

Original Article

7-Dehydrocholesterol-mediated ferroptosis is governed by glucose availability in pancreatic ductal adenocarcinoma

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Abstract

7-Dehydrocholesterol (7-DHC) has recently emerged as an endogenous suppressor of lipid peroxidation/ferroptosis; however, its function in nutrient-poor microenvironments remains undefined. Here, we demonstrate that activated c-Myc directly occupies the *DHCR7* promoter in pancreatic ductal adenocarcinoma (PDAC) cells, transcriptionally upregulating *DHCR7*, which converts 7-DHC into cholesterol, thereby fuelling PDAC cell proliferation. Under glucose deprivation, *DHCR7* deletion triggers pronounced ferroptosis in PDAC cells, an effect that is sharply attenuated when glucose is supplemented. Mechanistically, *DHCR7* inhibition drives 7-DHC accumulation, which plays a dual role: it traps free radicals (anti-ferroptosis) and yet simultaneously depletes the antioxidant pool (pro-ferroptosis), with the net outcome dictated by glucose availability. The antioxidant pool maintained by glucose is required to counteract the pro-peroxidation effects of 7-DHC; once glucose is limited, the radical-trapping-mediated anti-ferroptosis function of 7-DHC becomes negligible. Furthermore, glucose restriction *in vitro* and a low-glucose diet *in vivo* both sensitized PDAC cells to *DHCR7* inhibition-triggered lipid peroxidation, which allows accelerated cell death or suppressed tumor growth. Collectively, 7-DHC, negatively regulated by the c-Myc/*DHCR7* axis, promotes lipid peroxidation and ferroptosis in glucose-deficient PDAC tumors, revealing a metabolic vulnerability that can be exploited for therapy.

Key words lipid peroxidation, glucose-deficient, 7-dehydrocholesterol, pancreatic ductal adenocarcinoma

Introduction

Cholesterol is essential for regulating membrane fluidity in mammalian cells. It is required in the maintenance of membrane-localized protein function, including transporters, receptors, and metabolic enzymes [1]. Cholesterol can be synthesized via the mevalonate pathway (endogenous synthesis) or following the dietary absorption of lipoproteins (exogenous synthesis) [2]. However, cholesterol biosynthesis activity varies across different tissues and cell types [3,4], which explains the inhibition or promotion of cholesterol biosynthesis in cells by low oxygen [5–7]. Although some cancer cells rely exclusively on exogenous cholesterol uptake for proliferation, cholesterol biosynthesis is

constitutively activated in cancer cells [8–10], indicating that cancer development is supported by metabolic intermediates from the cholesterol synthetic pathway rather than endogenous cholesterol.

De novo cholesterol biosynthesis and ketogenesis share the metabolic route of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA conversion. In the mevalonate pathway, 3-hydroxy-3-methylglutaryl-CoA is further reduced to yield mevalonate, a precursor of cholesterol and many other metabolites, such as ubiquinone, heme A, and geranylgeranyl pyrophosphate (GGPP) [2]. Cholesterol-derived oxysterols modulate immune function in the tumor microenvironment and regulate cancer progression [11]. In addition, metabolic intermediates from the cholesterol synthetic path-

way are also associated with tumorigenesis. Specifically, an increased level of GGPP activates autophagy flux and maintains the stemness of cancer cells, thereby promoting cancer cell survival during chemotherapy [12,13]. Moreover, the accumulation of squalene, a downstream metabolite of mevalonate, induces apoptosis and ferroptosis [14,15]. Other metabolic intermediates [e.g., lanosterol and 7-dehydrocholesterol (7-DHC)] are closely associated with innate immune responses [16,17] but were not comprehensively investigated in previous cancer studies. However, the mechanisms by which cholesterol biosynthetic enzymes and their upstream and downstream metabolites affect the fate of cancer cells remain largely unknown.

Cholesterol biosynthesis is regulated by sterol regulatory element-binding proteins (SREBPs) [18]. Loss of function in the tumor suppressor *TP53* and activation of the oncogene *c-Myc* both increase cholesterol biosynthesis via transcriptional upregulation of SREBPs [19,20]. *c-Myc* can also bind to and upregulate the transcription of the cholesterol biosynthesis gene *SQLE* [21]. *TP53* mutation and *c-Myc* overactivation are major events during the development of pancreatic ductal adenocarcinoma (PDAC) [22], indicating that cholesterol biosynthesis is highly activated in PDAC; however, targeting cholesterol biosynthesis has proven ineffective against PDAC [9].

Therefore, we hypothesize that, rather than cholesterol synthesis, the effective metabolism of metabolic intermediates in cholesterol biosynthesis plays a major role in PDAC development. Moreover, our previous research revealed that the rapid metabolism of anti-cancer metabolites and accumulation of oncometabolites are regulated by oncogenes [23]; therefore, we further hypothesize that the effective metabolism of cancer-associated metabolic intermediates in cholesterol biosynthesis is regulated by a specialized PDAC oncogene. Investigating these hypotheses will aid our understanding of the role of unexplored cholesterol biosynthesis in PDAC.

Materials and Methods

Cells and culture conditions

Human PDAC cells of MIA PaCa-2 and HEK 293T cells were purchased from the National Collection of Authenticated Cell Cultures, Chinese Academy of Sciences (Shanghai, China). Cell authentication was performed by Genetic Testing Biotechnology Corporation (Suzhou, China) using short tandem repeat loci analysis. Immortalized KPC cells were provided by Dr Tao Xia from Zhejiang Provincial People's Hospital (Hangzhou, China). The identity of KPC cells was verified by Sanger sequencing of *Kars* and *Trp53* (Supplementary Figure S1). Both MIA PaCa-2 and HEK 293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, USA) supplemented with 12% calf serum (Sigma-Aldrich), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Beyotime, Shanghai, China), and 2.5 ng/mL amphotericin B (Sangon Biotech, Shanghai, China). KPC cells were maintained in Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific, Waltham, USA), which contains calf serum, antibiotics, and amphotericin B as described above. Cells were determined to be free of *Mycoplasma* contamination using a MycoAlert PLUS *Mycoplasma* Detection Kit (Lonza Bioscience, Basel, Switzerland). All cells were cultured at 37°C in a cell culture incubator (Thermo Fisher Scientific) with 5% CO₂ and then cultured with a low concentration of *Mycoplasma* removal agent (0.5 µg/mL; Beyotime) according to the manufacturer's instructions. To culture

cells under glucose-deprived conditions, DMEM and calf serum were replaced with DMEM without glucose (Sigma-Aldrich) and dialyzed serum without glucose, respectively.

Preparation of dialyzed serum

Serum without glucose was prepared via dialysis using dialysis membranes with a 1-kDa molecular-weight cutoff (Spectrum Labs, Los Angeles, USA) at 4°C for 12 h. The dialyzing buffer (151.425 g Trizma base, 37.275 g potassium chloride, and 400.314 g NaCl, added to 5 L of deionized water; pH = 7.35, all from Sigma-Aldrich) was replaced every 1 h to strip off the low-molecular-weight metabolites, e.g., glucose. Lipoprotein-deficient serum (LPDS) was prepared as previously described [24]. Fetal bovine serum (Sigma-Aldrich) was mixed with di-isopropyl ether (Sigma-Aldrich) and n-butanol (Aladdin, Shanghai, China) at a ratio of 5:4:1 and stirred for 30 min. The mixture was then centrifuged at 4000 g for 15 min at 4°C. The lower phase was retrieved and mixed with di-isopropyl ether at a ratio of 1:1. The mixture was centrifuged again at 4000 g for 15 min at 4°C. To remove the organic solvents, the lower phase obtained from the second centrifuge round was dialyzed using a dialysis bag with a molecular-weight cutoff of 1 kDa at 4°C for 72 h. The dialyzed solution (9 g/L NaCl; Sigma-Aldrich) was replaced every 24 h. The complete removal of cholesterol in the LPDS was confirmed using a total cholesterol assay kit (NJJC BIO, Nanjing, China).

Expression vectors

Pre-designed shRNA sequences of *DHCR7* (sh*DHCR7*#1: 5'-ACTTCA AGCTGTTCTTCAATG-3'; sh*DHCR7*#2: 5'-ATTGCCAGCACAGACG GATTT-3'; and shControl: 5'-CAACAAGATGAAGAGCACC AA-3') were obtained from Sigma-Aldrich Advanced Genomics; clone IDs TRCN0000218635 and TRCN0000230565, respectively. Both shRNA sequences were synthesized by Tsingke (Beijing, China) and inserted into the lentiviral vector pLKO.1-puro (Sigma-Aldrich) following a standard protocol [25]. The overexpression vector of *DHCR7* (clone ID: 101930311, pLX304-Blast-V5) or shRNA expression vector of *GSR* (sh*GSR*: 5'-GCATGATAAGGTACTTAGA-3', pGIPZ) were obtained from the DNA library of Shanghai Jiao Tong University (Shanghai, China). The overexpression vector of *c-Myc* (NM_001354870.1, pLVX-puro) was purchased from GenScript (Nanjing, China).

Generation of cell lines

The expression vector and two lentiviral packaging vectors (pMD2G and pSPAX2, obtained from Wenzhou Medical University) were co-transfected into HEK 293T cells at a ratio of 1:1:2 using Lipofectamine™ 3000 (Thermo Fisher Scientific) according to the manufacturer's protocol. The medium containing viral particles was collected after 72 h and filtered through a 0.45-µm filter (JET Biofil, Guangzhou, China). Stable cells were generated by infecting the cells with viral particles for 48 h, followed by selection with either 3 µg/mL puromycin (Sangon Biotech) or 6 µg/mL blasticidin S HCl (Beyotime) for two weeks. Following the successful construction of cell models via drug screening, the culture was maintained with medium containing 1 µg/mL puromycin or 3 µg/mL blasticidin S HCl. Transient depletion of specialized gene expression in cultured cells was achieved by transfecting small-interfering RNA (siRNA) using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's protocol. A total of 10 siRNAs were synthesized by GenePharma (Shanghai, China), and all siRNA

sequences are listed in [Supplementary Table S1](#).

Quantitative reverse transcription PCR

Quantitative reverse transcription PCR was performed as previously described [26]. Briefly, total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific) and then subjected to reverse transcription using HiScript II Q RT SuperMix for qPCR (Novazan, Nanjing, China). qPCR experiments were conducted with ChamQ SYBR qPCR Master Mix (Novazan) on a Quantagene q225 system (KUBO-Tech AG, Effretikon, Switzerland). Primers for mRNA measurement are listed in [Supplementary Table S2](#). The mRNA levels of target genes were normalized against the expression of β -actin.

Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were performed as described previously [23]. Proteins were extracted using 1× RIPA buffer (Cell Signaling Technology, Beverly, USA), and electrophorized via SDS-PAGE, and then electroblotted onto a 0.22- μ m PVDF membrane (Bio-Rad, Hercules, USA). After blocking with 5% milk, membranes were probed using appropriate primary antibodies, followed by related anti-rabbit or mouse IgG secondary antibodies (7076 and 7074; Cell Signaling Technology). Primary antibodies against DHCR7 (ab103296), c-Myc (67447-1-Ig), and β -actin (sc-47778) were obtained from Abcam (Cambridge, UK), Proteintech (Chicago, USA), and Santa Cruz (Santa Cruz, USA), respectively. The signal was detected using the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). Quantification was performed using the Gel-Pro Analyzer 4.0 (Media Cybernetics, Rockville, USA).

