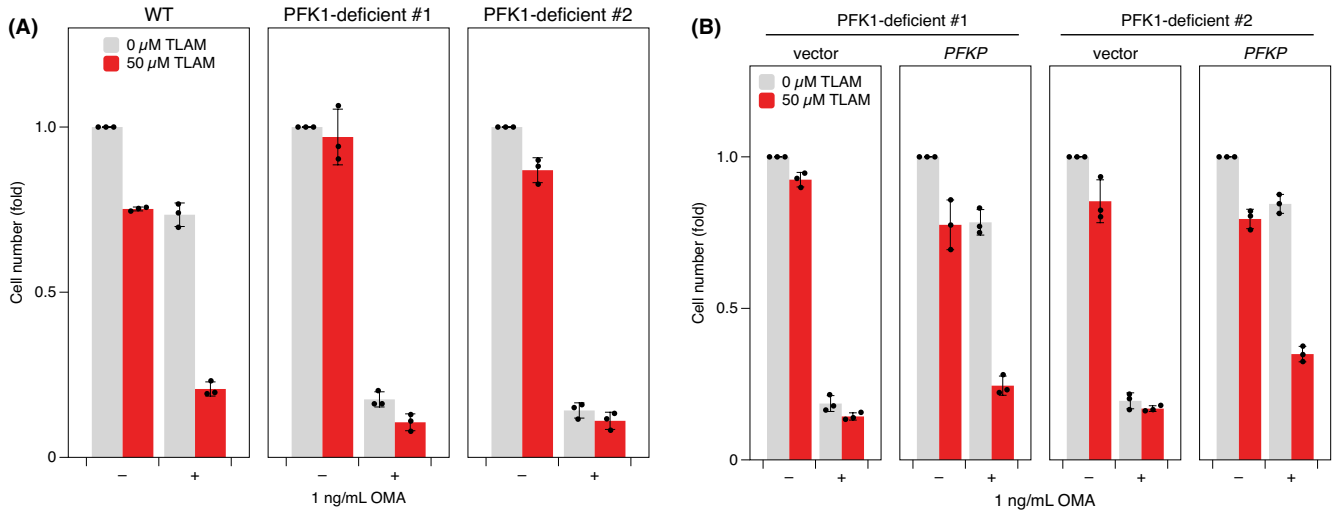


FIGURE 3 Validation of the synergistic interaction between phosphofructokinase-1 (PFK1) and ATP synthase using a genetic mutation of PFK1. (A–C) Effects of oligomycin A (OMA) on the numbers of HeLa cells and PFK1-deficient cells (A) or on two sets of PFK1-deficient cells and PFK1-restored cells (B) in the presence or absence of 50 μM tryptolinamide (TLAM). Throughout, data represent means ± SD of three independent experiments. WT.

3.4 | Characterization of the synthetic chemical–genetic interaction by simultaneous inhibition of PFK1 and ATP synthesis

Imaging analysis suggested that the combined treatment with OMA and TLAM induced a cytostatic effect but not a cytotoxic effect in HeLa cells and PFK1-deficient cells (Figure 4A,B). To validate this, we performed cell-cycle analysis by flow cytometry. Administration of OMA or TLAM alone did not affect the cell-cycle status in HeLa cells. However, co-treatment of these cells with OMA and TLAM induced G2/M arrest (Figures 4C and S1A). This was fully recapitulated when PFK1-deficient cells were used in place of TLAM; administration of OMA alone induced G2/M arrest in PFK1-deficient cells (PFK1-deficient#1/vector and PFK1-deficient#2/vector), whereas PFKP re-introduction reversed the cell cycle arrest (Figures 4D and S1B,C). These results show that simultaneous inhibition of PFK1 and ATP synthesis in HeLa cells induces a potent cytostatic effect but not cell death.

Next, to determine whether the synthetic effects of TLAM are selective in particular cancer cells, we investigated the efficacy of combined treatment with OMA and TLAM in several other cancer cell lines as well as normal fibroblast cell lines. As in the case of HeLa cells, TLAM potentiated OMA cytotoxicity in all the cancer cell lines tested, specifically epidermoid carcinoma A431 cells, non-small-cell lung carcinoma H1299 cells, renal carcinoma RXF-631L cells, and colorectal adenocarcinoma HT29 cells (Figure 4E). We noted that combined treatment with OMA and TLAM did not induce G2/M arrest in these cell lines (Figure S2). By contrast, TLAM barely potentiated the cytotoxicity of OMA in the normal fibroblast cell lines IMR90 and TIG3, although the administration of OMA alone resulted in higher cytotoxicity in normal fibroblasts than in cancer cells (Figure 4F), suggesting that the proliferative capacity of normal fibroblasts depends more on mitochondrial energy generation than that of cancer cells.

