

PBRM1 knockdown is associated with NRF2-dependent ALDOA upregulation and metabolic reprogramming in HCCC-9810 intrahepatic cholangiocarcinoma cells

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Abstract. Polybromo-1 (PBRM1) is a chromatin-remodeling factor that is altered in intrahepatic cholangiocarcinoma (ICC). The present study examined the effects of PBRM1 knockdown on tumor-related phenotypes and glucose metabolism in HCCC-9810 ICC cells. The present study also evaluated the roles of aldolase, fructose-bisphosphate A (ALDOA) and nuclear factor erythroid 2-related factor 2 (NRF2). Publicly available ICC mutation data were analyzed descriptively. The present study evaluated HCCC-9810 cells following PBRM1 knockdown using proliferation, colony formation, wound-healing, Transwell, metabolic, reverse transcription-quantitative PCR, western blotting, apoptosis and mitochondrial membrane-potential assays. ALDOA and NRF2 perturbation experiments were used to examine

their contribution to the PBRM1-knockdown phenotype. HCCC-9810-derived xenografts were used for *in vivo* assessment. PBRM1 knockdown increased proliferation, migration and invasion in HCCC-9810 cells and altered several glucose-metabolism readouts. ALDOA expression also increased following PBRM1 knockdown. ALDOA silencing attenuated several metabolic and survival-related changes. NRF2 overexpression increased ALDOA expression, whereas NRF2 knockdown reduced it. Co-silencing NRF2 attenuated the ALDOA increase associated with PBRM1 knockdown. In xenografts, PBRM1 knockdown was associated with greater tumor growth, higher ALDOA expression and a higher Ki67 signal. PBRM1 knockdown was associated with a tumor-promoting metabolic phenotype in HCCC-9810 cells, with ALDOA contributing to several metabolic and survival-related changes. NRF2 perturbation further supported an NRF2-dependent component of ALDOA regulation in this HCCC-9810 model; however, the upstream mechanism responsible for increased NRF2 after PBRM1 knockdown remains unresolved. These findings warrant validation in independent ICC models.

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Introduction

Intrahepatic cholangiocarcinoma (ICC) is a primary liver cancer characterized by frequent recurrence and limited treatment options (1-7). Metabolic adaptation is also a prominent feature of this disease (8-14). Such adaptation may help malignant cells tolerate nutrient limitation and treatment-related stress. Defining how tumor-suppressor alterations reshape these metabolic responses may improve our understanding of ICC progression.

Polybromo-1 (PBRM1) forms part of the PBAF branch of the SWI/SNF chromatin-remodeling machinery and participates in transcriptional and cell-cycle regulation (15-18). In clear cell renal cell carcinoma, Tang *et al* (19) linked PBRM1 deficiency to AKT-mTOR activation and enhanced aerobic glycolysis. PBAF loss has also been associated with defective

maintenance of the DNA-damage checkpoint (20). These observations arise from biological contexts distinct from ICC. Nevertheless, they raise the question of whether PBRM1 perturbation has comparable metabolic consequences in cholangiocarcinoma. This possibility needs to be tested directly because downstream effects of PBRM1 are likely to depend on tumor context.

Glycolysis provides ATP and biosynthetic intermediates for cancer cells (8-14). Several glycolytic enzymes can also contribute to tumor adaptation. In the present study, aldolase, fructose-bisphosphate A (ALDOA) showed a prominent change among the measured glycolysis-related genes following PBRM1 knockdown. Nuclear factor erythroid 2-related factor 2 (NRF2) is best known as a regulator of oxidative-stress responses, but it also participates in metabolic control. The present study therefore examined whether NRF2 contributes to ALDOA regulation in the PBRM1-knockdown setting.

Accordingly, the present study combined descriptive public-dataset analysis with functional and metabolic experiments in HCCC-9810 cells and an HCCC-9810-derived xenograft model. The present study examined whether PBRM1 knockdown altered tumor-associated phenotypes and glucose-metabolism readouts and then evaluated the contributions of ALDOA and NRF2 to these responses.

Materials and methods

Bioinformatic analysis of PBRM1 mutations in ICC. The present study interrogated cBioPortal for Cancer Genomics for somatic-variant information from intrahepatic cholangiocarcinoma (21). The cohort comprised 203 pathologically defined ICC cases with available mutation profiles; cases lacking somatic-variant data and duplicate tumor specimens were excluded. The descriptive summary covered mutation classes and types, single-nucleotide changes, the number of variants in each sample, commonly altered genes, and the positions of PBRM1 variants across its protein domains. Mutation frequencies were summarized for the cohort as a whole without survival or treatment-response modeling.

Cell culture and viral transduction. HCCC-9810 human ICC cells were obtained from Procell Life Science & Technology Co., Ltd. (cat. no. CL-0095). Routine culture used RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were kept at 37°C in humidified 5% CO₂. The standard RPMI-1640 formulation contained 2.0 g/l D-glucose, equivalent to ~11.1 mM (22). For experiments that altered glucose supply, the actual concentration is stated with the corresponding assay.

For viral transduction, 5x10³ cells were plated and allowed to attach for 18 h before exposure to the lentiviral constructs. A third-generation lentiviral packaging system was used. The 293T cells used for lentiviral packaging were obtained from ATCC (cat. no. CRL-3216). For transfection, 24 µg of lentiviral transfer plasmid was used, and the ratio of the lentiviral transfer, packaging and envelope plasmids was 12:4:4:4. Prepackaged short hairpin (sh)RNAs directed against the coding regions of PBRM1 (Sh-PBRM1), ALDOA (Sh-ALDOA), and NRF2/NFE2L2 (Sh-NRF2) were used, with a non-targeting construct serving as Sh-NC. Sh-PBRM1

or control virus was applied at a multiplicity of infection of 20-40 together with 2 µg/ml polybrene. The culture medium was renewed 24 h later, followed by a further 48 h of culture. GFP fluorescence provided the transduction-efficiency check, and downstream experiments were conducted 72 h after transduction when the transduction efficiency was >80%. NRF2 gain-of-function experiments used an overexpression (OE)-NRF2 construct and its matched empty-vector control (OE-NC). Samples for reverse transcription-quantitative PCR (RT-qPCR) and immunoblotting were collected at 0, 12, 24, and 48 h. Successful NRF2 knockdown and overexpression in cells transfected with Sh-NRF2 or OE-NRF2 alone, respectively, were verified by RT-qPCR and western blotting without additional treatments (Fig. S1). Table I summarizes the shRNA constructs, targeted regions and targeting sequences.

Immunoblotting. Cells were lysed in RIPA buffer (Beyotime Biotechnology; cat. no. P0013E) containing protease inhibitors, and protein concentrations were determined using the BCA method with a BCA Protein Assay Kit (Beyotime Biotechnology; cat. no. P0010). Equal amounts of protein (20 µg per lane) were resolved by 8-12% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked in 5% non-fat milk for 1 h at room temperature and then exposed overnight at 4°C to the relevant primary antibodies: ALDOA (Affinity Biosciences; cat. no. BF8051; 1:1,000), ENO1 (Affinity Biosciences; cat. no. DF6191; 1:1,000), GLUT1 (Affinity Biosciences; cat. no. AF5462; 1:1,000), NRF2 (Affinity Biosciences; cat. no. AF0639; 1:1,000), GAPDH (Affinity Biosciences; cat. no. T0004; 1:5,000), PBRM1 (Abcam; cat. no. ab196022; 1:5,000) or Tubulin (Abcam; cat. no. ab7291; 1:5,000), as required by the experiment. Following three rinses with TBST containing 1% (v/v) Tween-20, the appropriate HRP-linked goat anti-rabbit IgG (Bioss; cat. no. bs-0295G-HRP; 1:3,000) or goat anti-mouse IgG (Bioss; cat. no. bs-0296G-HRP; 1:3,000) was applied for 1 h at room temperature. Chemiluminescent signals were captured with a ChemiScope Mini 3300 system (Clinx Science Instruments Co., Ltd.). Quantity One software (version 4.6.8; Bio-Rad Laboratories, Inc.) was used for densitometry. Protein-band values were first normalized to the corresponding loading control and were then expressed relative to the designated control condition.