Chromatin immunoprecipitation (ChIP) and qPCR assay

ChIP was performed using the SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, 4×10^6 cells cultured in dishes were fixed with 1% formaldehyde (Sigma-Aldrich) and then neutralized with 1 mL of 10× glycine (Cell Signaling Technology) to crosslink the proteins to DNA. Cells were then harvested by adding 2 mL of phosphate-buffered saline (PBS) and 5 μ L of 200× Protease Inhibitor Cocktail (Cell Signaling Technology) to the dish, which was then centrifuged at 2000 *g* for 5 min at 4°C. Chromatin in the pellet was digested with micrococcal nuclease and sonicated with an ultrasonic homogenizer (Scientz-IID; Ningbo Scientz Biotechnology, Ningbo, China) as described previously [23]. The samples resolved in 1× ChIP buffer were incubated overnight at 4°C with either mouse IgG (Cell Signaling Technology) or anti-c-Myc antibodies (Cell Signaling Technology). Then, 30 μ L of protein G agarose beads was added to the sample and incubated at 65°C for another 30 min. Chromatins collected with antibody-linked beads were lysed, and the DNA was purified accordingly. Quantitative PCR was performed on the DNA fragment of the *DHCR7* promoter containing the c-Myc binding motif (−288 to −283 bp; CACGTG). The detailed primers are listed in [Supplementary Table S2](#).

Luciferase reporter assay

The transcriptional activity of c-Myc was quantified using a luciferase assay system, as described previously [23]. Briefly, a DNA fragment (1000 bp, −1000 to 0 bp region) containing the predicted c-Myc binding motif (−288 to −283 bp region) in the *DHCR7* promoter was synthesized by Tsingke and cloned and

inserted into the pLV (Exp) vector (Promega, Wisconsin, USA). HEK 293T cells cultured in a six-well plate with a confluence of 50% were transfected with pLV (Exp), pRLTK (an internal control vector containing a *Renilla* luciferase sequence; Promega), and either c-Myc siRNA or the overexpression vector of c-Myc. After 24 h of transfection, the cells were washed with PBS and lysed with luciferase detection reagent (Promega) accordingly. Firefly luciferase activity was boosted using the same kit according to the manufacturer's instructions and then monitored using a multi-functional plate reader (SpectraMax iD3; Molecular Devices, Silicon Valley, USA). Normalized luciferase activity is presented as a ratio relative to the activity of *Renilla* luciferase.

Human tumor samples and immunohistochemistry analysis

As described previously [27], a PDAC tissue microarray containing 11 tumors was generated by Dr Tao Xia at the Department of Pancreatic Surgery of Zhejiang Provincial People's Hospital using a protocol approved by the Ethics Committee of Zhejiang Provincial People's Hospital (approval number: 2022QT010). In this study, 23 PDAC tissues were successfully stained and analyzed. Anti-DHCR7 (PA5-48204; Thermo Fisher Scientific) or c-Myc (67447-1-Ig; Proteintech) antibodies were used for immunohistochemical staining, and nonspecific IgG (ZSGB-BIO, Beijing, China) was used as a negative control. For immunohistochemical staining, paraffin-embedded tissue sections were cut to a thickness of approximately 3 μ m. Subsequently, the sections were incubated with a diluted primary antibody overnight at 4°C and visualized using diaminobenzidine (Beyotime). The sections were then dehydrated with different concentrations of ethanol solution, cleared with xylene, and sealed with neutral resin. Protein expression was observed under a light microscope (Nikon Corporation, Tokyo, Japan), and the integrated optical density was calculated using ImagePro Plus 6.0 (Media Cybernetics). Finally, the mean optimal density, which represents the DHCR7 or c-Myc intensity, was calculated as the integrated optical density/area.

Transcriptome profiling

A total of 2×10^6 cells with or without *DHCR7* depletion were harvested from a 100-mm² dish, which were resuspended in TRIzol (Takara, Kyoto, Japan). The cells were then sent to Novogene (Beijing, China) for transcriptome profiling. Sequencing was performed on Illumina instruments (Illumina, San Diego, USA). The raw data were formatted as fastq and subjected to quality control using fastp software (<https://github.com/OpenGene/fastp>). The sequencing data were compared to the genome (*Homo sapiens*, GRCh38) using HISAT2 (<https://ccb.jhu.edu/software/hisat2>). Genes or transcripts were assembled and quantified using FPKM [FPKM = total_exon_fragments/mapped_reads (millions) × exon_length (kb)] and the R package edgeR (<https://bioconductor.org/packages/release/bioc/html/edgeR.html>) to analyze differential genes between samples. Gene set enrichment analysis was performed with the Kyoto Encyclopedia of Genes and Genomes (KEGG) with DAVID software (<https://david.ncicrf.gov/>). A bar plot was generated using the Bioinformatics cloud platform (<https://www.bioinformatics.com.cn/>).

Untargeted metabolic profiling

Cells (5×10^6) were rinsed with cold PBS three times, pelleted at

1000 g for 5 min at 4°C, and frozen in liquid nitrogen. Subsequently, the samples were sent to Calibra (Hangzhou, China) on dry ice for untargeted metabolic profiling. The data were analyzed using MultiQuant 3.0.3 software.

Targeted metabolic determination of 7-DHC

Cells (1×10^6) were trypsinized and rinsed with cold PBS five times. Then, the cells were pelleted at 1000 g for 5 min at 4°C and snap frozen with liquid nitrogen. Cell pellets were sent to Calibra on dry ice for the targeted metabolic determination of 7-DHC based on a previously published protocol [28]. Absolute 7-DHC levels were determined according to the standard curve generated by 7-DHC (Sigma-Aldrich). An isotope-labeled 7-DHC was used as the internal control. The data were analyzed using MultiQuant 3.0.3 software.

Cholesterol measurement

Cholesterol was measured using Filipin III Ready Made Solution (Sigma-Aldrich), as described previously [26]. Cells (1.5×10^4) were plated in triplicate on coverslips (WHB, Shanghai, China). After 48 h, the cells were washed twice with PBS and fixed with 4% paraformaldehyde (Beyotime), followed by incubation with 50 µg/mL Filipin III Ready Made Solution in the dark for 2 h at room temperature. After adding 5 µL of antifade Mounting Medium (Beyotime) to the slide, the slide was sealed with neutral resin. Fluorescence images were captured using a confocal laser scanning microscope (Nikon Corporation), with at least 100 cells captured from each biological replicate. The intensity of the fluorescence signal was analyzed using ImageJ software (1.54j).

Bioinformatics analysis of cholesterol metabolic genes

Gene expression data related to cholesterol synthesis and transportation pathways in pancreatic cancer tissues and normal pancreatic tissues were downloaded from the TCGA (The Cancer Genome Atlas, <https://portal.gdc.cancer.gov>) and GTEx (<https://gtexportal.org>) databases, respectively. Gene expression data of human tumor and normal pancreatic ductal cells (GSE number 93326), KPC mice with normal pathology, pancreatic intraepithelial neoplasia (PanIN), and PDAC cells (GSE207943) at the single-cell level were downloaded from the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>). Data were analyzed using SPSS 22.0 (IBM, New York, USA) and plotted using Prism 10.0.0 (GraphPad, San Diego, USA).

Transcription factor and binding site prediction

To predict *c-Myc* binding sites at the promoters of cholesterol metabolism-related genes, promoter sequences (−2000 bp) were obtained from the UCSC database (<http://genome.ucsc.edu>). LASAG-NA-Search 2.0 (<http://biogrid-lasagna.engr.uconn.edu/lasagna-search/>) was employed to predict the binding sites of *c-Myc* and *SREBP1*. The transcription factors and their binding sites were further verified using JASPAR (<https://jaspar2020.genereg.net/about/>).

Lipid peroxidation assay

Lipid peroxidation assays were performed using a BODIPY™ 581/591 C11 Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Cells cultured in a six-well plate that reached 70%–80% confluence were trypsinized and rinsed with pre-warmed PBS. Cell pellets were then resuspended in pre-warmed PBS containing 5 µM BODIPY™ 581/591 C11 and incubated at 37°C

for 30 min. Finally, the cells were washed with PBS and analyzed using a NovoCyte flow cytometer (Agilent, Santa Clara, USA).

Analysis of apoptosis

The apoptosis rate was measured using a propidium iodide (PI)/Annexin V apoptosis detection kit (Beyotime) according to the manufacturer's instructions. Briefly, cells were cultured in a six-well plate to reach 70%–80% confluence. Then, all cells, including those floating in the medium, were harvested and rinsed with pre-warmed PBS. Cells were incubated with 5 µM PI and 5 µM Annexin V for 20 min in the dark. Apoptosis was then analyzed using a NovoCyte flow cytometer.

Cell cycle assay

The cell cycle was analyzed as described previously [23,25]. Accordingly, cells cultured in a six-well plate to 70%–80% confluence were trypsinized and rinsed with pre-warmed PBS. During vortexing, 1 mL of cold 70%–80% ethanol was added drop-wise to the cell pellet, and the mixture was incubated at −20°C for 2 h. Next, the cells were washed twice with PBS to remove the ethanol. Pelleted cells were then incubated with 0.5 mL of PI/RNase Staining Buffer (BD Bioscience, San Diego, USA) at room temperature for 15 min in the dark. The cell cycle was analyzed using a NovoCyte flow cytometer.

Cell proliferation and cell survival assays

Cell proliferation and survival assays were performed as described previously [25]. For the cell proliferation assay, 1×10^4 cells were plated in triplicate in 12-well plates; after 24 h, the culture medium was replaced with customized culture medium supplemented with or without chemicals [AY9944, RSL3, 7-DHC, N-acetylcysteine (NAC), or Fer-1, all from Sigma-Aldrich]. After the indicated time described in the results, the cells were then collected for cell counting. For the cell survival assay under glucose-deprived conditions, 1×10^5 cells were plated in triplicate in a 12-well plate. After 24 h, the medium was replaced with glucose-deprived medium, and cell counting was performed for MIA PaCa-2 and KPC cells after an additional 24 and 20 h, respectively. All cells were counted using a NovoCyte flow cytometer.

H₂O₂ measurement

The intracellular H₂O₂ concentration was measured using a Hydrogen Peroxide Assay Kit (Beyotime) according to the manufacturer's instructions. Briefly, 1×10^6 cells cultured in a six-well plate were trypsinized and rinsed with cold PBS. H₂O₂ was extracted and measured at 560 nm with a multifunctional plate reader. The measured values were normalized to cell number.

NADP⁺/NADPH measurement

The ratio of NADP⁺/NADPH was determined using an NADP⁺/NADPH Assay Kit (Beyotime) according to the manufacturer's instructions. Briefly, 1×10^6 cells cultured in a six-well plate were trypsinized and rinsed with cold PBS and then lysed with the lysis buffer supplied in the kit. NADP⁺ and NADPH were extracted and then measured at 450 nm with a multifunctional plate reader.

GSH/GSSG measurement

The ratio of GSH/GSSG was determined using a GSH and GSSG Assay Kit (Beyotime) according to the manufacturer's instructions.

Briefly, 1×10^6 cells cultured in a six-well plate were trypsinized and rinsed with cold PBS and then lysed with the lysis buffer supplied in the kit. The mixture was centrifuged at 12,000 *g* for 10 min at 4°C, and the protein was removed from the pellets with a protein removal solution. GSH and GSSG were determined using a protein-free fraction at 412 nm with a multifunctional plate reader.