3.5 | PFK1 contributes to elevated glycolytic capacity in cancer cells

A remaining issue was the known importance of PFK1 in cancer malignancy. Because both mitochondrial ATP synthase and PFK1 contribute to cellular energy production, we addressed this issue by analyzing energy metabolism. Our previous study revealed that PFK1 regulates the balance between glycolysis and OXPHOS; when PFK1 is inhibited, AMPK is activated to promote fatty acid oxidation.[9] From the opposite perspective, we expected that PFK1 would help buffer ATP content when mitochondrial ATP synthesis declined. Therefore, we examined the effect of PFK1 activity on glycolytic capacity in HeLa cells; this capacity can be defined as the maximum glycolytic flux reached in response to the inhibition of mitochondrial ATP synthesis. As anticipated, inhibition of PFK1 activity by TLAM decreased the glycolytic capacity (Figure 5). While PFK1-deficient HeLa cells (PFK1-deficient #1/vector and PFK1-deficient #2/vector) had low glycolytic capacities, similar to TLAM-treated wild-type cells, the reintroduction of PFKP into PFK1-deficient cells reversed the decrease in capacity (Figure 5). These results suggested that PFK1 contributes to increasing glycolytic capacity to compensate for defective mitochondrial ATP synthesis. In addition, the differences in glycolytic capacities between PFK1-deficient cells and PFKP-restored cells were similar to those between TLAM-treated and vehicle-treated PFKP-restored cells, indicating that TLAM can be used to estimate the contribution of PFK1 toward cellular glycolytic capacity.

4 | DISCUSSION

Cancer-specific metabolism has been recognized as an important therapeutic target.[32,33] In this study, to investigate the impact of PFK1, a gatekeeper of glycolysis, on cancer malignancy, we screened