CCK-8 cell viability. For the CCK-8 assay, logarithmically growing Sh-NC and Sh-PBRM1 HCCC-9810 cells were distributed into 96-well plates at 1,000 cells in 100 µl per well and six wells were used for each group. Absorbance-based viability was assessed at 0, 24, 48, 72, and 96 h. At each measurement, 10 µl of CCK-8 reagent was added and the plate was incubated at 37°C for 1 h before the optical density at 450 nm was recorded. The glucose-concentration experiments followed the same procedure under the specified glucose conditions.

Clonogenic assay. For clonogenic assessment, stable Sh-NC or Sh-PBRM1 cells were placed in 6-cm dishes at 1,500 cells per dish in 4 ml complete RPMI-1640. Cultures were maintained for 7-10 days, and fresh medium was supplied every 2-3 days. After colonies had formed, the dishes were rinsed with PBS and fixed in 4% paraformaldehyde for 10 min. The colonies were then stained with 0.1% crystal violet, washed, air-dried,

images captured and the colonies counted. Only discrete colonies containing ≥ 50 cells were included; diffuse background staining was excluded from the count.

Scratch-wound assay. Scratch assays were initiated when control and PBRM1-shRNA cultures reached 90-100% confluence. A sterile 10- μ l pipette tip was used to create a linear wound. Detached material was removed with two PBS rinses, after which serum-free RPMI-1640 was added. Phase-contrast images were taken immediately and again at 24 h. ImageJ (National Institutes of Health) was used to measure scratch width. Wound closure (%) was calculated as $[(\text{initial width} - \text{width at 24 h}) / \text{initial width}] \times 100$.

Transwell migration and invasion. Transwell assays were used to assess cell migration and invasion. For migration, a suspension containing 5×10^4 cells/ml in serum-free medium was prepared, and 200 μ l was placed in the upper compartment. The lower compartment received 600 μ l complete medium. After 48 h, cells that had migrated were fixed in 4% paraformaldehyde for 10 min at room temperature (RT; 20-25°C) and stained with 0.1% crystal violet for 10 min at RT; three randomly chosen microscopic fields were counted. For invasion experiments, 40 μ l Matrigel was added to the upper chamber and allowed to set at 37°C for 5 h before the cells were introduced; all later steps matched the migration protocol.

F-actin immunofluorescence. Phalloidin staining was used to characterize F-actin morphology. HCCC-9810 cells were cultured overnight and rinsed twice with PBS. The cells were fixed in 4% paraformaldehyde for 10 min at room temperature (RT; 20-25°C), permeabilized with 0.5% Triton X-100 for 5 min at RT, and blocked with 3% BSA for 30 min at 37°C. FITC-conjugated phalloidin (100 nM; Sigma-Aldrich) was then applied for 30 min at 37°C in the dark. After three PBS washes, samples were mounted in antifade medium and imaged at a magnification of x400.

Measurement of intracellular reactive oxygen species (ROS). Intracellular ROS was quantified with a fluorescent probe kit from Nanjing Jiancheng Bioengineering Institute (cat. no. E004-1-1). HCCC-9810 cells were detached with trypsin and pelleted at $300 \times g$ for 5 min at room temperature (RT; 20-25°C). The cells were incubated with 10 μ M DCFH-DA (4.87 μ g/ml) at 37°C for 30 min with gentle intermittent mixing and were subsequently washed three times with PBS to clear extracellular probe. Fluorescence was acquired on a BD FACSCanto II flow cytometer (BD Biosciences) and processed in FlowJo software (version 10.10.1; BD Biosciences). Mean fluorescence intensity served as the ROS measure.

ATP assay. ATP was determined with the Nanjing Jiancheng Bioengineering Institute ATP assay kit (cat. no. A095-1-1). HCCC-9810 cells were plated at 1×10^4 cells/well and maintained for 48 h. Each well was extracted with 400 μ l ice-cold lysis buffer. Lysates remained on ice for 10 min and were then centrifuged at $12,000 \times g$ for 10 min at 4°C. The resulting supernatant was mixed with the kit reaction solution and processed according to the manufacturer's procedure. Absorbance was

read at 650 nm. Values were interpolated from the standard curve and expressed relative to total protein as μ mol/g protein.

Lactate assay. Lactate was assayed with kit A019-2-1 (Nanjing Jiancheng Bioengineering Institute Co., Ltd.). HCCC-9810 cells were seeded at 1×10^4 cells/well and incubated for 48 h. Cell extracts were prepared in 200 μ l extraction buffer and clarified by centrifugation at $12,000 \times g$ for 10 min at 4°C. A 50- μ l aliquot of sample supernatant or standard was mixed with 100 μ l working reagent. After incubation at 37°C for 10-15 min, absorbance was recorded at 530 nm. Lactate concentrations were derived from the calibration curve and normalized to the total protein content.

Pyruvate assay. Pyruvate was measured after 48 h of culture. HCCC-9810 cells were collected and extracted with the assay buffer provided in the pyruvate kit. After centrifugation at $12,000 \times g$ for 10 min at 4°C, 50 μ l of sample supernatant or standard was reacted with 100 μ l chromogenic solution. The reaction proceeded for 30 min at 37°C, and absorbance was then read at 520 nm. Sample concentrations were obtained from the corresponding standard curve.

RT-qPCR. Total RNA was extracted from 1×10^6 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA quantity and purity were checked on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.), while agarose-gel electrophoresis was used to examine RNA integrity. For reverse transcription, 2 μ g of RNA from each sample was processed with the PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd.). qPCR reactions used TB Green Premix Ex Taq II (Takara Biotechnology Co., Ltd.) on either a Bio-Rad CFX96 or ABI 7500 platform. RNA extraction, cDNA synthesis and qPCR were performed according to the respective manufacturers' protocols. The PCR cycling conditions were 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. Relative transcript abundance was determined by the $2^{-\Delta\Delta C_q}$ method (23), using TUBB as the reference gene. RT-qPCR experiments were performed in six independent biological samples. The primer sequences used for RT-qPCR are listed in Table II.

Apoptosis analysis by flow cytometry. At 48 h, cells were prepared for Annexin V-FITC/propidium iodide (PI) staining using the Beyotime apoptosis kit (Beyotime Biotechnology; cat. no. C1062). After two washes with cold PBS, cells were adjusted to $\sim 1 \times 10^6$ cells/ml in 500 μ l precooled 1X binding buffer. Annexin V-FITC (5 μ l) was added for 15 min at room temperature (RT; 20-25°C), followed by 2.5 μ l PI for a further 5 min at RT in darkness. A minimum of 10,000 events was acquired for each sample using a BD FACSCanto II flow cytometer (BD Biosciences). FlowJo software (version 10.10.1; BD Biosciences) was used for analysis and the reported total apoptotic fraction combined early- and late-apoptotic populations.

JC-1 assessment of mitochondrial membrane potential. For JC-1 imaging, HCCC-9810 cells were grown on sterile coverslips in 24-well plates until they reached 60-70% confluence.

Table I. Lentiviral shRNA constructs used for gene silencing.

Construct	Target gene	Targeted region Targeting sequence (5'-3')	Experimental role
Sh-PBRM1	PBRM1	Coding region (CDS) TCCAATCTTCCAAGTGTAGAT	PBRM1 knockdown
Sh-ALDOA	ALDOA	Coding region (CDS) TCTACCTGGAAGGCACCTTGC	ALDOA knockdown
Sh-NRF2	NRF2	Coding region (CDS) ACGGTATGCAACAGGACATTG	NRF2 knockdown
Sh-NC	None	Non-targeting control sequence TTCTCCGAACGTGTCACGT	Negative control

Sh, short hairpin; PBRM1, polybromo-1; ALDOA, aldolase, fructose-bisphosphate A; NC, negative control; CDS, coding DNA sequence.

Table II. Primer sequences for reverse transcription-quantitative PCR.