Animal experiments

Nude mice (BALB/C nude, male, four weeks old) were procured from Zhejiang Vital River Experimental Animal Technology Co., Ltd. (Hangzhou, China) and housed in the Animal Experiment Center of Wenzhou Medical University under specific-pathogen-free conditions. This study was approved by the Ethics Committee of Wenzhou Medical University (wydw2023-0106). Cells were resuspended in a mixture of PBS and Matrigel (Corning, New York, USA) at a 1:1 ratio and then injected subcutaneously into the shoulder of four-week-old mice, following a previously described protocol [23]. For dietary intervention, the regular diet was formulated using an Ain-93G standard, whereas the low-carbohydrate customized diet (Xietong Shengwu, Haimen, China) was modified from Ain-93G by reducing the proportion of carbohydrates from 63.95% to 19.72% and increasing the proportion of protein to ensure a consistent energy supply between diets. After 2 weeks of subcutaneous tumorigenesis, mice maintained on either a regular or low carbohydrate diet were subjected to injections of AY9944 (25 mg/kg) every two days or were left untreated. Once the tumors were established, tumor sizes were measured using a Vernier caliper, and tumor volume was calculated according to the following formula: volume = (length $\times$ width $\times$ height) $\times$ 0.5236. Mice were sacrificed using cervical dislocation and dissected to obtain tumors for weighing and other specialized experiments.

Malondialdehyde determination

The level of malondialdehyde (MDA) was measured using an MDA Assay Kit (TBA method; NJCBIO, Nanjing, China) according to the manufacturer's instructions. Briefly, approximately 5 mg tissue was homogenized with a tissue grinder (Wheaton, Millville, USA) and then lysed at 95°C for 40 min using a commercial lysis buffer supplied in the kit. After centrifuging at 3500 *g* for 10 min, the absorbance of the MDA-containing supernatant was measured at 532 nm using a multifunctional plate reader. The MDA signals from different samples were normalized against the protein concentration.

Statistical analysis

Quantitative results are presented as the mean $\pm$ standard error of the mean (SEM), and all experiments were independently repeated more than three times. Statistical analysis was performed using one-way ANOVA to compare the results from more than two groups, and the independent two-tailed Student's *t* test was applied to compare the results from two groups. All data in this study were analyzed using SPSS 22.0 (IBM) and plotted using Prism 10.0.0. *P* < 0.05 was considered statistically significant.

Results

***c-Myc* transcriptionally upregulates DHCR7 in PDAC cells**
Cholesterol metabolism is intimately linked to PDAC pathogenesis [29,30]; therefore, we speculate that heightened cholesterol biosynthesis driven by the *c-Myc*/SREBP1 axis—and/or *c-Myc*-

controlled mevalonate flux—is indispensable for tumor development [31,32]. Consistent with this notion, *c-Myc* is markedly overactivated in PDAC [33,34], and both cholesterol biosynthesis-related (Figure 1A) and cholesterol transport-related genes (Supplementary Figure S2A) are significantly upregulated in PDAC tissues compared with normal pancreas. The increased expression of cholesterol metabolic genes in tumor ductal cells was confirmed via single-cell sequencing (Figure 1B and Supplementary Figure S2B). Although *c-Myc*-regulated SREBP1/2 is predicted to bind to the promoters of *DHDDS* and *ABCA1/VAPA/SQLE*, which are involved in cholesterol biosynthesis and transportation (Supplementary Table S3), respectively, we found that *c-Myc*-, rather than *c-Myc*-regulated SREBP1/2, binds to the promoters of *DHCR7*, *SC5D*, and *DHDDS* for cholesterol biosynthesis (Figure 1C), as well as *APOC1*, *ABCA1*, and *VAPA* for cholesterol transportation (Supplementary Figure S2C). Of these six genes, only the expression of *DHCR7* (Figure 1D,E) and *APOC1* (Supplementary Figure S2D,E) was downregulated by *c-Myc* depletion in both PDAC and human embryonic kidney (HEK 293T) cells. Although *c-Myc* depletion led to decreased expression of *SREBP1*, as previously described [20] (Supplementary Figure S2F), *SREBP1* depletion did not affect the expression of *DHCR7* or *APOC1* (Supplementary Figure S2G). This indicates that *c-Myc* regulates cholesterol metabolism in both SREBP1-dependent and SREBP1-independent manners. *DHCR7* catalyzes the final step of cholesterol biosynthesis by converting 7-DHC to cholesterol (Figure 1F), and its loss is a well-established driver of the inherited metabolic disorder Smith-Lemli-Opitz syndrome [35,36]. As shared metabolic dysregulation is frequently detected between inherited metabolic disease and many other cancer types [37], it is highly likely that *DHCR7* is involved in the development of different types of cancer.

ChIP was used to reveal the regulatory role of *c-Myc* in *DHCR7* expression. *c-Myc* was able to bind to the −500 to 0 bp region of the *DHCR7* promoter (Figure 1G). Luciferase reporter assays in HEK 293T cells revealed that *c-Myc* knockdown suppressed, whereas *c-Myc* overexpression potentiated, *DHCR7*-driven luciferase activity (Figure 1H,I). Furthermore, the regulation of *DHCR7* expression by *c-Myc* was confirmed at the protein level in MIA PaCa-2 cells and HEK 293T cells (Figure 1J–M). These results indicate that *c-Myc* is a transcription factor of *DHCR7*.

A positive correlation between *c-Myc* expression and the cholesterol biosynthesis gene *DHCR7* was confirmed by reanalyzing the transcriptome data of normal pancreas tissues from the GTEx (<https://xena.ucsc.edu/>) (Figure 1N), reanalyzing the single-cell transcriptome data of PDAC cells (Figure 1O; GSE 93326) [38], and performing immunohistochemical staining of *c-Myc* and *DHCR7* in a local PDAC tissue microarray (Figure 1P). Together, these results strongly suggest that the tumorigenic role of *c-Myc* in PDAC can be at least partly attributed to the transcriptional upregulation of *DHCR7*.

The *c-Myc*/DHCR7 axis promotes cholesterol biosynthesis to support PDAC cell proliferation

To determine whether *c-Myc* regulates *DHCR7* to promote cholesterol biosynthesis, we determined cholesterol levels via Filipin III staining in MIA PaCa-2 cells and found that *c-Myc* depletion could not alter the cholesterol level in PDAC cells cultured in regular medium (Figure 2A). Although *DHCR7* deficiency can reduce intracellular cholesterol levels, the difference is minimal (Figure 2B). This negligible role of *DHCR7* in the regulation of cellular

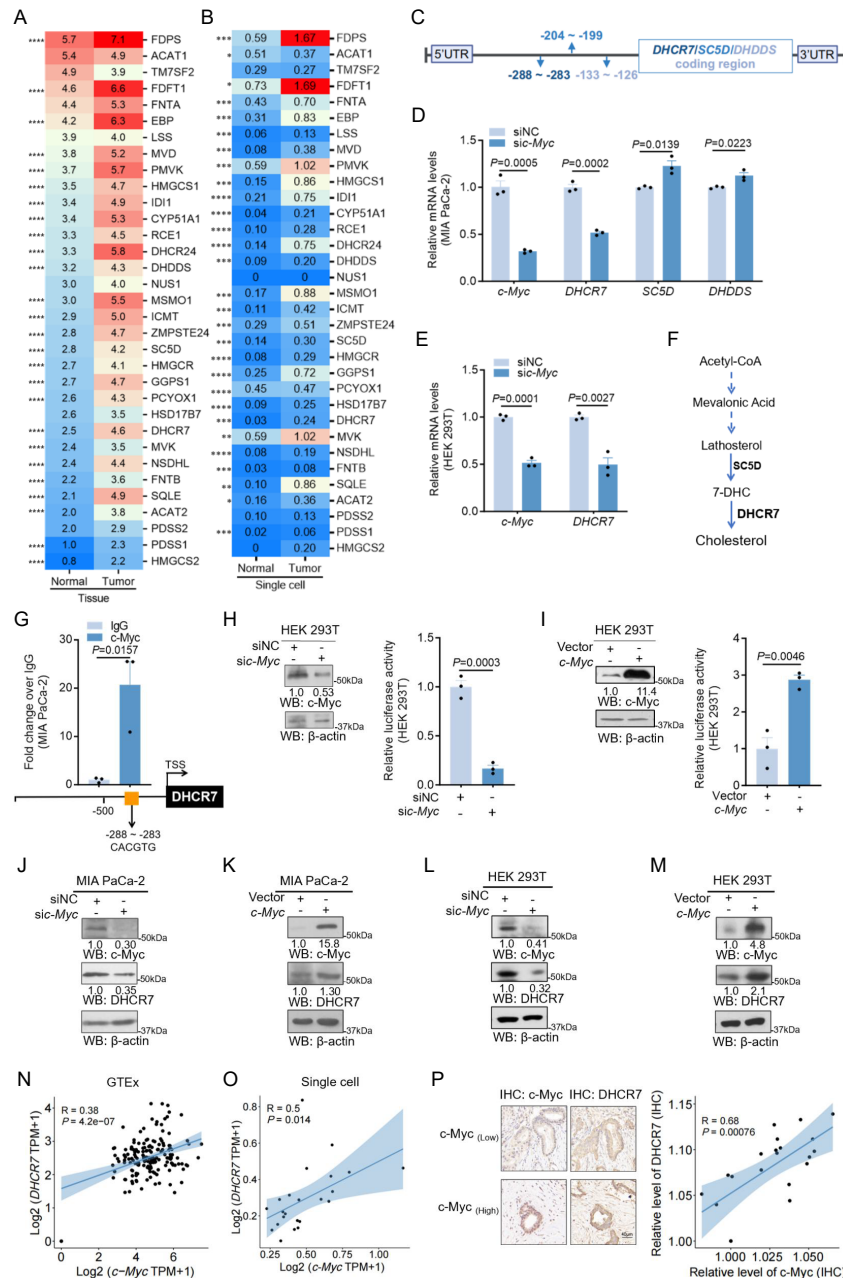


Figure 1. c-Myc promotes *DHCR7* expression in PDAC cell (A) Comparison of the transcription levels of cholesterol biosynthesis-related genes between normal pancreatic tissues from the databases of GTEx (<https://gtexportal.org>) and pancreatic cancer tissues from TCGA (<https://portal.gdc.cancer.gov>). (B) Comparison of the transcription levels of cholesterol biosynthesis-related genes between normal and tumor pancreatic ductal cells at the single-cell level (GEO accession number: 93326). (C) Bioinformatic prediction of c-Myc-binding sites at the promoters of *DHCR7*, *SC5D*, and *DHDDS* via combined use of two transcription factor predictors (LASAGNA-Search 2.0, http://biogrid-lasagna.engr.uconn.edu/lasagna_search/; JASPAR, <https://jaspar2020.genereg.net>). (D,E) mRNA levels of *c-Myc*, *DHCR7*, *SC5D*, and *DHDDS* in MIA PaCa-2 cells (D), as well as *c-Myc* and *DHCR7* in HEK 293T cells (E), with or without *c-Myc* depletion. β -Actin was used as an internal control. (F) Schematic diagram of the cholesterol biosynthesis pathway in humans. (G) qPCR analyses of DNA fragments co-immunoprecipitated with IgG or c-Myc in MIA PaCa-2 cells via ChIP assay (upper panel). qPCR primers were designed to amplify regions containing predicted binding sites of c-Myc in the *DHCR7* promoter (lower panel). TSS, transcription start site. (H,I) Luciferase reporter assay in HEK 293T cells with different expression levels of c-Myc. Western blot analysis of c-Myc in HEK 293T cells with or without *c-Myc* depletion for 24 h (H, left panel) or in cells with or without c-Myc overexpression for 24 h (I, left panel). β -Actin was used as an internal control. Fluorescence intensity was determined in genetically modified HEK 293T cells co-transfected with a luciferase reporter vector containing the *DHCR7* promoter (right panel). (J-M) Western blot analysis of c-Myc and DHCR7 in MIA PaCa-2 and HEK 293T cells with or without *c-Myc* depletion (J,L) or in cells with or without c-Myc overexpression (K,M). β -Actin was used as an internal control. (N, O) Co-expression analyses of *c-Myc* and *DHCR7* in normal pancreatic tissues (GTEx database, $n = 167$) (N) and tumor pancreatic ductal cells (GEO accession number: 93326, $n = 23$) (O). (P) Immunohistochemistry analyses of c-Myc and DHCR7 co-expression in local PDAC tissue ($n = 20$). Data are presented as the mean $\pm$ SEM for bar graphs, and representative immunoblot images were quantified from at least three independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t* test for (B,D,E,G-I), the Mann-Whitney *U* test for (A) and Person's correlation coefficient test for (N-P). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

cholesterol levels was further confirmed by directly measuring the cellular cholesterol level (Supplementary Figure S3A). However, for PDAC cells cultured in medium containing lipoprotein-deficient serum (LPDS), *c-Myc*, and *DHCR7* depletion led to a substantial decrease in the cholesterol level in both MIA PaCa-2 (67.26% for *c-Myc* depletion, 32.76% for *DHCR7* depletion; Figure 2C,D) and KPC cells (31.94% for *c-Myc* depletion, 31.35% for *DHCR7* depletion; Supplementary Figure S3B,C), indicating that exogenous rather than endogenous cholesterol is the major cholesterol source in PDAC cells and that the *c-Myc*/*DHCR7* axis plays an important role in regulating endogenous cholesterol biosynthesis.