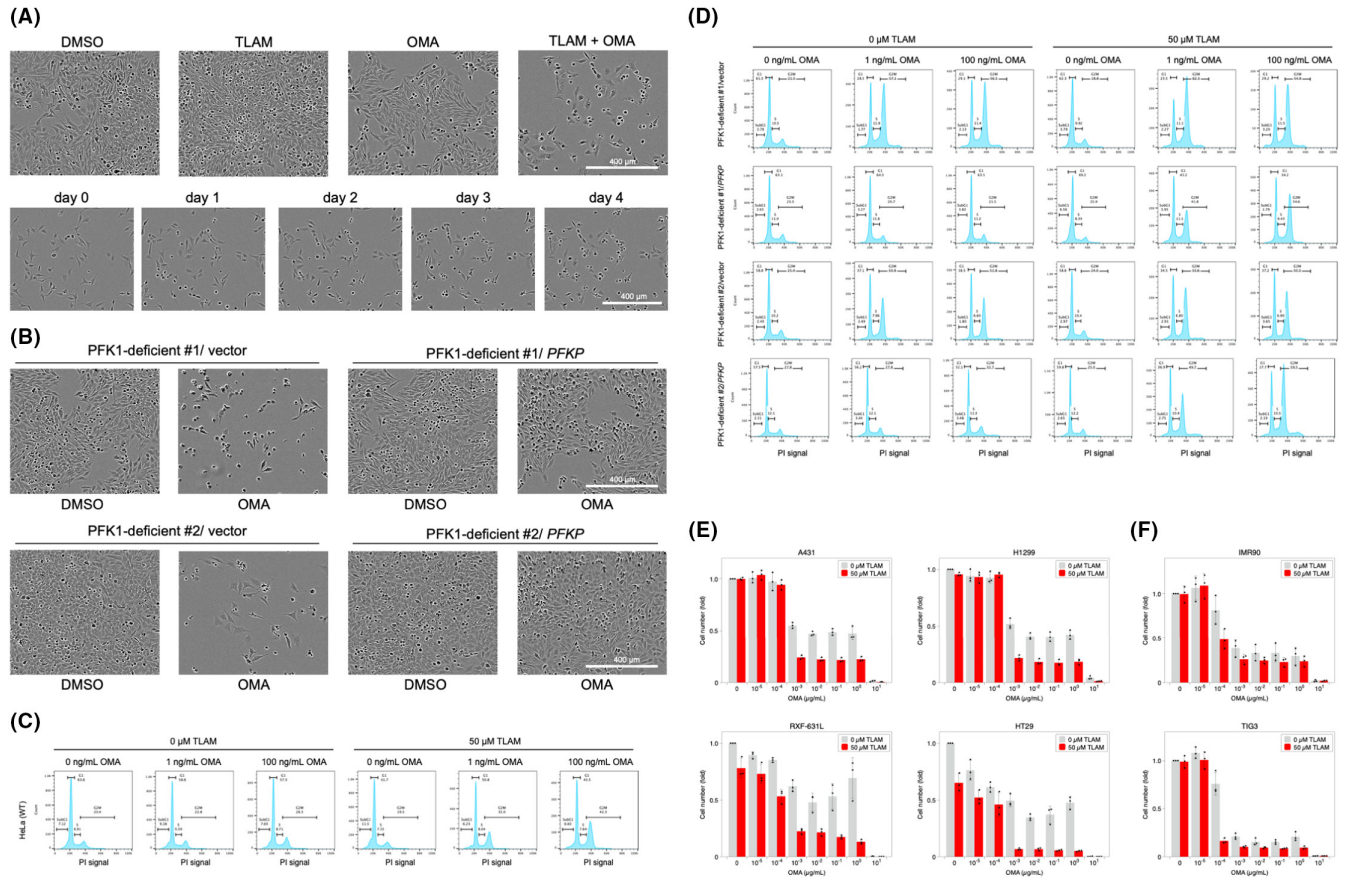


FIGURE 4 Characterization of synergism resulting from simultaneous inhibition of phosphofructokinase-1 (PFK1) and ATP synthesis. (A) Representative images of cells treated with DMSO, tryptolinamide (TLAM) (50 μM), oligomycin A (OMA) (1 ng/ml), or TLAM (50 μM) + OMA (1 ng/ml) for 4 days (upper), or cells treated with TLAM (50 μM) + OMA (1 ng/ml) for the periods indicated (lower). (B) Representative images of cells treated with DMSO or OMA (1 ng/ml). Images in (A) and (B) are from single experiments that are representative of three independent experiments. Scale bar, 400 μm. (C, D) Flow cytometry cell-cycle analysis of HeLa cells (C) or two sets of PFK1-deficient cells and PFK1-restored cells (D), treated with OMA and/or TLAM for 4 days. Quantified and statistically analyzed data are included in Figure S1E,F. Effects of OMA in cancer cell lines (E) and normal fibroblast cell lines (F) in the presence or absence of 50 μM TLAM. Data represent means ± SD of three independent experiments.

the determinants of sensitivity to TLAM and found that certain genes encoding subunits of ATP synthase (mitochondrial respiratory chain complex V) showed synthetic chemical-genetic interactions with PFK1 inhibition. Subsequent validation using ATP synthesis inhibitors and genetic mutations of PFK1 revealed that ATP synthase and PFK1 showed synthetic lethal interaction in cancer cells. Glycolysis and mitochondrial respiratory complex V serve as alternative and complementary means of achieving ATP synthesis; simultaneous inhibition of both pathways should decrease cellular ATP content and thereby induce a stimulatory cytostatic effect. While TLAM enhanced the efficacy of OMA by 13,000 times, its ability to enhance cell growth inhibition that was induced by RTN or AMA was much less remarkable. Among mitochondrial respiratory complexes, complex V is solely responsible for the direct generation of cellular energy, whereas respiratory complexes I and III share the function in proton-pumping activity. Therefore, the depletion of ATP on complex V inhibition may be more rapid and more severe than that by the inhibition of complexes I or III, which can explain why complex V but not complex I or III did show a strong synthetic interaction with PFK1. The G2/M arrest in HeLa cells induced by combined treatment with OMA and TLAM is probably due to human papillomavirus-derived proteins E6 and E7, which degrade p53 and inhibit pRB, respectively.[34] Both p53 and pRB function as checkpoints at G1/S phase transition. Simultaneous loss of both *TP53* and *RB1* alters the cell cycle progression, thereby inducing switching from G1 arrest to G2/M arrest in response to the anticancer drug,[35] similar to the case of the forced expression of E6 and E7.[36] By contrast, *TP53* is mutated in A431, H1299, and HT29 cells, whereas *RB1* is intact in these cells.[37,38]