Gene	Direction	Sequence (5'-3')	Product length (bp)
<i>GLUT1</i>	Forward	TCCTATCTGAGCATCGTGGC	204
	Reverse	GTAGGGACCACACAGTTGCT	
<i>GPI</i>	Forward	GTGACTTCCTCATCCCGGTC	213
	Reverse	TGGTTGGGCGATTTCCTTCA	
<i>PFK</i>	Forward	GGCATCTTCGACAGCAGGAA	227
	Reverse	TCAGCCACTGGTTGGAAGAC	
<i>ALDOA</i>	Forward	CTTGCTGAAGCCCAACATGG	197
	Reverse	GCAGGGGGCACTTGTTAATG	
<i>GAPDH</i>	Forward	GACCACAGTCCATGCCATCA	213
	Reverse	GTCAGGTCCACCACTGACAC	
<i>PGK</i>	Forward	AGAGGGAGCCAAGATTGTCA	227
	Reverse	ACACAATCTGCTTAGCCCGA	
<i>ENO1</i>	Forward	ACCCAAAGAGGATCGCCAAG	215
	Reverse	AGTCTTGATCTGCCCAGTGC	
<i>TUBB</i>	Forward	TGTTCACTGGTACGTTGGGG	171
	Reverse	AGCAGCACCTTTGTGACGTT	

Following 48 h of treatment, the cells were rinsed with PBS and exposed to 500 μ l JC-1 working solution for 20 min at 37°C in the dark. Two washes with JC-1 buffer were performed before mounting in DAPI-containing antifade medium. Images were acquired immediately on a Zeiss Axio Imager fluorescence microscope (Carl Zeiss AG). The relative appearance of red aggregates and green monomers was used qualitatively to assess mitochondrial membrane-potential status.

Immunohistochemical staining and image quantification. Tumor tissues were fixed in 4% paraformaldehyde, dehydrated through graded ethanol, cleared, embedded in paraffin and sectioned at 4 μ m. Sections were deparaffinized in xylene and rehydrated through graded ethanol. No permeabilization step was performed. Sections were exposed to 3% hydrogen peroxide for 15 min at room temperature (RT; 20-25°C) to suppress endogenous peroxidase activity, followed by heat-mediated antigen retrieval in citrate buffer (pH 6.0)

for 10 min. After blocking with goat serum (Beyotime Biotechnology; cat. no. C0265) for 30 min at RT, slides were incubated at 4°C overnight with anti-ALDOA (Affinity Biosciences; cat. no. BF8051; 1:100) or anti-Ki67 (Abcam; cat. no. ab16667; 1:200). The slides were then washed in PBS and incubated for 30 min at RT with HRP-conjugated goat anti-mouse IgG(H+L) (Beyotime Biotechnology; cat. no. A0216; 1:50) or HRP-conjugated goat anti-rabbit IgG(H+L) (Beyotime Biotechnology; cat. no. A0208; 1:50), as appropriate. Staining was developed using an Enhanced DAB Horseradish Peroxidase Color Development Kit (Beyotime Biotechnology; cat. no. P0201S), followed by hematoxylin counterstaining for 10 min at RT. Images were captured using a light microscope at x400 magnification with unchanged microscope and camera settings. From each section, five non-overlapping fields at x400 magnification were randomly sampled within viable tumor tissue; areas showing necrosis, hemorrhage, folding, or tissue edges were omitted. Semi-automated quantification

was carried out in Image-Pro Plus 6.0 (Media Cybernetics, Inc.). For each marker, one color-segmentation threshold was defined and kept constant for all images in the same staining batch. Viable tumor tissue constituted the region of interest (ROI). Integrated optical density (IOD) within that ROI was divided by ROI area for group comparison, in line with optical-density-based IHC quantification methods (24). Then, five-field values were averaged to obtain one result per tumor and the mouse was the biological replicate.

Subcutaneous xenograft model. Male BALB/c nude mice aged 5 weeks (17–19 g; n=12) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed under specific-pathogen-free conditions at 22±2°C and 55±5% relative humidity on a 12-h light/dark cycle, with free access to food and water. Mice were acclimated for 7 days before the experiment and allocated to two groups (n=6 per group). HCCC-9810 cells carrying stable Sh-NC or Sh-PBRM1 were prepared at 1×10⁵ cells and implanted subcutaneously in the dorsal flank. Caliper monitoring began once tumors were palpable. Tumor volume was calculated as 0.5 × length × width² (25). The maximum tumor volume observed during the study was 454 mm³, and the maximum measured tumor diameter was 12 mm. For procedures requiring anesthesia during the experiment, anesthesia was induced with 3% isoflurane in an induction chamber and maintained with 1.5% isoflurane via a nose cone; anesthetic depth was monitored by the toe-pinch reflex. At the predefined endpoint, mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (150 mg/kg). Following loss of the toe-pinch reflex, cervical dislocation was immediately performed to ensure death before tissue collection. Excised tumors were weighed and allocated for immunoblotting and immunohistochemistry. The protocol complied with institutional animal-care requirements and was approved by the Animal Ethics Committee of Shandong Medical and Pharmaceutical University Hospital (approval no. 2024-G013-01).

Statistical methods. GraphPad Prism 10.1.2 (Dotmatics) was used for data analysis and figure preparation. Unless specified otherwise, results are reported as mean ± SD from three independent biological experiments. Simple end-point comparisons between two independent groups used two-sided unpaired Student's t-tests. For the CCK-8 time series in Fig. 1K, treatment and time were entered as factors in a two-way ANOVA, followed by Šídák-adjusted pairwise comparisons. The glucose experiments in Fig. 2A–C were evaluated with a second two-way ANOVA using PBRM1 status and glucose concentration as factors, again with Šídák adjustment. In Fig. 3B, control and Sh-PBRM1 values were compared separately for each glycolysis-related gene using multiple unpaired Student's t-tests followed by Holm-Šídák multiple-comparison correction. The four-group analyses in Figs. 3E–G, 4B–E, H, and 6A–C used one-way ANOVA with Šídák correction for the prespecified contrasts displayed in the figures. For the NRF2 overexpression and knockdown time courses in Fig. 5, one-way ANOVA was followed by Dunnett comparisons against 0 h. Xenograft end points in Fig. 7 were assessed with two-sided unpaired Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

PBRM1 mutation profiling and PBRM1-knockdown phenotypes in HCCC-9810 cells. Fig. 1A–G presents the mutation profile of the 203-case public ICC cohort. Panels A–E summarize the overall distribution of mutation classes, variant types, single-nucleotide substitutions, variants per case, and related classification frequencies. The 20 most commonly altered genes are shown in panel F; PBRM1 alterations occurred in ~5% of the cohort. Panel G maps the detected PBRM1 variants across the protein domain structure.

Immunoblotting and densitometry confirmed lower PBRM1 abundance after Sh-PBRM1 transduction (Fig. 1I and J). Fig. 1H shows F-actin morphology in control and PBRM1-knockdown cells. Compared with Sh-NC cells, PBRM1-knockdown cells had higher CCK-8 signals, formed more colonies, showed greater 24-h wound closure, and displayed increased migration and invasion in Transwell assays (Fig. 1K–Q). Together, these assays indicate enhanced proliferative and motile behavior in HCCC-9810 cells following PBRM1 knockdown.

PBRM1 knockdown is associated with altered glucose-metabolism readouts in HCCC-9810 cells. Control and PBRM1-knockdown HCCC-9810 cells were studied under 11.1, 5, 0.5 or 0 mM glucose. PBRM1 knockdown increased CCK-8 viability signals under reduced-glucose conditions (Fig. 2A), with the clearest separation at 0.5 and 0 mM glucose. ROS was analyzed as an additional redox readout. In Fig. 2B, significant increases in the Sh-PBRM1 group were observed at 0.5 and 0 mM glucose.

ATP levels also differed between groups at the glucose concentrations marked as significant in Fig. 2C. PBRM1-knockdown cells showed higher lactate and pyruvate levels than control cells (Fig. 2D–E). These results indicated altered glucose-dependent viability and metabolic end points after PBRM1 knockdown.

PBRM1 knockdown is associated with increased ALDOA expression. RT-qPCR of the glycolysis-related genes shown in Fig. 3A and B demonstrated higher ALDOA and ENO1 mRNA levels in PBRM1-knockdown cells, whereas the other measured transcripts did not show significant changes. Western blotting showed corresponding increases in ALDOA and ENO1 protein, while GAPDH and GLUT1 were not notably changed (Fig. 3C and D). These findings identified ALDOA as a candidate downstream factor associated with PBRM1 knockdown in HCCC-9810 cells.