To determine whether endogenous cholesterol synthesis regulated by *DHCR7* is sufficient to impact PDAC cell proliferation, MIA PaCa-2 and KPC cells were cultured with or without depletion of cholesterol-enriched low-density lipoprotein receptor (LDLR), the principal gateway for cholesterol uptake. The results showed that cell proliferation arrest was greater in PDAC cells with double depletion of *LDLR* and *DHCR7* than in those with single *DHCR7* depletion (Figure 2E and Supplementary Figure S3D). This indicates that both endogenous and exogenous cholesterol contributed to PDAC cell proliferation.

A previous study using *Dhcr7*-knockout mice revealed that a loss of *Dhcr7* led to oxidative stress accumulation, which may boost stress-induced cell death [35]. Therefore, we analyzed cell death, as represented by apoptosis and lipid peroxidation, and found that neither process was affected in PDAC cells with *DHCR7* depletion, and cell morphology was unchanged in *DHCR7*-depleted cells (Figure 2F–H and Supplementary Figure S3E–G). Likewise, cell cycle profiles were unaltered in the two PDAC lines (Figure 2I for MIA PaCa-2 cells and Supplementary Figure S3H for KPC cells), collectively demonstrating that *DHCR7* loss is non-toxic *in vitro*. Nevertheless, supplying exogenous cholesterol to LPDS-cultured MIA PaCa-2 cells only partially rescued the proliferation defect caused by *DHCR7* depletion (Figure 2J), indicating that the growth impairment extends beyond a simple shortage of cholesterol. Consistently, transcriptome profiling revealed broad dysregulation of multiple proliferation-related signaling pathways upon *DHCR7* depletion (Figure 2K and Supplementary Dataset S1). Together, these results reveal that *DHCR7* depletion plays an additional role in PDAC cell proliferation in addition to cholesterol biosynthesis.

c-Myc is a master transcription factor of many metabolic genes [39]; therefore, it is reasonable that *c-Myc* depletion led to suppressed cell proliferation regardless of the availability of exogenous cholesterol (Figure 2L,M). However, the rescue of cell proliferation was more efficient in LPDS-containing medium (29% to 61%) than in regular medium (21% to 31%) when over-expressing *DHCR7* in *c-Myc*-depleted MIA PaCa-2 cells (Figure 2L, M). These results strongly suggest that *c-Myc* regulates *DHCR7* to promote PDAC cell proliferation, at least partially through the synthesis of cholesterol when exogenous cholesterol is limited.

DHCR7 protects cells from oxidative stress-induced cell death under glucose-deprived conditions

The PDAC tumor microenvironment is notoriously nutrient-poor, leaving tumor cells acutely vulnerable to oxidative stress because they are deprived of key antioxidant precursors such as glucose and glutamine [40]. Given that the loss of *Dhcr7* led to the accumulation of oxidative stress in mice [41], we believe that increased *DHCR7*

levels in human (Figure 1A,B) and mouse (Supplementary Figure S4A) PDAC cells may protect against oxidative stress-induced cell death, which may explain why *DHCR7* is an unfavorable prognostic marker in PDAC (Supplementary Figure S4B). To test this hypothesis, PDAC cells were cultured in glucose-deprived medium. *DHCR7* depletion in both MIA PaCa-2 (Figure 3A) and KPC cells (Supplementary Figure S4C) resulted in decreased cell survival compared to that of control cells. For cells cultured in glucose-deprived medium, increased apoptosis and lipid peroxidation were consistently observed in MIA PaCa-2 (Figure 3B,C) and KPC cells (Supplementary Figure S4D,E) because of *DHCR7* depletion. Moreover, H_2O_2 levels and NADP⁺/NADPH ratios were higher in two *DHCR7*-depleted PDAC cells cultured in glucose-deprived medium than in matched control cells (Figure 3D,E for MIA PaCa-2 cells and Supplementary Figure S4F,G for KPC cells); however, these differences were abolished when glucose was added to the culture medium (Figure 3F,G for MIA PaCa-2 cells and Supplementary Figure S4H,I for KPC cells). Moreover, a decreased GSH/GSSG ratio was observed in *DHCR7*-depleted MIA PaCa-2 cells cultured in glucose-deprived medium rather than in regular medium compared with matched control cells (Figure 3H,I). Gene set enrichment analysis further confirmed that ferroptosis was activated in *DHCR7*-depleted MIA PaCa-2 cells cultured in glucose-deprived medium (normalized $P = 0.0027$; Figure 3J and Supplementary Figure S4J), in contrast to those cultured in regular medium (normalized $P = 0.5536$; Figure 3K and Supplementary Datasets S1,2). These results strongly indicate that glucose deprivation predisposes *DHCR7*-depleted PDAC cells to oxidative stress-induced cell death, particularly lipid peroxidation and ferroptosis.

To determine whether alleviating oxidative stress can at least partially protect cells from death, we performed exogenous supplementation with an antioxidative compound, which prevented lipid peroxidation and cell death due to *DHCR7* depletion under glucose-deprived conditions (Figure 3L,M for MIA PaCa-2 cells and Supplementary Figure S4K,L for KPC cells). Moreover, treating MIA PaCa-2 cells and KPC cells with a lipid reactive oxygen species scavenger prevented cell death induced by *DHCR7* depletion (Figure 3N and Supplementary Figure S4M). Together, our results strongly indicate that *DHCR7* depletion increases oxidative stress and lipid peroxidation in PDAC cells, which promotes PDAC cell death under glucose deprivation.

To further investigate whether the regulatory role of *c-Myc* in cellular oxidative stress is mediated at least partially by *DHCR7*, exogenous *DHCR7* was expressed in PDAC cells with *c-Myc* depletion. *DHCR7* overexpression prevented lipid peroxidation (Figure 3O), H_2O_2 accumulation (Figure 3P), and excessive cell death (Figure 3Q) induced by *c-Myc* depletion in MIA PaCa-2 cells cultured with glucose-deprived medium. These results strongly suggest that *c-Myc* protects cells from oxidative stress, at least partly by the transcriptional regulation of *DHCR7* in PDAC cells with limited glucose supplementation.

DHCR7 depletion drives 7-DHC accumulation, which precipitates PDAC cell death under glucose-deprived conditions

As 7-DHC and its derived oxysterols are oxidative inducers, it is considered a cytotoxic metabolite [41,42]. *DHCR7* is required for 7-DHC metabolism during cholesterol biosynthesis; therefore, we hypothesized that *DHCR7* or *c-Myc* inhibition promotes cell death