Our metabolic analysis revealed that PFK1 contributes to an increase in glycolytic capacity that compensates for mitochondrial energy generation. This increased glycolytic capacity probably confers a survival advantage to tumor cells in their oxygen-limited environment. In this regard, dual inhibition of PFK1 and mitochondrial ATP synthase may be an attractive strategy for developing new anticancer therapeutics because of the wide range of effective concentrations of ATP synthesis inhibitors in the context of PFK1 inhibition. In glioma, cancer stem cells (CSCs) mainly rely on

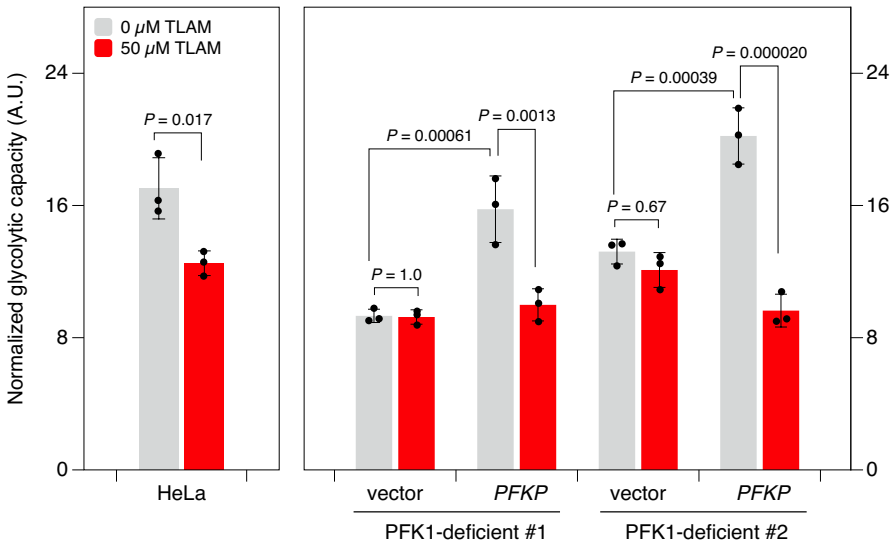


FIGURE 5 Cellular phosphofructokinase-1 (PFK1) activity elevates glycolytic capacity. Effects of pharmacological (left) or genetic (right) perturbation of PFK1 activity on glycolytic capacity. Data represent means ± SD of three independent experiments. *P* values were calculated using Student's two-tailed *t*-test (left, $n = 3$) and ANOVA with Tukey's multiple comparison test (right, $n = 3$). A.U., arbitrary unit.

mitochondrial OXPHOS but have metabolic plasticity and can also use glycolysis.[39] Notably, CSCs in lung cancer,[40] pancreatic cancer[41] and leukemia[42] also rely on mitochondrial OXPHOS. These CSCs are likely vulnerable to the aforementioned combined treatment strategy. Although the use of mitochondrial ATP synthesis inhibitors raises concerns about adverse effects on normal organs (Figure 4F), pharmacological inhibition of ATP synthase did not exert cytotoxic effects in in vivo xenograft models.[43] Thus, further investigation into the pharmacological targeting of PFK1 and ATP synthase is required for the development of anticancer therapeutics.

AUTHOR CONTRIBUTIONS

H.K. designed and carried out the experiments with technical assistance from H.N., and drafted the manuscript. S.T. and K.M performed and analyzed RNAi screening experiments. H.H. helped write the manuscript. M.Y. coordinated the study and helped write the manuscript. All authors gave final approval for publication.

ACKNOWLEDGMENTS

We are grateful to Dr. Yoshihiro Hayashi (Tokyo University of Pharmacy and Life Sciences) for help with the enrichment analysis. This work was inspired by the JSPS Asian Chemical Biology Initiative.