ALDOA-targeting shRNA reduced ALDOA mRNA and protein in the PBRM1-knockdown setting (Fig. 3E–G). These experiments supported a functional association between PBRM1 knockdown and ALDOA expression.

ALDOA knockdown attenuates phenotypes associated with PBRM1 knockdown. ALDOA was co-silenced in PBRM1-knockdown HCCC-9810 cells to assess its contribution to the observed phenotype. Compared with PBRM1 knockdown alone, co-silencing ALDOA reduced colony formation and attenuated the increases in lactate, ROS and ATP shown in Fig. 4A–E. These findings indicated that

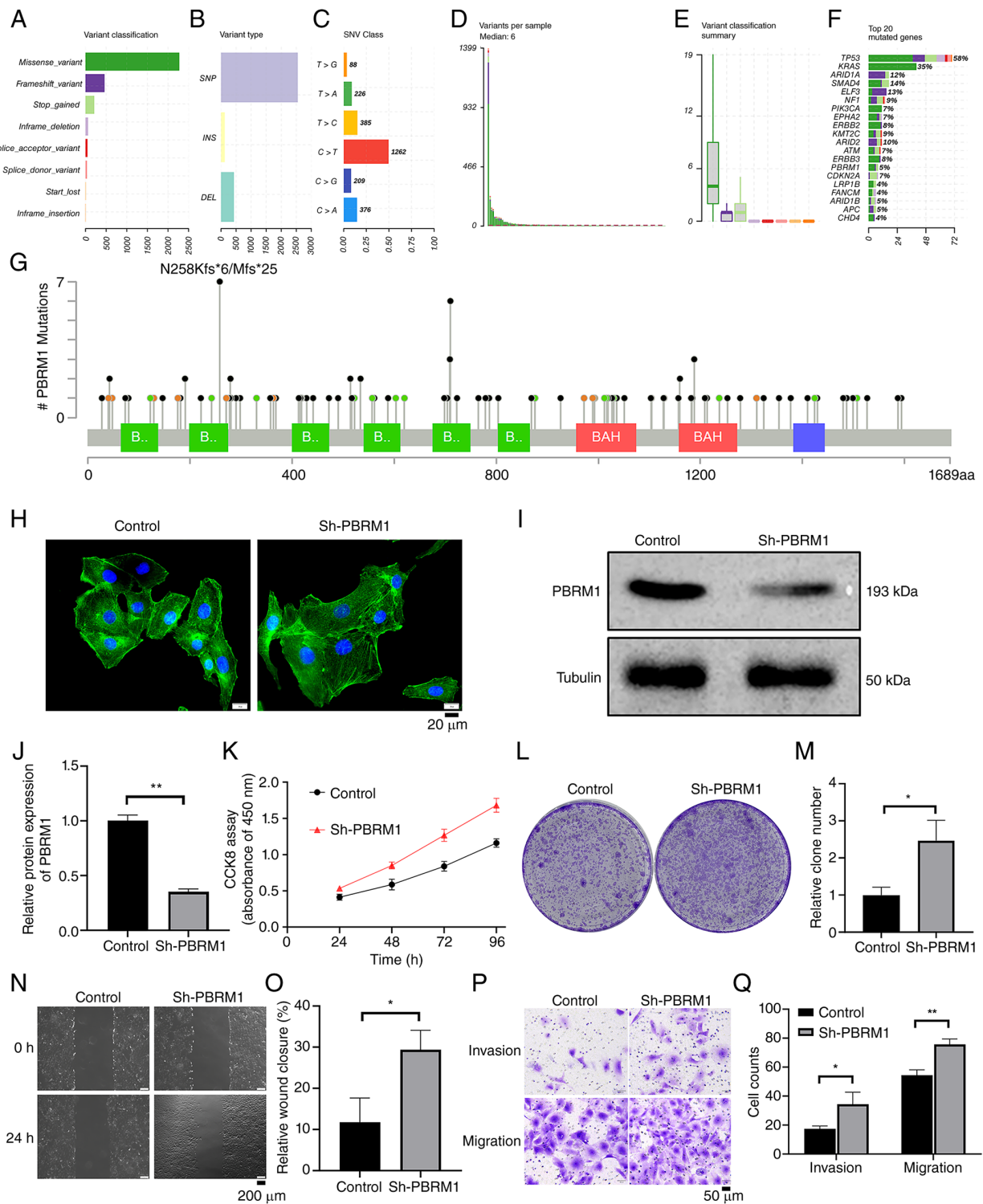


Figure 1. PBRM1 mutation profiling and PBRM1-knockdown phenotypes in HCCC-9810 cells. Cohort-wide summary of (A) variant classification, (B) variant type, (C) single-nucleotide substitution class, (D) variants per sample and (E) variant-classification distribution in the 203-case public ICC dataset. (F) Top 20 mutated genes in the cohort; PBRM1 was altered in ~5% of cases. (G) Distribution of observed PBRM1 variants across the protein domain structure. (H) Representative F-actin staining (green) with DAPI nuclear counterstaining (blue) in control and PBRM1-knockdown cells. (I) Representative western blotting and (J) densitometric quantification of PBRM1. (K) CCK-8 viability over 0-96 h; data were analyzed by two-way ANOVA followed by Šidák's multiple-comparisons test. (L) Colony formation and (M) quantification. (N) Wound-healing assay and (O) quantification at 24 h. Transwell (P) invasion and migration assays and (Q) quantification. Data are mean $\pm$ SD. Two-group endpoint comparisons used two-tailed unpaired Student's t-tests. * $P < 0.05$; ** $P < 0.01$. PBRM1, polybromo-1; SNV, single nucleotide variant; ICC, intrahepatic cholangiocarcinoma.

ALDOA contributes to several metabolic and growth-related changes associated with PBRM1 knockdown.

JC-1 imaging revealed a shift in the relative red and green signals among the experimental groups (Fig. 4F), consistent

with altered mitochondrial membrane-potential status. Annexin V/PI analysis provided a separate apoptosis readout. The reduced apoptotic fraction observed after PBRM1 knockdown increased after ALDOA co-silencing (Fig. 4G and H).