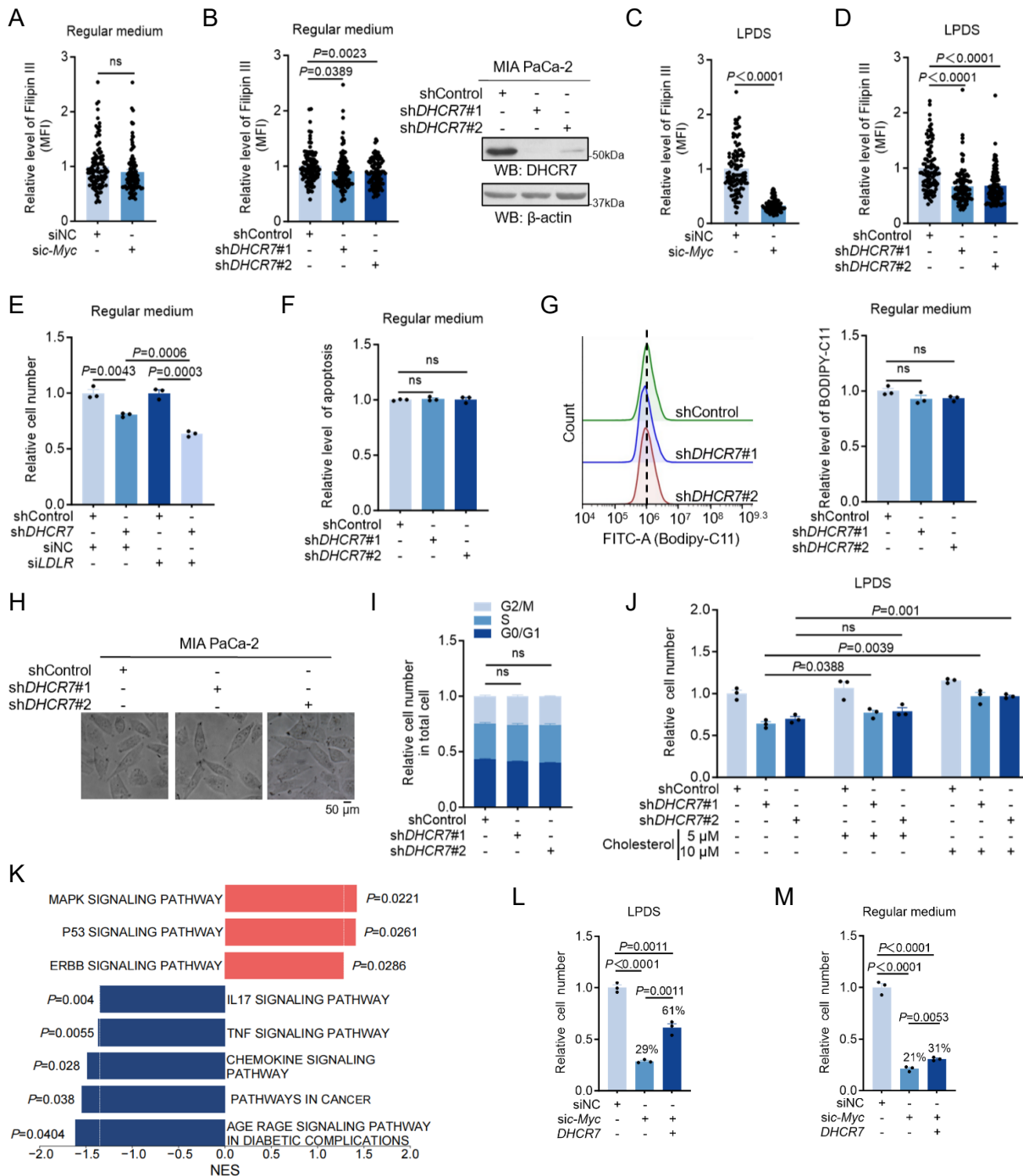


Figure 2. c-Myc-regulated DHCR7 promotes endogenous cholesterol biosynthesis and supports PDAC cell proliferation (A,B) Confocal analyses of Filipin III-stained cholesterol levels in MIA PaCa-2 cells with or without *c-Myc* depletion (A) and in cells with or without *DHCR7* depletion (B). Western blot analysis of the indicated proteins were performed for MIA PaCa-2 cells with or without *DHCR7* depletion. (C,D) Confocal analyses of filipin III-stained cholesterol levels in MIA PaCa-2 cells cultured in lipoprotein-deficient serum (LPDS) with or without *c-Myc* depletion (C) or in cells with or without *DHCR7* depletion (D). (E) Relative cell proliferation of MIA PaCa-2 cells in regular medium (three days) with or without *DHCR7* depletion and then with or without *LDLR* depletion. (F,G) Relative apoptosis (F) and lipid peroxidation (G) levels in MIA PaCa-2 cells with or without *DHCR7* depletion. (H) Representative images of the cell morphology of MIA PaCa-2 cells with or without *DHCR7* depletion. Scale bar = 50 μ m. (I) Cell cycle analysis in MIA PaCa-2 cells with or without *DHCR7* depletion. (J) Relative cell proliferation of MIA PaCa-2 cells cultured in LPDS-containing medium with or without *DHCR7* depletion, followed by exogenous cholesterol supplementation at the indicated concentrations. (K) KEGG enrichment signal transduction pathway analysis of Gene Set Enrichment Analysis between control MIA PaCa-2 cells and cells with *DHCR7* depletion ($n=3$). (L,M) Relative cell proliferation of MIA PaCa-2 cells cultured in LPDS-containing (L) or regular medium (M), with or without *c-Myc* depletion followed by *DHCR7* overexpression. Data are presented as the mean $\pm$ SEM for bar graphs from at least three independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t* test for (E–G,I,J,L,M) and the Mann-Whitney *U* test for (A–D). ns, not significant.

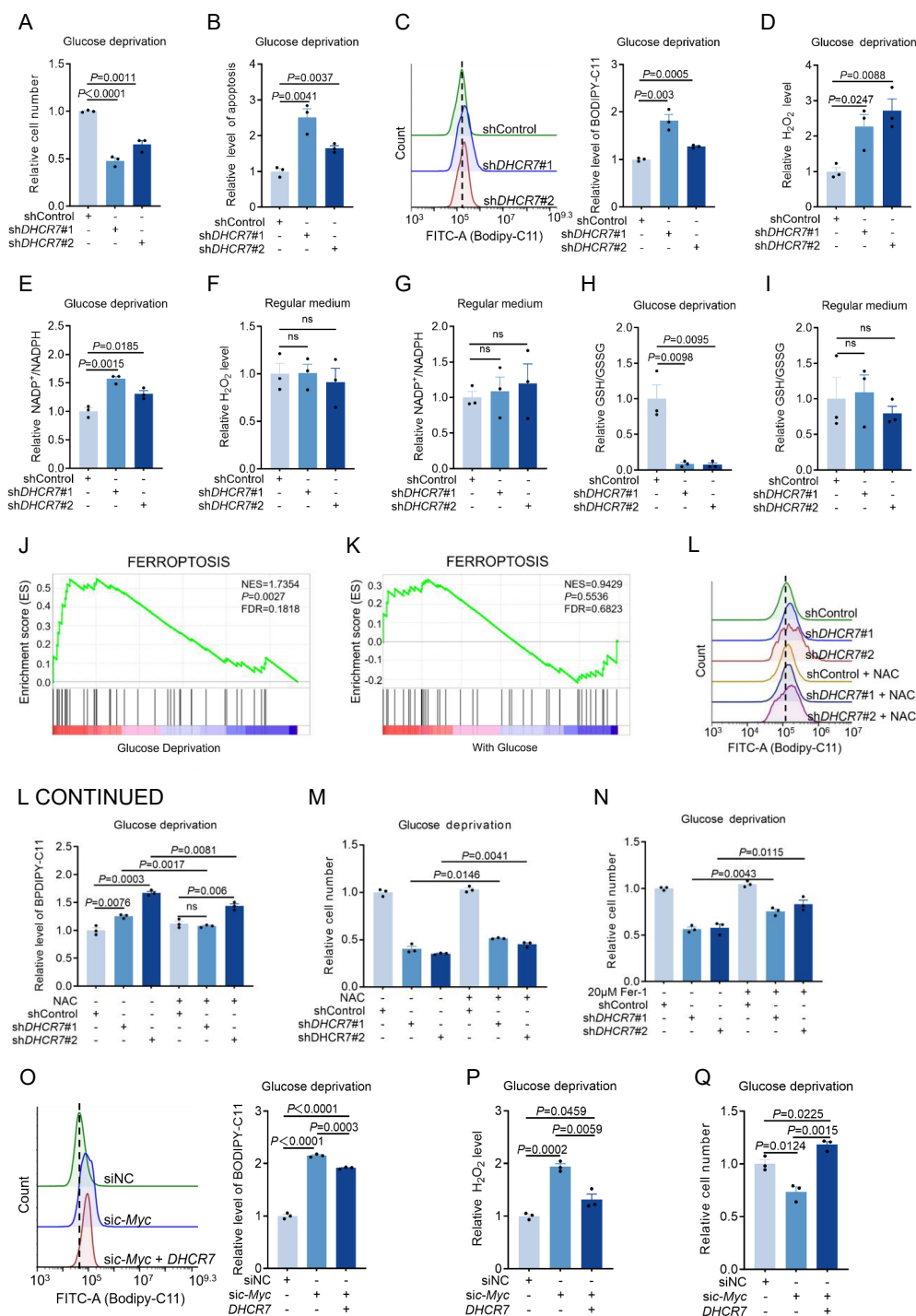


Figure 3. DHCRT7 protects PDAC cells from glucose deprivation-related lipid peroxidation and cell death (A) Relative survival of MIA PaCa-2 cells with or without *DHCRT7* depletion cultured in glucose-deprived medium for 24 h. (B,C) Apoptosis (B) and relative lipid peroxidation (C) levels of MIA PaCa-2 cells with or without *DHCRT7* depletion cultured in glucose-deprived medium for 24 h. (D–G) Relative H_2O_2 level and NADP⁺/NADPH ratio in MIA PaCa-2 cells with or without *DHCRT7* depletion, followed by 24 h glucose deprivation (D,E) or regular culture (F,G). (H,I) Relative GSH/GSSG ratio in MIA PaCa-2 cells with or without *DHCRT7* depletion, followed by 24 h of glucose deprivation (H) or regular culture (I). (J,K) Gene Set Enrichment Analysis of ferroptosis based on the KEGG database in MIA PaCa-2 cells with or without *DHCRT7* depletion ($n = 3$) and cultured either with (J) or without glucose deprivation (K). (L,M) Relative lipid peroxidation level (L) and relative survival (M) of MIA PaCa-2 cells cultured in glucose-deprived medium (24 h) with or without *DHCRT7* depletion and then with or without NAC (0.3 mM) supplementation. MIA PaCa-2 cells were pretreated with 1 mM NAC for 24 h before culture in glucose-deprived medium. (N) Relative surviving cell number of MIA PaCa-2 cells cultured in glucose-deprived medium (24 h) with or without *DHCRT7* depletion, followed by Fer-1 (20 μ M) supplementation. (O–Q) Relative lipid peroxidation level (O), H_2O_2 level (P), and survival (Q) of MIA PaCa-2 cells cultured in glucose-deprived medium (24 h) with or without *c-Myc* depletion, followed by *DHCRT7* overexpression in *c-Myc*-depleted cells. Data are presented as the mean $\pm$ SEM from at least three independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t* test. ns, not significant.

under glucose deprivation, at least partially via 7-DHC accumulation.

Untargeted metabolomic profiling showed that the 7-DHC level was significantly higher in *DHCR7*-depleted MIA PaCa-2 cells than in matched control cells with (approximately 5-fold increase) or without (approximately 6-fold increase) glucose deprivation (Supplementary Figure S5A,B). Exogenous 7-DHC supplementation not only mimicked the effect of *DHCR7* depletion-induced 7-DHC accumulation in MIA PaCa-2 cells (Figure 4A) but also exacerbated the death of cells cultured in glucose-deprived medium in a dose-dependent manner (Figure 4B). Notably, although cells treated with a low concentration of 7-DHC (10 μ M) did protect cells from glucose deprivation-induced cell death (Figure 4B), only a minimal increase in 7-DHC was detected in the cells (339 to 390 μ g/million cells; $\sim$ 15% increase) (Figure 4A), which was much lower than the 7-DHC increase caused by *DHCR7* depletion (Supplementary Figure S5A). Additionally, the lowest concentration of exogenous 7-DHC that can induce MIA PaCa-2 cell death under glucose-deprived conditions ($\sim$ 60 μ M) led to a similar accumulation of 7-DHC (approximately 6-fold) to that observed in cells with *DHCR7* depletion. Moreover, the toxic effect of 7-DHC was also observed in KPC cells cultured under glucose-deprived conditions (Figure 4C); however, limited and weaker cellular toxic effects were observed in two PDAC cells cultured with glucose-containing medium (Supplementary Figure S5C,D). Furthermore, treating MIA PaCa-2 cells with AY9944, a specific *DHCR7* inhibitor, mimicked the cell death-promoting role in cells cultured with glucose deprivation rather than glucose-containing medium (Figure 4D and Supplementary Figure S5E). In contrast, inhibiting 7-DHC generation from lathosterol by depleting *SC5D* in *DHCR7*-depleted MIA PaCa-2 cells (Figure 1F and Supplementary Figure S5F) partially restored the cell survival rate under glucose-deprived conditions (Figure 4E). Moreover, decreased 7-DHC levels (Figure 4F), reduced lipid peroxidation (Figure 4G), and increased cell survival (Figure 4H) were observed in MIA PaCa-2 cells overexpressing *DHCR7* compared with control cells cultured under glucose deprivation, which further indicates that 7-DHC is positively associated with ferroptosis under glucose deprivation conditions. Together, these data highlight that 7-DHC accumulation in *DHCR7*-depleted PDAC cells accelerates cell death under glucose deprivation.

As 7-DHC can be further oxidized into multiple types of oxysterols (Figure 4I) [43–45], it is reasonable that higher oxysterol levels were detected in *DHCR7*-depleted MIA PaCa-2 cells than in matched control cells with (Figure 4J,K) or without (Supplementary Figure S5G,H) glucose deprivation. Oxysterols, together with 7-DHC, are responsible for increased oxidative stress [46]; therefore, MIA PaCa-2 cells treated with oxysterols, including 7-ketocholesterol, a precursor of 7-hydroxycholesterol and 4-cholesten-3-one, had a lower cell survival rate than matched control cells cultured with glucose-deprived medium (Figure 4L,M). As observed in 7-DHC-treated cells, PDAC cells were less affected by oxysterols when cultured with glucose-containing medium (Supplementary Figure S5I,J). Collectively, our data demonstrate that c-Myc drives *DHCR7* expression to convert 7-DHC into cholesterol, thereby limiting oxysterol formation and shielding PDAC cells from death during nutrient stress, such as glucose deprivation.