FUNDING INFORMATION

This work was supported in part by a Grant-in-Aid for Scientific Research (S) (JP19H05640; M.Y.), Grants-in-Aid for Young Scientists (JP19K16728; H.K.) from the Japan Society for the Promotion of Science, and an SGH Cancer Research Grant from the SGH Foundation (to H.K.).

CONFLICT OF INTEREST

The authors have no conflict of interest. Minoru Yoshida is an Editorial Board Member of Cancer Science.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed consent: N/A.

Registry and the registration no. of the study/trial: N/A.

Animal studies: N/A.

ORCID

Hiroki Kobayashi https://orcid.org/0000-0002-3785-2317
Shohei Takase https://orcid.org/0000-0002-5868-4519
Ken Matsumoto https://orcid.org/0000-0002-7864-3394
Hironori Harada https://orcid.org/0000-0001-9401-5470
Minoru Yoshida https://orcid.org/0000-0002-4376-5674

REFERENCES

1. Warburg O. On the origin of cancer cells. *Science*. 1956;123:309-314.
2. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer*. 2011;11:85-95.
3. San-Millán I, Brooks GA. Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg effect. *Carcinogenesis*. 2017;38:119-133.
4. Tanner LB, Goglia AG, Wei MH, et al. Four key steps control glycolytic flux in mammalian cells. *Cell Syst*. 2018;7:49-62.e48.
5. Tian RF, Li XF, Xu C, et al. SiRNA targeting PFK1 inhibits proliferation and migration and enhances radiosensitivity by suppressing glycolysis in colorectal cancer. *Am J Transl Res*. 2020;12:4923-4940.
6. Lee JH, Liu R, Li J, et al. Stabilization of phosphofructokinase 1 platelet isoform by AKT promotes tumorigenesis. *Nat Commun*. 2017;8:949.
7. Moon JS, Kim HE, Koh E, et al. Krüppel-like factor 4 (KLF4) activates the transcription of the gene for the platelet isoform of phosphofructokinase (PFKP) in breast cancer. *J Biol Chem*. 2011;286:23808-23816.
8. Wang J, Zhang P, Zhong J, et al. The platelet isoform of phosphofructokinase contributes to metabolic reprogramming and maintains cell proliferation in clear cell renal cell carcinoma. *Oncotarget*. 2016;7:27142-27157.
9. Kobayashi H, Hatakeyama H, Nishimura H, et al. Chemical reversal of abnormalities in cells carrying mitochondrial DNA mutations. *Nat Chem Biol*. 2021;17:335-343.