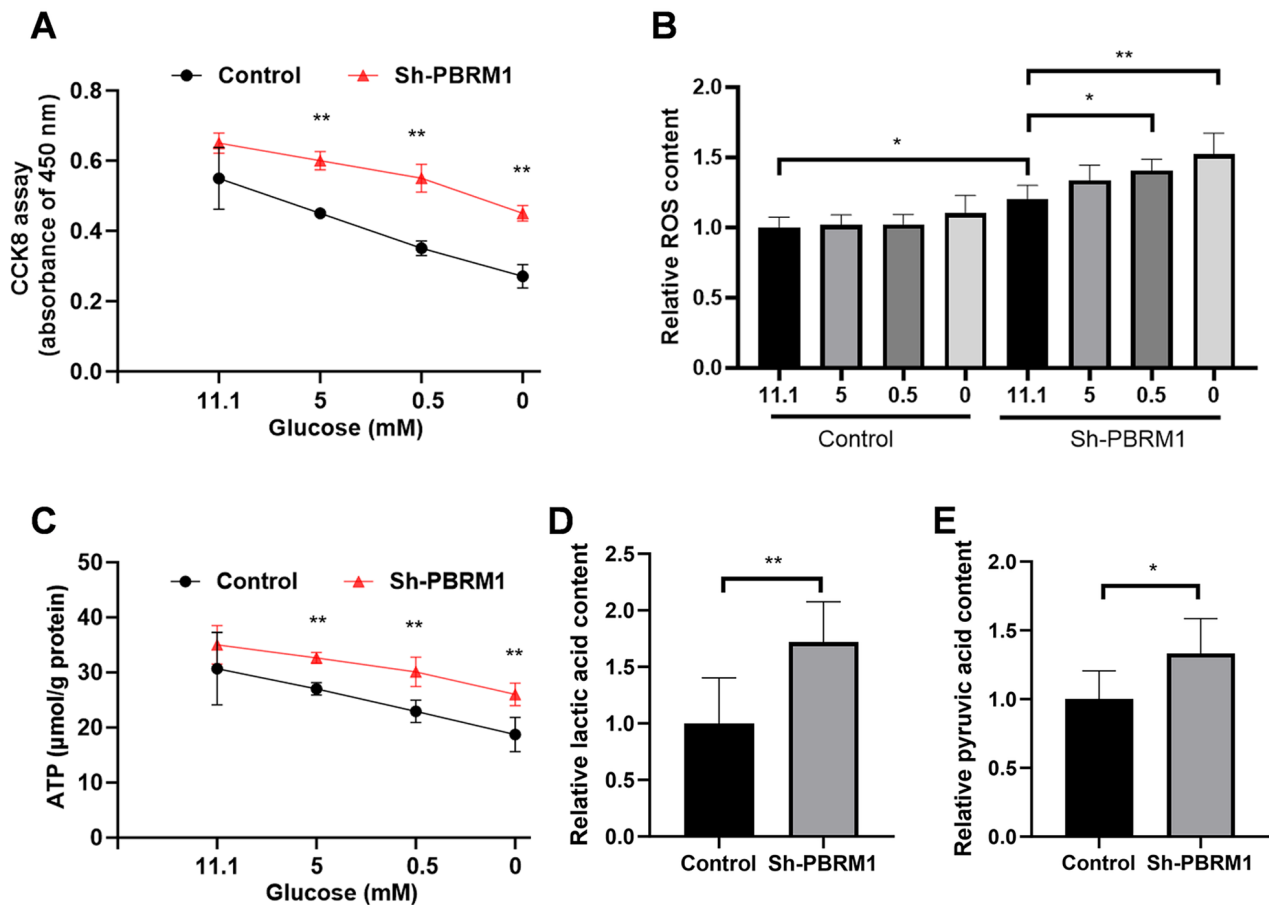


Figure 2. PBRM1 knockdown is associated with altered glucose-metabolism readouts in HCCC-9810 cells. (A) CCK-8 viability under 11.1, 5, 0.5 or 0 mM glucose. (B) Intracellular ROS measurements and (C) ATP measurements under the same glucose concentrations. A-C were analyzed by two-way ANOVA with PBRM1 status and glucose concentration as factors, followed by Šidák's multiple-comparisons test. (D) Lactate and (E) pyruvate measurements were analyzed using two-tailed unpaired Student's t-tests. Data are mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. PBRM1, polybromo-1; Sh, short hairpin; ROS, reactive oxygen species.

Together, these findings supported a functional contribution of ALDOA to the metabolic and survival-related phenotype associated with PBRM1 knockdown.

NRF2 perturbation alters ALDOA expression in HCCC-9810 cells. Prior to assessing ALDOA responses, successful NRF2 knockdown and overexpression were confirmed in HCCC-9810 cells transfected with Sh-NRF2 or OE-NRF2 alone, respectively. RT-qPCR and western blotting showed lower NRF2 abundance following Sh-NRF2 transfection and higher NRF2 abundance after OE-NRF2 transfection compared with their corresponding controls (Fig. S1). To examine whether NRF2 regulates ALDOA expression, ALDOA mRNA and protein were measured following NRF2 overexpression or knockdown. NRF2 overexpression produced a time-dependent increase in ALDOA mRNA and protein, whereas NRF2 knockdown progressively reduced ALDOA expression (Fig. 5A-F). Together, these results supported an association between NRF2 activity and ALDOA expression in HCCC-9810 cells.

NRF2 knockdown attenuates ALDOA upregulation associated with PBRM1 knockdown. RT-qPCR showed that PBRM1 knockdown was associated with increased NRF2 and ALDOA mRNA levels, whereas NRF2 knockdown reduced ALDOA expression (Fig. 6A). When NRF2 and PBRM1 were co-silenced, the increase in ALDOA observed with PBRM1

knockdown alone was attenuated. Western blotting showed the same overall pattern at the protein level (Fig. 6B and C): PBRM1 knockdown was associated with higher NRF2 and ALDOA abundance, whereas NRF2 co-silencing reduced ALDOA. These findings supported an NRF2-dependent contribution to ALDOA upregulation in PBRM1-knockdown HCCC-9810 cells, but they did not define the upstream mechanism responsible for the increase in NRF2.

PBRM1 knockdown is associated with increased tumor growth and higher ALDOA and Ki67 expression in vivo. HCCC-9810-derived xenografts with stable PBRM1 knockdown showed greater tumor weight and volume than control xenografts (Fig. 7A-C). Tumor-tissue western blotting showed lower PBRM1 and higher ALDOA abundance in the PBRM1-knockdown group (Fig. 7D and E). IHC also showed higher ALDOA and Ki67 staining (Fig. 7F and G). Semi-automated analysis of five non-overlapping viable-tumor fields per tumor showed higher normalized IOD for both markers. NRF2 was not measured in xenograft tissues; therefore, these *in vivo* data do not test or establish a PBRM1-NRF2 relationship.

Discussion

The present study showed that PBRM1 knockdown is associated with a tumor-promoting metabolic phenotype in HCCC-9810