7-DHC accelerates lipid peroxidation under glucose-deprived conditions

7-DHC promotes the generation of cellular reactive oxygen species

and increases the oxidation of membrane lipids [42]. Here, we systematically assessed lipid peroxidation signatures in PDAC cells treated with 7-DHC and cultured in either complete or glucose-deprived medium. Exogenous 7-DHC supplementation resulted in an increased NADP⁺/NADPH ratio (Figure 5A), decreased GSH/GSSG ratio (Figure 5B), and boosted lipid peroxidation in MIA PaCa-2 cells cultured with glucose-deprived medium (Figure 5C). Consistent results were also found in KPC cells treated with 7-DHC and cultured with glucose-deprived medium (Supplementary Figure S6A–C). In contrast, the NADP⁺/NADPH ratio, GSH/GSSG ratio, and lipid peroxidation were minimally affected by 7-DHC in both MIA PaCa-2 (Figure 5D–F) and KPC cells (Supplementary Figure S6D–F) cultured with glucose-containing medium. Exposure of MIA PaCa-2 cells to oxysterols generated from 7-DHC under glucose-deprived conditions likewise triggered lipid peroxidation (Figure 5G,H), whereas lipid peroxidation was less affected by those oxysterols in cells cultured in glucose-containing medium (Supplementary Figure S6G,H).

Two previous studies reported that 7-DHC protects cells from lipid peroxidation through its radical trapping role [47,48]. Although low doses of 7-DHC suppress ferroptosis and protect cells from death induced by RSL3, a ferroptosis activator (Supplementary Figure S7A), this protective effect was abolished when glucose was deprived in cells treated with RSL3, and even low doses of 7-DHC were sufficient to accelerate cell death (Supplementary Figure S7B). Moreover, increased cell death was observed when a cross-titration of 7-DHC and RSL3 was used in MIA PaCa-2 cells cultured in glucose-deprived medium (Supplementary Figure S7C). In cells cultured with RSL3 in glucose-deprived medium, lipid peroxidation increased following the exogenous addition of AY9944 (Figure 5I). In contrast, *DHCR7* overexpression protected cells from RSL3-induced lipid peroxidation when MIA PaCa-2 cells were cultured in glucose-deprived medium (Supplementary Figure S7D). To verify the effect of 7-DHC on key ferroptosis regulators, we examined the expression levels of glutathione peroxidase 4 (GPX4), acyl-CoA synthetase long-chain family member 4 (ACSL4), and ferroptosis suppressor protein 1 (FSP1) following *DHCR7* inhibition or 7-DHC treatment. Our results demonstrated that, similar to RSL3, *DHCR7* inhibition by AY9944 or 7-DHC treatment under glucose-deprived conditions upregulates the ferroptosis driver ACSL4 while down-regulating the ferroptosis inhibitors GPX4 and FSP1.

Notably, exogenous supplementation with an antioxidative compound partially inhibited lipid peroxidation and restored the cell survival rate in both MIA PaCa-2 (Figure 5J,K) and KPC cells (Figure 5L,M) treated with 7-DHC and cultured in glucose-deprived medium. Together, our results strongly indicate that 7-DHC and its oxysterol derivatives induce lipid peroxidation and may serve as anti-cancer metabolites in glucose-deficient PDAC cells.

Glucose limitation sensitizes cells to *DHCR7* inhibition-induced lipid peroxidation

A low-carbohydrate diet is a promising strategy for treating cancer cells sensitive to glucose limitation [49,50]. Because either 7-DHC accumulation or *DHCR7* inhibition selectively triggers PDAC cell death under glucose deprivation, we investigated whether this effect is tightly regulated by glucose availability. In MIA PaCa-2 cells, inhibiting *DHCR7* with AY9944 (Figure 6A,B) or shRNA-mediated *DHCR7* depletion (Figure 6C,D) led to arrested cell proliferation and increased lipid peroxidation, which was alleviated

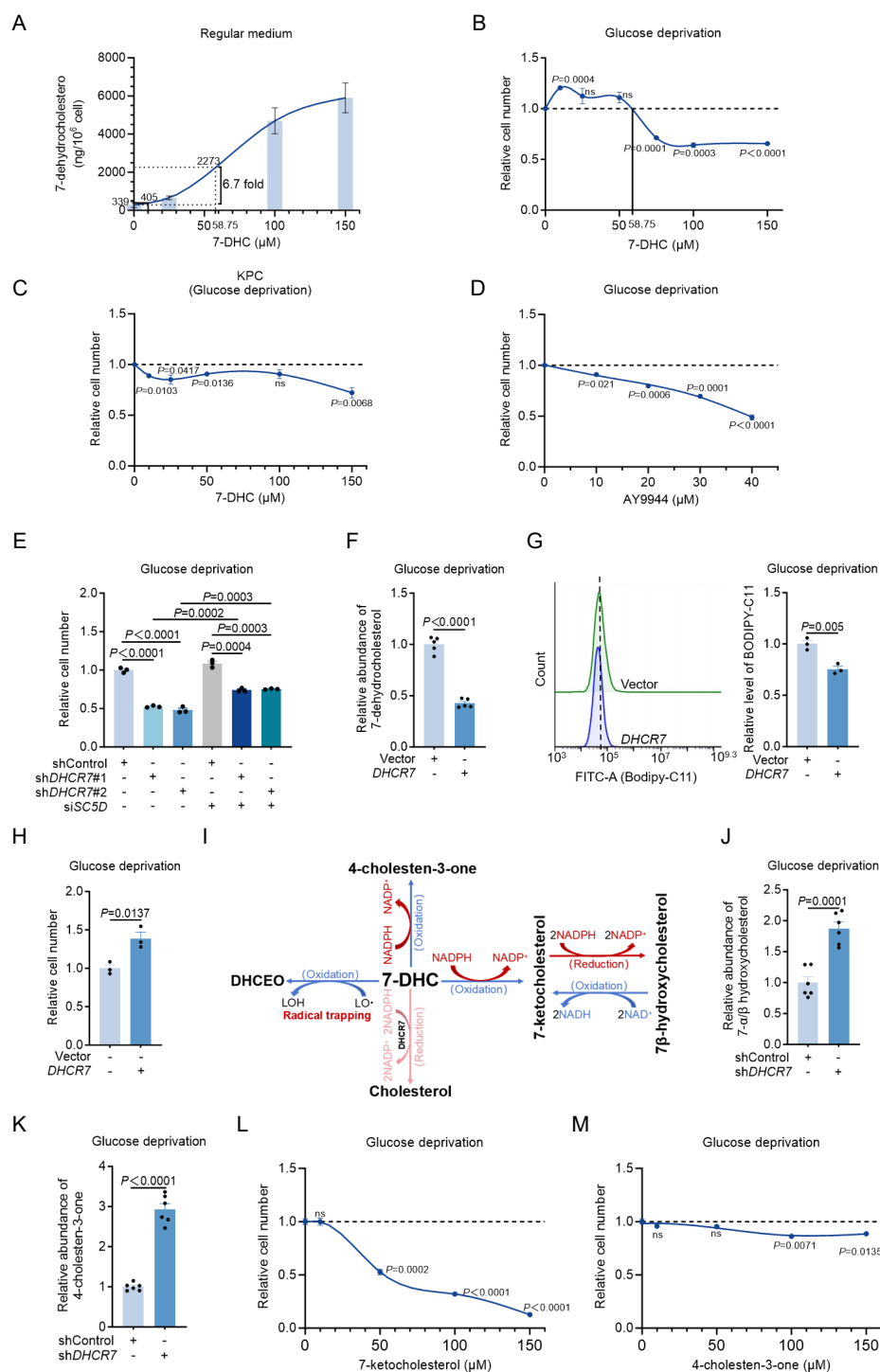


Figure 4. 7-DHC promotes PDAC cell death under glucose-deprived conditions (A) Absolute level of 7-DHC in MIA PaCa-2 cells treated with titrated exogenous 7-DHC, as indicated, for 72 h. The endogenous 7-DHC concentration curve according to the change in titrated exogenous 7-DHC was generated via Prism. (B,C) Relative survival of MIA PaCa-2 cells (B) and KPC cells (C) cultured in glucose-deprived medium (24 h for MIA PaCa-2 cells; 20 h for KPC cells) with 7-DHC treatment at the indicated concentrations. (D,E) Relative survival of MIA PaCa-2 cells cultured in glucose-deprived medium (24 h) with AY9944 treatment at the indicated concentrations (D) or with or without *DHCR7* depletion and then with or without *SC5D* depletion (E). (F–H) Relative abundance of 7-DHC (F) relative lipid peroxidation levels (G) and relative survival (H) of MIA PaCa-2 cells with or without *DHCR7* overexpression and cultured in glucose-deprived medium for 24 h. (I) Schematic diagram of 7-DHC metabolic pathways. (J,K) Relative levels of 7- α/β hydroxycholesterol (J) and 4-cholesten-3-one (K) in MIA PaCa-2 cells cultured in glucose-deprived medium (24 h) with or without *DHCR7* depletion. (L,M) Relative survival of MIA PaCa-2 cells cultured in glucose-deprived medium (24 h), with or without treatment with 7-ketocholesterol (L) or 4-cholesten-3-one (M) as indicated. Data are presented as the mean $\pm$ SEM from at least three independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t* test. ns, not significant.