10. Yi W, Clark PM, Mason DE, et al. Phosphofructokinase 1 glycosylation regulates cell growth and metabolism. *Science*. 2012;337:975-980.
11. Yamamoto T, Takano N, Ishiwata K, et al. Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway. *Nat Commun*. 2014;5:3480.
12. Kim NH, Cha YH, Lee J, et al. Snail reprograms glucose metabolism by repressing phosphofructokinase PFKP allowing cancer cell survival under metabolic stress. *Nat Commun*. 2017;8:14374.
13. Wang H, Nicolay BN, Chick JM, et al. The metabolic function of cyclin D3-CDK6 kinase in cancer cell survival. *Nature*. 2017;546:426-430.
14. O'Neil NJ, Bailey ML, Hieter P. Synthetic lethality and cancer. *Nat Rev Genet*. 2017;18:613-623.
15. Kaelin WG. The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer*. 2005;5:689-698.
16. Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med*. 2009;361:123-134.
17. Lord CJ, Ashworth A. PARP inhibitors: synthetic lethality in the clinic. *Science*. 2017;355:1152-1158.
18. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*. 2005;434:913-917.
19. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434:917-921.
20. McDonald ER, de Weck A, Schlabach MR, et al. Project DRIVE: a compendium of cancer dependencies and synthetic lethal relationships uncovered by large-scale, deep RNAi Screening. *Cell*. 2017;170:577-592.e510.
21. Matsumoto K, Yoshida M. Mammalian chemical genomics towards identifying targets and elucidating modes-of-action of bioactive compounds. *Chembiochem*. 2022;23:e202100561.
22. Kobayashi H, Nishimura H, Matsumoto K, Yoshida M. Identification of the determinants of 2-deoxyglucose sensitivity in cancer cells by shRNA library screening. *Biochem Biophys Res Commun*. 2015;467:121-127.
23. Takase S, Kurokawa R, Arai D, et al. A quantitative shRNA screen identifies ATP1A1 as a gene that regulates cytotoxicity by aurilide B. *Sci Rep*. 2017;7:2002.
24. Takase S, Kurokawa R, Kondoh Y, et al. Mechanism of action of prethioviridamide, an anticancer ribosomally synthesized and post-translationally modified peptide with a polythioamide structure. *ACS Chem Biol*. 2019;14:1819-1828.
25. Chen EY, Tan CM, Kou Y, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics*. 2013;14:128.
26. Kuleshov MV, Jones MR, Rouillard AD, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res*. 2016;44:W90-W97.
27. Xie Z, Bailey A, Kuleshov MV, et al. Gene set knowledge discovery with Enrichr. *Curr Protoc*. 2021;1:e90.
28. Lardy HA, Johnson D, McMurray WC. Antibiotics as tools for metabolic studies. I. a survey of toxic antibiotics in respiratory, phosphorylative and glycolytic systems. *Arch Biochem Biophys*. 1958;78:587-597.
29. Symersky J, Osowski D, Walters DE, Mueller DM. Oligomycin frames a common drug-binding site in the ATP synthase. *Proc Natl Acad Sci U S A*. 2012;109:13961-13965.
30. Kanai M, Iba S, Okada R, Tashiro E, Imoto M. Oligomycin induced the proteasomal degradation of cyclin D1 protein. *J Antibiot (Tokyo)*. 2009;62:425-429.
31. Kuroki S, Kobayashi M, Tani H, et al. Selective growth inhibition by suppression of F1Fo ATPase in canine malignant melanoma cell lines. *J Vet Pharmacol Ther*. 2017;40:101-104.
32. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100:57-70.
33. Luengo A, Gui DY, Vander Heiden MG. Targeting metabolism for cancer therapy. *Cell Chem Biol*. 2017;24:1161-1180.
34. Pal A, Kundu R. Human papillomavirus E6 and E7: the cervical cancer hallmarks and targets for therapy. *Front Microbiol*. 2019;10:3116.
35. Nyquist MD, Corella A, Coleman I, et al. Combined TP53 and RB1 loss promotes prostate cancer resistance to a spectrum of therapeutics and confers vulnerability to replication stress. *Cell Rep*. 2020;31:107669.
36. DeWeese TL, Walsh JC, Dillehay LE, et al. Human papillomavirus E6 and E7 oncoproteins alter cell cycle progression but not radiosensitivity of carcinoma cells treated with low-dose-rate radiation. *Int J Radiat Oncol Biol Phys*. 1997;37:145-154.
37. Kawabe S, Nishikawa T, Munshi A, Roth JA, Chada S, Meyn RE. Adenovirus-mediated mda-7 gene expression radiosensitizes non-small cell lung cancer cells via TP53-independent mechanisms. *Mol Ther*. 2002;6:637-644.
38. Barretina J, Caponigro G, Stransky N, et al. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012;483:603-607.
39. Vlashi E, Lagadec C, Vergnes L, et al. Metabolic state of glioma stem cells and nontumorigenic cells. *Proc Natl Acad Sci U S A*. 2011;108:16062-16067.
40. Ye XQ, Li Q, Wang GH, et al. Mitochondrial and energy metabolism-related properties as novel indicators of lung cancer stem cells. *Int J Cancer*. 2011;129:820-831.
41. Sancho P, Burgos-Ramos E, Tavera A, et al. MYC/PGC-1α balance determines the metabolic phenotype and plasticity of pancreatic cancer stem cells. *Cell Metab*. 2015;22:590-605.
42. Kuntz EM, Baquero P, Michie AM, et al. Targeting mitochondrial oxidative phosphorylation eradicates therapy-resistant chronic myeloid leukemia stem cells. *Nat Med*. 2017;23:1234-1240.
43. Shi Y, Lim SK, Liang Q, et al. Gboxin is an oxidative phosphorylation inhibitor that targets glioblastoma. *Nature*. 2019;567:341-346.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kobayashi H, Takase S, Nishimura H, Matsumoto K, Harada H, Yoshida M. RNAi screening reveals a synthetic chemical–genetic interaction between ATP synthase and PFK1 in cancer cells. *Cancer Sci*. 2023;114:1663-1671. doi:10.1111/cas.15713