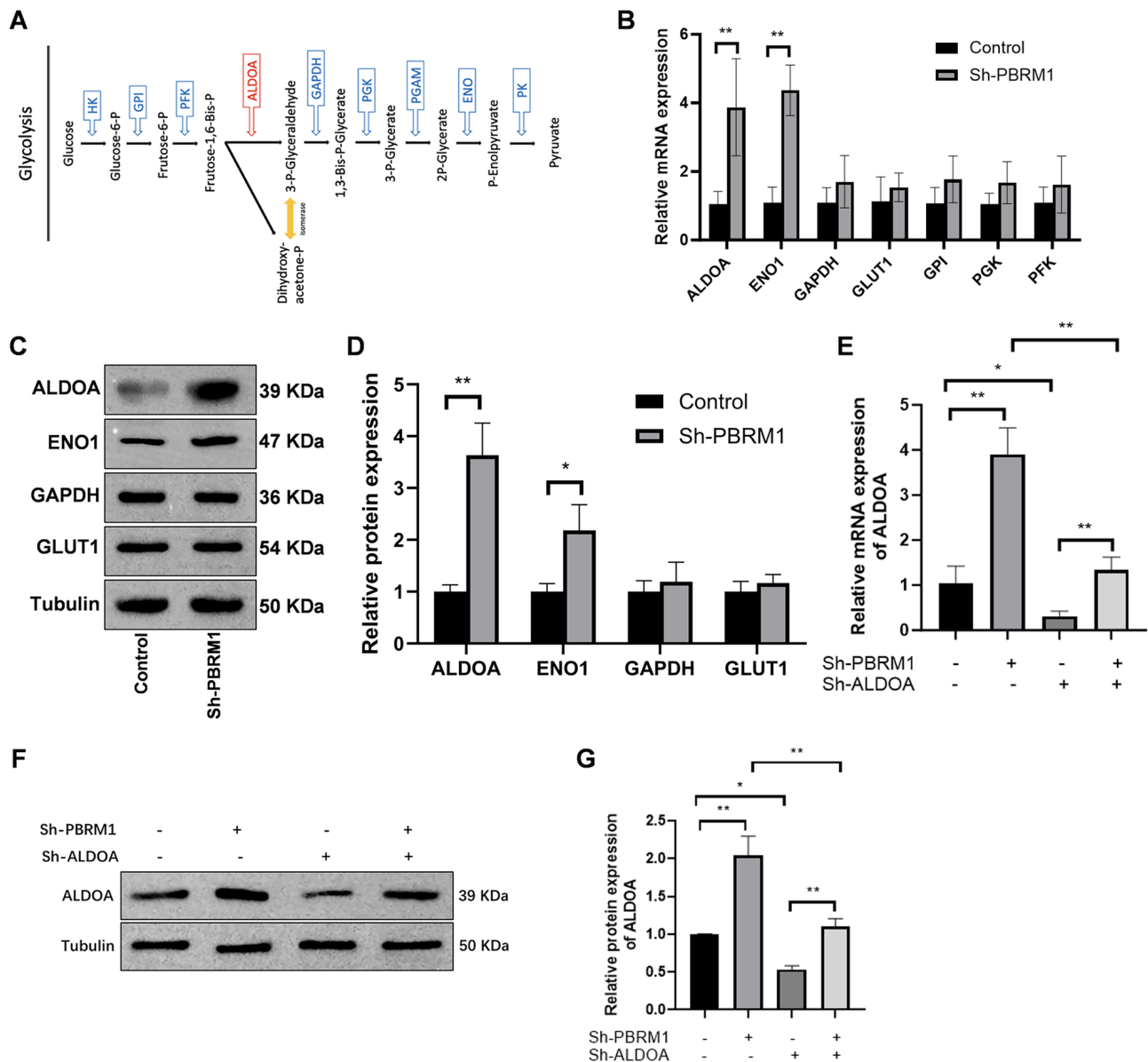


Figure 3. PBRM1 knockdown is associated with increased ALDOA expression in HCCC-9810 cells. (A) Glycolysis-pathway schematic. (B) RT-qPCR analysis of the indicated glycolysis-related genes; control and Sh-PBRM1 values for each gene were compared using multiple unpaired t-tests with Holm-Šidák correction. (C) Western blotting and (D) densitometric quantification. (E) RT-qPCR analysis of ALDOA in the indicated Sh-PBRM1/Sh-ALDOA groups. (F) Western blotting and (G) densitometric quantification of ALDOA. Four-group comparisons in E and G were analyzed by one-way ANOVA followed by Šidák's multiple-comparisons test for the comparisons shown. Data are mean $\pm$ SD from three independent biological experiments. * $P < 0.05$; ** $P < 0.01$. PBRM1, polybromo-1; ALDOA, aldolase, fructose-bisphosphate A; RT-qPCR, reverse transcription-quantitative PCR; Sh, short hairpin.

ICC cells. ALDOA contributes to several of these changes. NRF2 perturbation altered ALDOA expression, and NRF2 co-silencing attenuated the ALDOA increase associated with PBRM1 knockdown. These findings supported an NRF2-dependent component of ALDOA regulation in this experimental model.

Evidence from clear cell renal cell carcinoma motivated the assessment of PBRM1-associated metabolism. Tang *et al* (19) showed that loss of PBRM1 in that tumor setting coincided with activation of AKT-mTOR signaling and greater aerobic glycolysis. The present study asked a related question in an ICC model without assuming that the same mechanism would apply across tumor types. In HCCC-9810 cells, PBRM1 knockdown modified viability during glucose restriction and altered ATP, lactate, pyruvate and ROS measurements. These observations

are compatible with metabolic reprogramming, although they do not provide a direct measurement of metabolic flux.

Among the glycolysis-related genes examined, ALDOA showed a prominent increase after PBRM1 knockdown. ALDOA is a canonical glycolytic enzyme. In the present study, ALDOA knockdown attenuated several growth, metabolic, mitochondrial and apoptosis-related changes associated with PBRM1 knockdown. ALDOA therefore represents one functional contributor to the PBRM1-knockdown phenotype in this experimental system.

NRF2 coordinates cellular responses to oxidative stress and can also influence metabolic pathways (26-30). Manipulating NRF2 alone changed ALDOA expression, indicating that the NRF2-ALDOA relationship was

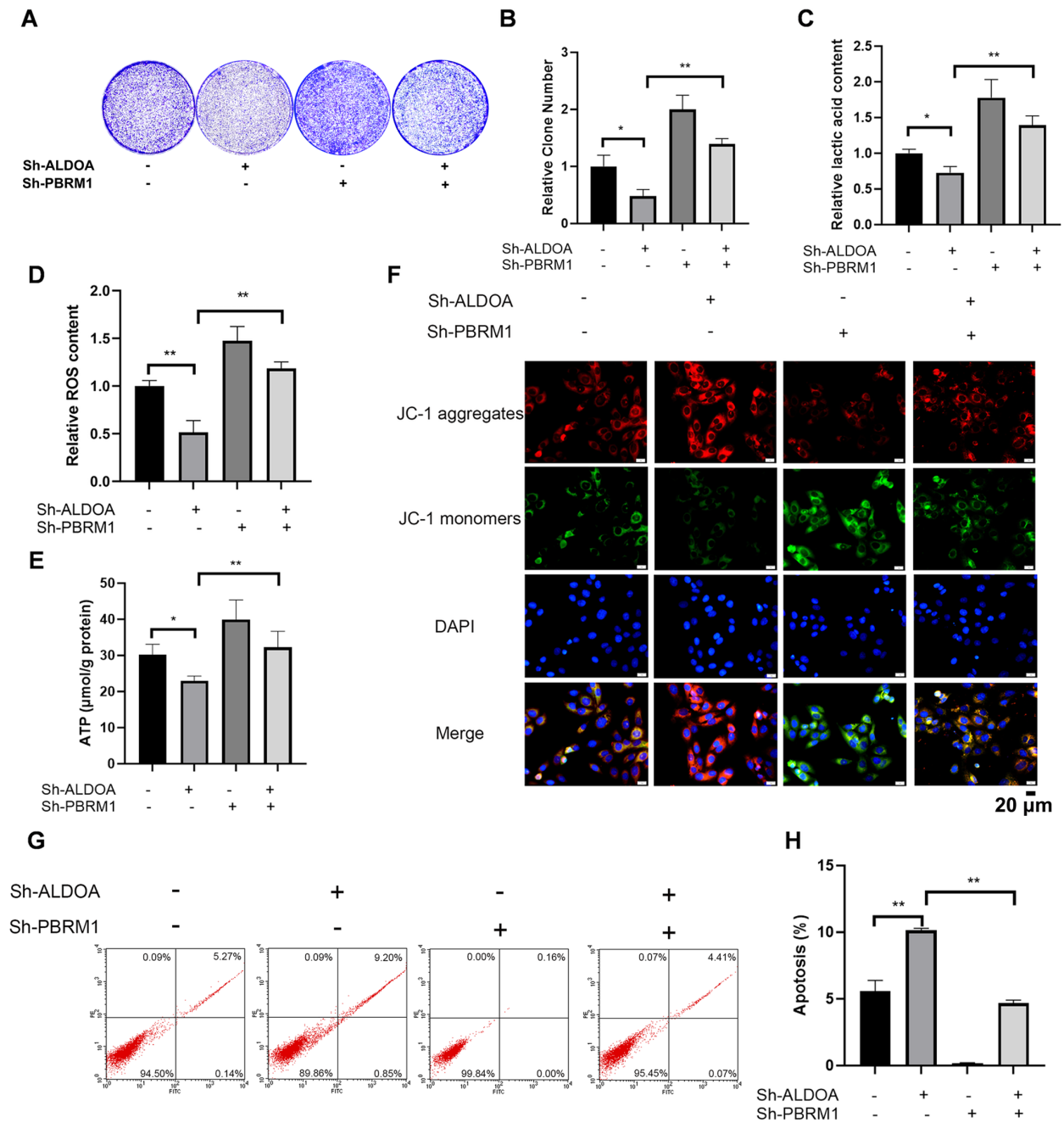


Figure 4. ALDOA knockdown attenuates phenotypes associated with PBRM1 knockdown. (A) Colony formation and (B) quantification. (C) Lactate, (D) ROS and (E) ATP measurements in the indicated groups. (F) JC-1 staining showing red aggregates, green monomers and DAPI counterstaining. (G) Annexin V/PI apoptosis analysis and (H) quantification. Quantitative comparisons were analyzed by one-way ANOVA followed by Šidák's multiple-comparisons test for the prespecified comparisons shown. Data are mean $\pm$ SD from three independent biological experiments. * $P < 0.05$; ** $P < 0.01$. ALDOA, aldolase, fructose-bisphosphate A; PBRM1, polybromo-1; RT-qPCR, reverse transcription-quantitative PCR; Sh, short hairpin; ROS, reactive oxygen species.

not restricted to the PBRM1 co-knockdown experiment. Reducing NRF2 also attenuated the ALDOA increase observed after PBRM1 knockdown. The upstream basis for increased NRF2 following PBRM1 knockdown remains unresolved and may involve transcriptional regulation, KEAP1-dependent protein stabilization, chromatin accessibility, or other mechanisms. The present data therefore established only that NRF2 abundance increased following PBRM1 knockdown; they do not distinguish among these potential upstream mechanisms.