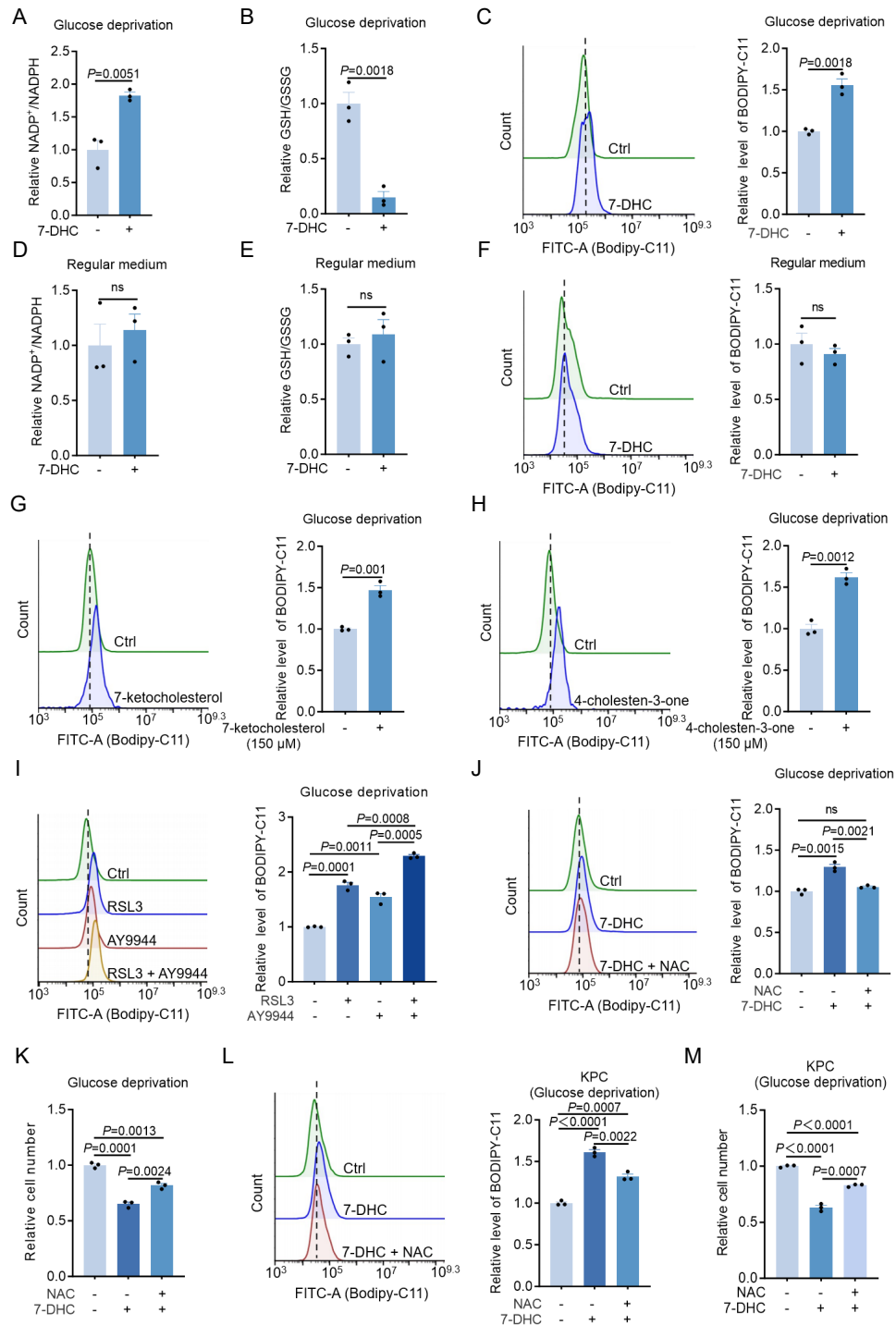


Figure 5. 7-DHC promotes lipid peroxidation-induced PDAC cell death under glucose-deprived conditions (A–C) Relative NADP⁺/NADPH ratio (A), GSH/GSSG ratio (B), and lipid peroxidation (C) in MIA PaCa-2 cells cultured in glucose-deprived medium (24 h), with or without 150 μ M 7-DHC treatment. (D–F) Relative NADP⁺/NADPH ratio (D), GSH/GSSG ratio (E), and lipid peroxidation (F) in MIA PaCa-2 cells with or without 150 μ M 7-DHC treatment for 24 h. (G,H) Relative levels of lipid peroxidation in MIA PaCa-2 cells cultured in glucose-deprived medium (24 h), with or without treatment with 7-ketocholesterol (G) or 4-cholesten-3-one (H) as indicated. (I,J) Relative levels of lipid peroxidation in MIA PaCa-2 cells cultured in glucose-deprived medium (24 h), with or without RSL3 treatment (10 μ M) then with or without treatment with AY9944 (30 μ M) (I) or 7-DHC (150 μ M) (J). (K) Relative cell survival of MIA PaCa-2 cells. Cells were cultured in glucose-deprived medium (24 h) with or without treatment with 7-DHC (150 μ M) and with co-treatment with NAC (0.3 mM) and 7-DHC (150 μ M). (L,M) Relative lipid peroxidation (L) and survival (M) of KPC cells cultured in glucose-deprived medium (20 h) with or without treatment with 7-DHC (150 μ M) and with co-treatment with NAC (0.3 mM) and 7-DHC (150 μ M). Data are presented as the mean $\pm$ SEM from at least three independent experiments. MIA PaCa-2 cells and KPC cells cultured under glucose-deprived conditions were seeded with 1×10^5 cells. Statistical significance was determined by unpaired two-tailed Student's *t* test. ns, not significant.

with increased glucose availability in the culture medium. Thus, we hypothesized that the tumor suppression effect of low-carbohydrate diets may be closely associated with DHCR7 activity. To test this hypothesis, we examined the tumor suppression effect of a low-carbohydrate diet in mice via the subcutaneous injection of MIA PaCa-2 cells with or without DHCR7 inhibition. Inhibiting DHCR7 function with either shRNA-mediated DHCR7 depletion or the DHCR7-specific inhibitor AY9944 led to suppressed tumor progression in mice fed a high- or low-carbohydrate diet (Figure 6E–G), indicating an overall tumor inhibition role of DHCR7 depletion in PDAC. Moreover, although the inhibition of tumor volume was not significantly affected by carbohydrate availability in DHCR7-inhibited tumors (Figure 6H), which was probably due to the difficulty of precise volume calculation, the tumor suppression effect represented by tumor weight was enhanced in DHCR7-inhibited PDAC cells fed a low-carbohydrate diet (Figure 6I). Accordingly, DHCR7 depletion in PDAC tumors under a low-carbohydrate diet led to an increase in the lipid peroxidation level, which was minimally affected in DHCR7-depleted tumors under a regular diet (Figure 6J). Together, these results strongly indicate that a combined low-carbohydrate diet and DHCR7 inhibition may be an effective treatment for PDAC.

Discussion

c-Myc, which is frequently activated during the development of PDAC, controls 10%–15% of genes in humans [51,52]. In addition to many downstream target genes involved in cell cycle and apoptosis regulation, c-Myc controls several essential metabolic pathways for cancer cell proliferation, such as glucose, glutamine, serine, and fatty acid metabolic pathways [53,54]. c-Myc regulates cholesterol biosynthesis by controlling the transcription of *SREBP1* [20]; however, a recent study revealed that c-Myc can also directly bind to the cholesterol biosynthesis gene *SQLE* [21]. Here, we revealed that DHCR7, a key enzyme for cholesterol biosynthesis from 7-DHC, is transcriptionally controlled by c-Myc. In PDAC tumors with sufficient glucose supplementation, c-Myc drives DHCR7 expression and accelerates endogenous cholesterol biosynthesis from 7-DHC, thereby supporting PDAC cell proliferation. Moreover, although a slight increase in 7-DHC protects cells from ferroptosis, PDAC cell survival, as measured by apoptosis and lipid peroxidation, is minimally affected by the rapid catabolism of 7-DHC because of the activation of c-Myc/DHCR7, even in conditions of induced ferroptosis. Conversely, in the PDAC tumor environment with limited glucose, activation of the c-Myc/DHCR7 axis promotes the catabolism of 7-DHC and diminishes the ferroptosis of PDAC cells induced by 7-DHC. Our results highlight the distinct role of 7-DHC in lipid peroxidation/ferroptosis in PDAC tumors, which is largely dependent on glucose availability in the tumor microenvironment (Figure 7).

Loss-of-function mutation in *DHCR7* was first described in Smith-Lemli-Opitz Syndrome [36]. In addition to an insufficient supply of cholesterol, which is essential for growth and development [55], Smith-Lemli-Opitz Syndrome is also attributed to 7-DHC accumulation, which induces cell death through elevated cellular oxidative stress [42,56,57]. In this study, although DHCR7-regulated endogenous cholesterol synthesis promoted PDAC cell growth, targeting endogenous cholesterol biosynthesis had minimal effects, whereas blocking cholesterol-enriched lipoprotein uptake appears to be a promising strategy for PDAC treatment [9,58]. This indicates

that PDAC may favor exogenous rather than endogenous cholesterol as major biosynthetic molecules for PDAC cell proliferation. However, the different impacts of exogenous and endogenous cholesterol on PDAC cell proliferation and survival require further *in vivo* investigation.

Metabolic intermediates and the regulation of endogenous cholesterol biosynthesis pathways, independent of the cholesterol biosynthesis role, are frequently associated with various types of cancers. One of the most studied mechanisms is 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and its downstream target, geranylgeranyl pyrophosphate (GGPP), which is essential for protein geranylgeranylation [59]. HMGCR blockage inhibits cholesterol biosynthesis; however, the anti-cancer role of HMGCR blockage is mediated by increased protein geranylgeranylation rather than impaired cholesterol biosynthesis [12,59]. We believe that protein geranylgeranylation may also be affected in PDAC cells with DHCR7 depletion because GGPP targets small GTPases for geranylgeranylation, and the small GTPase Rap1 pathway was altered in this study (Figure 2L) [60]. Moreover, SREBP1, a master regulator of cholesterol biosynthesis, can also be hijacked to boost the signaling pathways related to cancer development [30]. Thus, exogenous cholesterol supplementation can only partially restore PDAC cell proliferation if the endogenous cholesterol biosynthesis pathway is not adjusted, for example, through DHCR7 depletion. Nevertheless, the type of cholesterol biosynthetic intermediates and the mechanisms by which they alter cell proliferation-related signaling pathways remain unclear.

It has been reported that DHCR7 expression is significantly elevated in liver metastases compared to paired primary pancreatic cancer lesions [61]. Analysis of pancreatic cancer samples and matched normal tissues from the TCGA database revealed significantly elevated DHCR7 mRNA levels in tumors. Kaplan-Meier survival analysis with log-rank tests further demonstrated that high DHCR7 expression was associated with markedly reduced disease-free survival (DFS) and overall survival (OS) in pancreatic cancer patients. These findings identify DHCR7 as a potential prognostic biomarker and therapeutic target. Additionally, our experimental data demonstrate that DHCR7 alleviates RSL3-induced ferroptosis in glucose-starved PDAC cells, suggesting that DHCR7 may play a pivotal role in ferroptosis-mediated drug resistance.

Oncogenic amplification of genes, including *c-Myc*, has been shown to shape PDAC phenotypes. c-Myc is overexpressed in 42% of cases, primarily driven by KRAS activation, copy number gains, and high-level amplification [62]. As a downstream effector of c-Myc, DHCR7-mediated cholesterol metabolism is also activated in the majority of PDAC tumors. Notably, targeting DHCR7 may offer therapeutic benefits for patients with elevated c-Myc levels.

DHCR7 depletion regulates PI3K-AKT [17,63], possibly because the accumulation of 7-DHC, an upstream substrate of DHCR7, alters membrane stability [17]. 7-DHC was initially considered to induce oxidative stress and promote lipid peroxidation by increasing NADPH oxidase activity [42] or indirectly altering GPx and SOD activities [46]. However, recent studies have revealed that at low dosages, 7-DHC is an anti-ferroptosis/anti-lipid peroxidation metabolite because of its higher radical trapping activity than other membrane lipids [47,48,64,65]. Notably, these studies were performed in cells cultured with glucose. In this study, we confirmed that a low dose of exogenous 7-DHC (10–25 μ M)

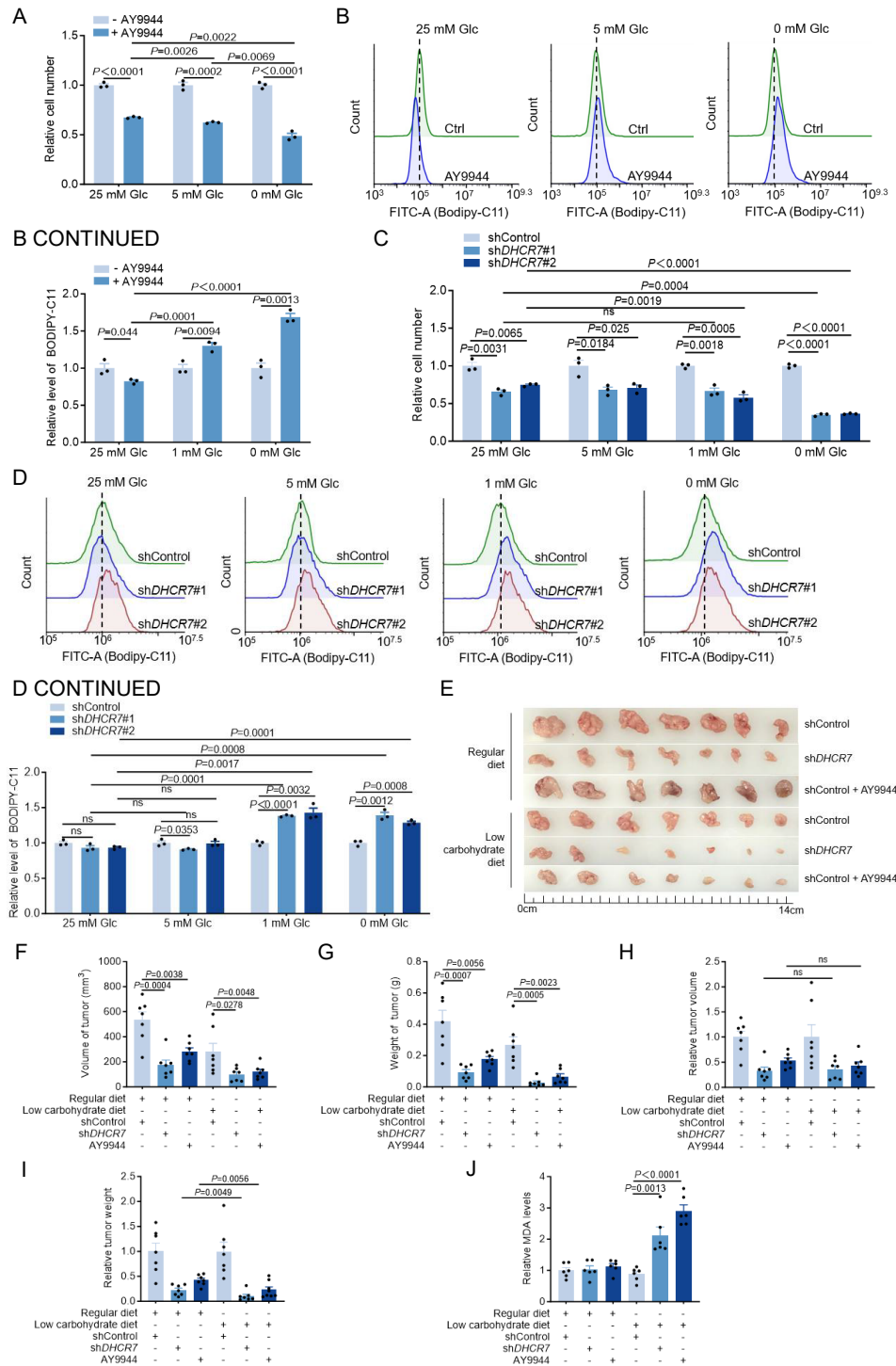


Figure 6. Glucose limitation sensitizes DHC7 inhibition-induced lipid peroxidation and tumor growth (A,B) Relative survival (A) and lipid peroxidation (B) of MIA PaCa-2 cells with or without AY9944 (30 μ M) treatment cultured in medium with the indicated titrated glucose concentrations (24 h). (C,D) Relative survival (C) and lipid peroxidation (D) of MIA PaCa-2 cells with or without DHC7 depletion cultured in medium with the indicated titrated glucose concentrations (24 h). (E–G) Images of PDAC tumors (E), tumor volumes (F), and tumor weights (G) from mice with subcutaneous injection of MIA PaCa-2 cells (5 $\times$ 10⁶ cells) with or without DHC7 depletion. After 2 weeks of subcutaneous tumorigenesis, mice maintained on either a regular or low carbohydrate diet were subjected to injections of AY9944 (25 mg/kg) every two days or were left untreated. Following a five-week observation period, the mice were humanely euthanized (n = 7 per group). (H,I) Relative tumor volumes (H) and tumor weights (I) in the flank regions from athymic nude mice with subcutaneous injection. (J) Relative malondialdehyde (MDA, cellular lipid peroxidation marker) content in tumors derived from (E). Data are presented as the mean $\pm$ SEM from at least three independent experiments. MIA PaCa-2 cells cultured under glucose-deprived conditions were seeded with 1×10^5 cells. Statistical significance was determined by unpaired two-tailed Student's t test.

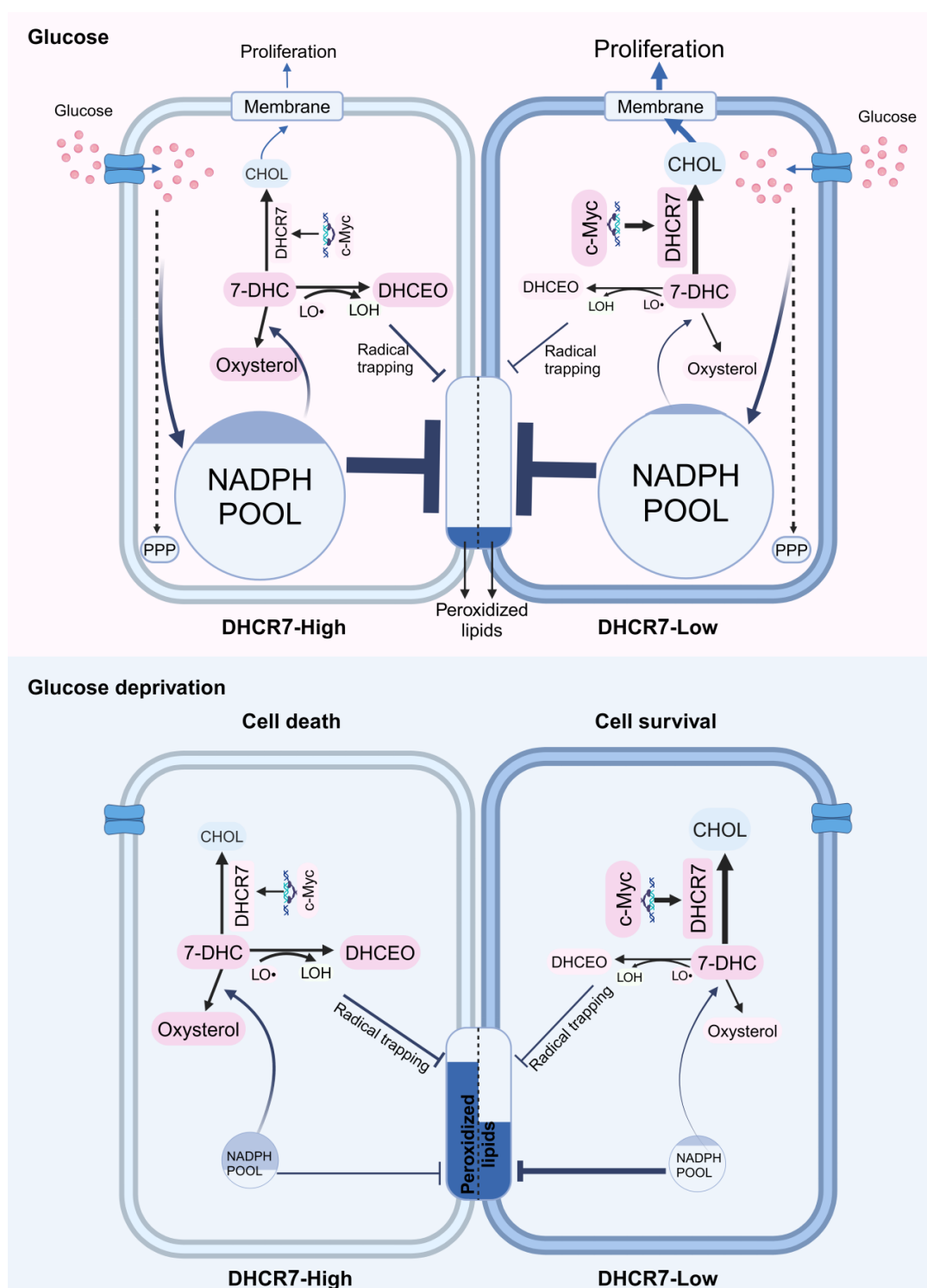


Figure 7. Schematic diagram depicting the mechanism by which c-Myc/DHCR7 regulates 7-DHC in cell survival and proliferation In a glucose-enriched tumor microenvironment, due to the large NADPH pool maintained by glucose, only a small proportion of NADPH is consumed by 7-DHC and its oxysterol derivatives in PDAC cells with both high and low levels of DHCR7. The anti-lipid peroxidation potential provided by 7-DHC-related radical trapping is negligible compared to the robust anti-oxidative NADPH pool. Therefore, increased cholesterol biosynthesis due to c-Myc-regulated DHCR7 upregulation supports PDAC cell proliferation (upper panel). In cells with a small NADPH pool due to low glucose supplementation, high levels of 7-DHC and its oxysterol derivatives lead to exhaustion of the NADPH pool, and the anti-lipid peroxidation potential provided by 7-DHC-related radical trapping is insufficient to overcome the pro-lipid peroxidation induced by NADPH exhaustion. Therefore, increased cholesterol biosynthesis from 7-DHC in PDAC cells with activated c-Myc/DHCR7 preserves the NADPH pool and protects cells from lipid peroxidation-induced cell death (lower panel). Images were created with a drawing software (BioRender).

protected PDAC cells from RSL3-induced lipid peroxidation and cell death in cells cultured with glucose. However, when PDAC cells were challenged with glucose deprivation, 7-DHC induced rather than suppressed ferroptosis. Therefore, we suggest that the opposing effect of 7-DHC in lipid peroxidation can be attributed to the involvement of glucose in other recent studies [47,48,64,65]. Although we agree that the oxidation of 7-DHC leads to radical trapping and protects the membrane lipid from attack by free radicals, the oxysterols derived from 7-DHC require further reduction via the oxidation of NADPH, such as 7-ketocholesterol to 7 β -hydroxycholesterol reduction [66]. These oxysterols are known for their cytotoxicity, which disrupts membrane fluidity and signaling pathways, causing cellular damage by increasing ROS levels [67–70]. We hypothesize that ferroptosis induced by 7-DHC accumulation is primarily driven by NADPH consumption and is subsequently amplified by oxysterol-mediated oxidative stress.

Beyond its role in metabolism and derivative generation, 7-DHC possesses additional functions. Notably, 7-DHC oxidation is catalyzed by the reduced form of CYP7A1, an enzyme that requires NADPH oxidation to maintain its reduced form from oxidized CYP7A1 [71–73]. Finally, 7-DHC itself is an inhibitor of GPx/SOD and an activator of NADPH oxidase; therefore, a robust antioxidative pool is required to buffer the rapid loss of NADPH due to 7-DHC accumulation. As the majority of cellular NADPH is generated from glucose via the pentose phosphate pathway [74], the oxidative challenge caused by a low dose of exogenous 7-DHC treatment can be efficiently cleared. Conversely, in cells lacking glucose, limited NADPH is hijacked by 7-DHC metabolism, and a minimal amount of NADPH is available for the anti-lipid peroxidation of membrane lipids. Our results therefore highlight that 7-DHC plays distinct roles in ferroptosis, depending on a glucose-rich or glucose-poor tumor environment (Figure 7). Previous research has shown that a low-carbohydrate diet is an efficient dietary perturbation in cancer treatment [49,75]; however, our results further highlight that patients with higher levels of 7-DHC, which is represented by lower DHCR7 expression, may gain greater benefits from a low-carbohydrate diet. Nevertheless, additional clinical translational studies are warranted to confirm these findings.

In summary, c-Myc/DHCR7 activation promotes PDAC cell proliferation under glucose-sufficient conditions, independent of cholesterol. However, under glucose deprivation, DHCR7 activation protects PDAC cells from ferroptosis by rapidly reducing 7-DHC levels.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Data Availability

The metabolomics data reported in this paper have been deposited in the China National Center for Bioinformation (CNCB) under accession number OMIX016316. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2025) in National Genomics Data Center (Nucleic Acids Res 2025), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA019122) that are publicly accessible at <https://ngdc.cncb.ac.cn/gsa-human>. All other data supporting the findings of this study, including raw data and original images, are available from the corresponding author upon

reasonable request.

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Author contributions

H.F., Y.Z., and M.J. conceived and designed the study. M.J., X.Z., X.R., S.X., K.H., Y.D., and X.Y. performed the experiments. M.J. and Z.Y. performed the metabolic analysis. X.Z. provided IHC analysis. X.Y. assisted in the transcriptional analysis. H.F., M.J., Y. Z., X.Z., and X.R., drafted the paper. M.J., X.Z., Z.Y., and X.R. analyzed and finalized the data. J.L. provided guidance on revising the article. All the authors have read and approved the paper.

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Conflict of Interest

The authors declare they have conflict of interest.

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