Taken together, the data supported a model-specific relationship in which NRF2 contributes to ALDOA upregulation

in PBRM1-knockdown HCCC-9810 cells, while other transcriptional and signaling inputs may also participate; they do not establish a linear or fully resolved PBRM1-NRF2-ALDOA mechanism. PBRM1-dependent chromatin regulation at the NRF2 or ALDOA loci was not directly examined.

Clinical interpretation should remain limited at this stage. The present study did not analyze patient survival, treatment response, or other outcome-linked clinical variables. Consequently, the present data do not establish PBRM1, NRF2, or ALDOA as prognostic or predictive biomarkers in ICC. Their potential clinical value will require testing in ICC cohorts with detailed annotation and in additional preclinical systems.

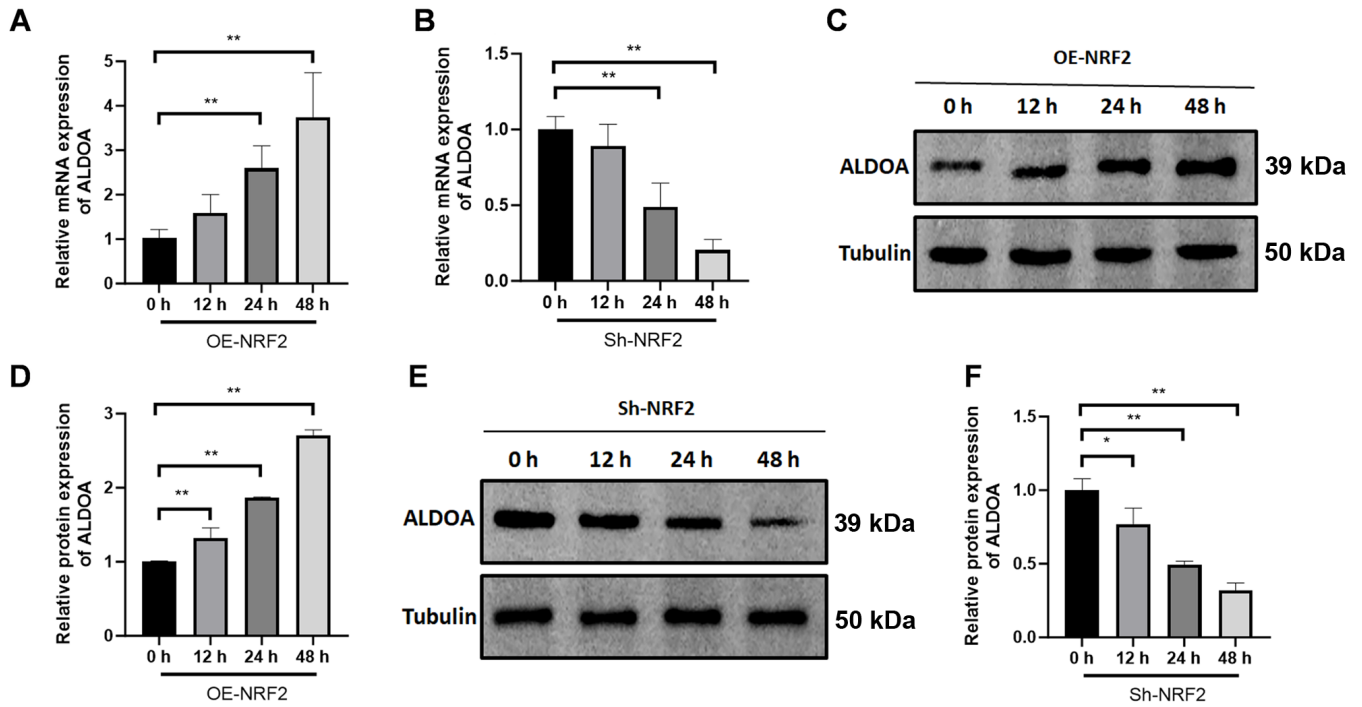


Figure 5. NRF2 perturbation alters ALDOA expression in HCCC-9810 cells. RT-qPCR analysis of ALDOA mRNA following NRF2 (A) overexpression or (B) knockdown at 0, 12, 24 and 48 h. (C) Western blotting and (D) densitometric quantification of ALDOA after NRF2 overexpression. (E) Western blotting and (F) densitometric quantification of ALDOA after NRF2 knockdown. Tubulin served as the loading control. For each time course, one-way ANOVA followed by Dunnett's multiple-comparisons test vs. 0 h was used. Data are mean $\pm$ SD from three independent biological experiments. * P <0.05; ** P <0.01. NRF2, nuclear factor erythroid 2-related factor 2; ALDOA, aldolase, fructose-bisphosphate A; RT-qPCR, reverse transcription-quantitative PCR.

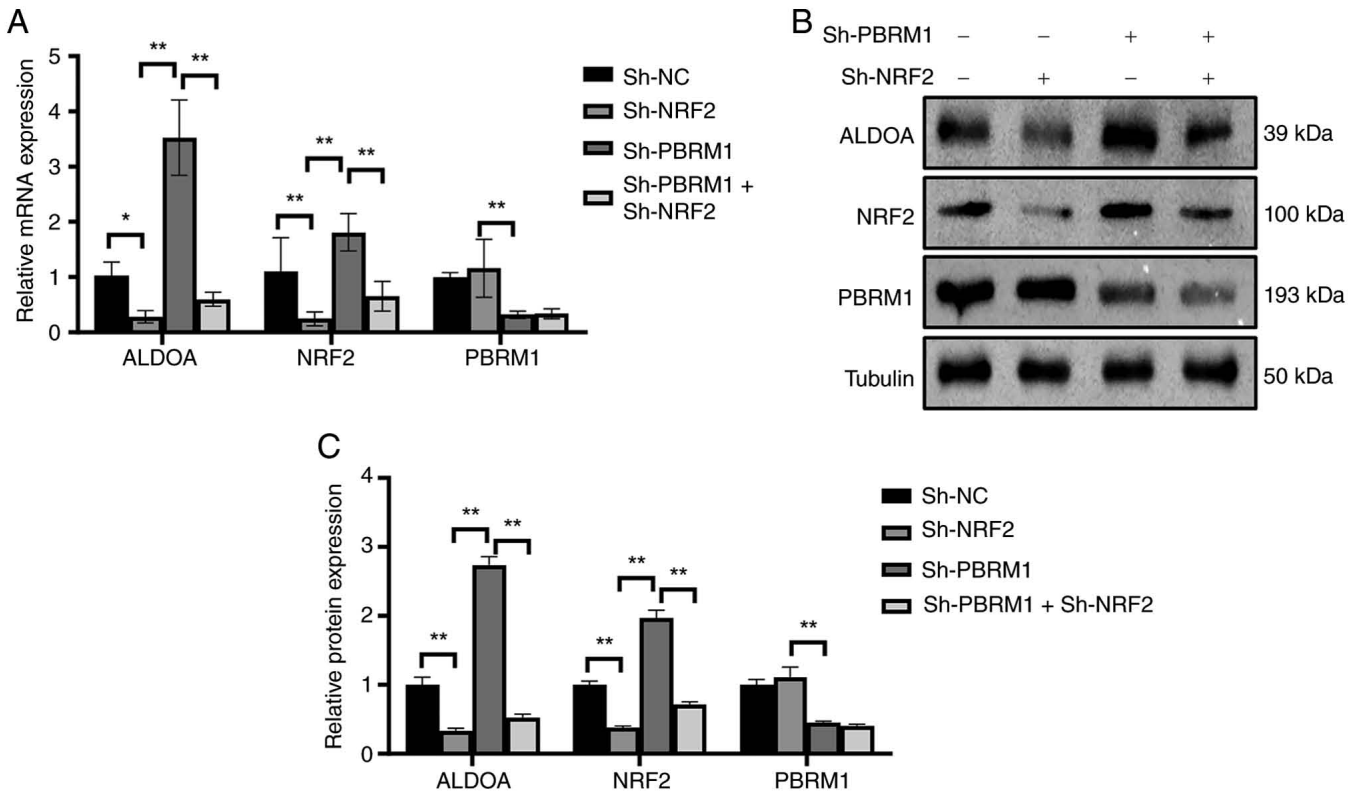


Figure 6. NRF2 knockdown attenuates ALDOA upregulation associated with PBRM1 knockdown. (A) RT-qPCR analysis of ALDOA, NRF2 and PBRM1 mRNA in Sh-NC, Sh-NRF2, Sh-PBRM1 and combined Sh-PBRM1/Sh-NRF2 groups. (B) Representative western blotting and (C) densitometric quantification of ALDOA, NRF2 and PBRM1 in the same groups. Tubulin served as the loading control. For each analyte, one-way ANOVA followed by Šidák's multiple-comparisons test was used for the prespecified comparisons shown. Data are mean $\pm$ SD from three independent biological experiments. * P <0.05; ** P <0.01. NRF2, nuclear factor erythroid 2-related factor 2; ALDOA, aldolase, fructose-bisphosphate A; PBRM1, polybromo-1; RT-qPCR, reverse transcription-quantitative PCR; Sh, short hairpin; NC, negative control.

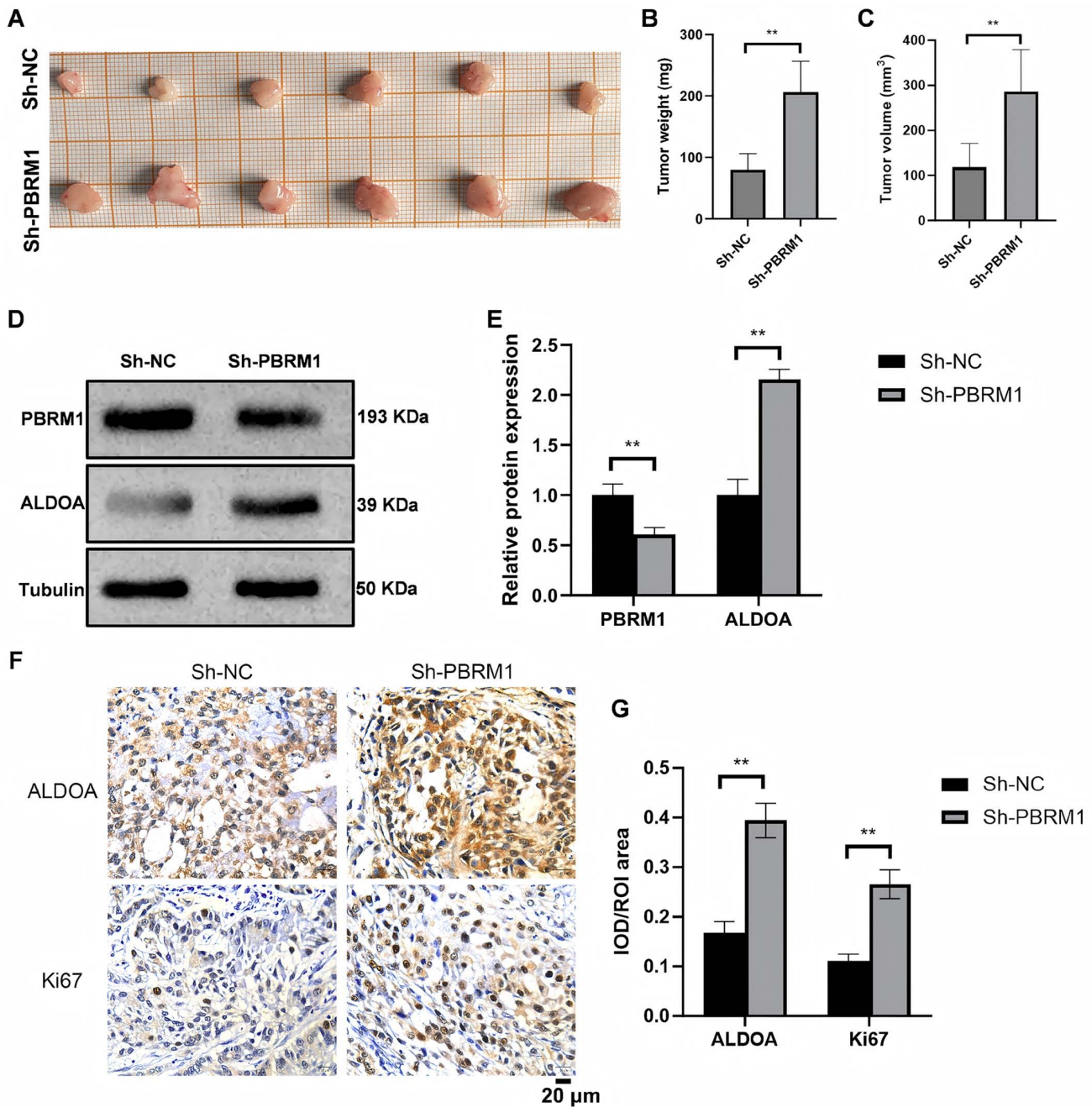


Figure 7. PBRM1 knockdown is associated with increased xenograft growth and higher ALDOA and Ki67 staining. (A) Representative xenograft tumors derived from HCCC-9810 cells expressing Sh-NC or Sh-PBRM1 at the study endpoint. (B) Tumor weight and (C) volume. (D) Western blotting and (E) densitometric quantification of PBRM1 and ALDOA in tumor tissue. (F) Representative ALDOA and Ki67 immunohistochemistry. (G) Semi-automated Image-Pro Plus 6.0 quantification of staining based on five non-overlapping x400 viable-tumor fields per tumor; IOD was normalized to the corresponding ROI area. Each mouse was treated as one biological replicate. Data are mean $\pm$ SD; two-group comparisons used two-tailed unpaired Student's t-tests. **P<0.01. PBRM1, polybromo-1; ALDOA, aldolase, fructose-bisphosphate A; Sh, short hairpin; NC, negative control; IOD, integrated optical density; ROI, region of interest.

The study has several limitations. Mechanistic experiments were performed in a single ICC cell line and used one PBRM1-targeting shRNA. Accordingly, the PBRM1-associated NRF2/ALDOA findings should be interpreted as model-specific observations in HCCC-9810 cells rather than as a general mechanism of ICC and validation in independent ICC models and with complementary genetic approaches is warranted. The upstream mechanism linking PBRM1 knockdown to increased NRF2 remains to be defined and direct NRF2 occupancy at the ALDOA promoter was not assessed using a DNA-binding assay. In addition, NRF2 protein was not

quantified in xenograft tissues; accordingly, the PBRM1-NRF2 relationship is not extended to the *in vivo* setting, and the xenograft conclusions are limited to the endpoints directly measured: tumor growth, PBRM1, ALDOA and Ki67. The public mutation cohort was clinically heterogeneous and was analyzed descriptively rather than for prognostic or predictive associations.

Overall, PBRM1 knockdown in HCCC-9810 cells enhanced tumor-associated behaviors and altered glucose-related metabolic readouts. ALDOA contributed functionally to part of this phenotype, and NRF2 manipulation supported an NRF2-dependent component of ALDOA

regulation. Together, these findings supported a model-specific association among PBRM1 knockdown, increased NRF2 abundance and NRF2-dependent ALDOA regulation in HCCC-9810 cells, rather than a generalized or fully resolved PBRM1-NRF2-ALDOA mechanism in ICC and provided a basis for validation in additional ICC systems.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LZ was responsible for study conception and design. DZ and DL carried out the experiments and acquired the data. Data analysis and figure preparation were performed by DZ, SY and ZZ. DZ and XZ prepared the initial manuscript draft. LZ and FZ provided critical intellectual revision. DZ, DL and LZ confirm the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The Animal Ethics Committee of Shandong Medical and Pharmaceutical University Hospital approved the animal protocol (approval no. 2024-G013-01). All procedures were performed under the institution's requirements for laboratory-animal care and use.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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