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FOXC1-mediated serine metabolism reprogramming enhances colorectal cancer growth and 5-FU resistance under serine restriction

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Abstract

Colorectal cancer (CRC) is the most common gastrointestinal malignancy, and 5-Fluorouracil (5-FU) is the principal chemotherapeutic drug used for its treatment. However, 5-FU resistance remains a significant challenge. Under stress conditions, tumor metabolic reprogramming influences 5-FU resistance. Serine metabolism plasticity is one of the crucial metabolic pathways influencing 5-FU resistance in CRC. However, the mechanisms by which CRC modulates serine metabolic reprogramming under serine-deprived conditions remain unknown. We found that exogenous serine deprivation enhanced the expression of serine synthesis pathway (SSP) genes, which in turn supported CRC cell growth and 5-FU resistance. Serine deprivation activate the ERK1/2-p-ELK1 signaling axis, leading to upregulated FOXC1 expression in CRC cells. Elevated FOXC1 emerged as a critical element, promoting the transcription of serine metabolism enzymes PHGDH, PSAT1, and PSPH, which in turn facilitated serine production, supporting CRC growth. Furthermore, through serine metabolism, FOXC1 influenced purine metabolism and DNA damage repair, thereby increasing 5-FU resistance. Consequently, combining dietary serine restriction with targeted therapy against the ERK1/2-pELK1-FOXC1 axis could be a highly effective strategy for treating CRC, enhancing the efficacy of 5-FU.

Keywords Colorectal cancer, 5-Fluorouracil resistance, De novo serine synthesis, FOXC1, Metabolic reprogramming

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Introduction

Colorectal cancer (CRC) is the third most common cancer in incidence and the second leading cause of cancer-related deaths worldwide [1, 2]. Due to a lack of early screening and detection, the majority of CRC patients are diagnosed at an advanced stage, leading to poor prognoses [3]. Therefore, comprehensive treatment strategies for CRC are increasingly emphasized in recent research [4]. In these strategies, surgical resection and adjuvant chemotherapy are key components, yet tumor development and chemotherapy resistance remain major challenges in CRC clinical treatment [5]. This emphasizes the critical need for innovative therapeutic approaches and detailed research into the mechanisms of drug resistance. In the treatment of advanced CRC, 5-Fluorouracil (5-FU) is a key component of the FOLFOX chemotherapy regimen [6]. The primary mechanism of action of 5-FU involves disrupting nucleotide biosynthesis by targeting and inhibiting thymidylate synthase (TS). This inhibition is pivotal because thymidylate synthase is responsible for the production of thymidine monophosphate, a nucleotide vital for DNA replication and repair [7, 8]. However, the resistance rate to 5-FU in the treatment of colorectal cancer remains high, highlighting the need to further explore the mechanisms behind its resistance [9, 10].

Metabolic reprogramming plays a critical role in both tumor growth and chemotherapy resistance [11–14], among which serine metabolic reprogramming is one of the salient features of tumor metabolism [15–19]. Furthermore, serine metabolism is highly correlated with the development of CRC [20]. Serine, a non-essential amino acid crucial for cellular metabolism, is sourced through exogenous uptake and the de novo synthesis pathway (SSP) [15, 16]. Under conditions of exogenous serine deprivation, approximately 70% of the serine in cells is derived from the SSP [21]. The SSP begins with the glycolytic intermediate 3-phosphoglycerate, undergoing transformations via SSP enzymes, 3-phosphoglycerate dehydrogenase (PHGDH), phosphoserine transaminase (PSAT1), and phosphoserine phosphatase (PSPH), to generate serine [15–19]. Serine plays a pivotal role in one-carbon metabolism, contributing significantly to various cellular processes including nucleotide synthesis, glutathione production, regulation of the NADPH/NADP+ratio, and maintaining cellular redox balance [15–19, 22]. Therefore, serine is implicated in several drug resistance mechanisms, such as the maintenance of intracellular antioxidant capacity [13, 23] and the facilitation of nucleotide biosynthesis to mitigate DNA damage responses [20]. Resistance to 5-FU in CRC cells is attributed to serine-driven compartmentalization of one-carbon metabolism within mitochondria, which supports purine biosynthesis and subsequently enhances DNA damage repair [6, 20, 24]. Additionally, serine contributes to tumor proliferation by facilitating the metabolism of one-carbon units, as observed in CRC [19, 25–28]. Thus, serine enhances both tumor growth and 5-FU resistance, suggesting that serine-targeting strategies could be an effective cancer treatment strategy.

Studies have shown that depriving CRC cells of exogenous serine can inhibit tumor growth and reduce resistance to 5-FU [20, 24, 29–31]. However, the clinical efficacy of merely restricting dietary serine is considered suboptimal, largely because external deprivation triggers a compensatory activation of endogenous serine synthesis [29, 31–33]. Research indicates that these compensatory mechanisms are associated with the activation of specific signaling pathways and enhanced transcriptional activity of key transcription factors [34–40]. Similarly, CRC cells upregulate serine biosynthesis enzymes in response to deprivation of exogenous serine [37], yet the compensatory mechanisms remain unexplored. Our study explores how CRC cells adapt to serine deprivation, emphasizing the key role of the FOXC1 transcription factor. We discovered that serine deprivation activates the ERK1/2-p-ELK1 pathway, leading to increased FOXC1 expression in CRC cells. This upregulated FOXC1 promotes the transcription of serine metabolism enzymes (PHGDH, PSAT1, PSPH), which supports CRC growth by enhancing serine production. Furthermore, FOXC1 affects purine metabolism and DNA damage repair, thereby increasing resistance to 5-FU. Thus, combining dietary serine restriction with targeted therapy against the ERK1/2-pELK1-FOXC1 pathway could improve the effectiveness of 5-FU in CRC treatment.

Materials and methods

Cell culture, cell transfection, and small chemical compounds

The CRC cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Yuuta, Shanghai, China) supplemented with 10% fetal bovine serum (ExCell Bio, Shanghai, China) and maintained at 37 °C in a humidified 5% CO_2 atmosphere. These cell lines were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (CAS), Shanghai, China. For experiments involving serine and glycine deprivation, the cells were cultured in a specialized medium that, similar to DMEM, lacked only serine and glycine (Biosharp, Cat. No. BL301A, Shanghai, China), and also supplemented with 10% fetal bovine serum (ExCell Bio, Cat. No. FSD500, Shanghai, China) under the same conditions of temperature and CO_2 concentration.

Transient transfection was performed using reagents from HighGene (Cat. No. RM09014, ABclonal, Wuhan, China). Small interfering RNAs (siRNAs) targeting

FOXC1, along with plasmids overexpressing FOXC1, were sourced from Gene Pharma (Shanghai, China). Similarly, siRNAs directed against PHGDH, PSAT1, and PSPH were obtained from Genomeditech (Shanghai, China). Lentiviral vectors, LV-Control and LV-shFOXC1, were acquired from Genechem Biotechnology (Shanghai, China). After transduction, cell lines exhibiting stable gene expression were selected and cultured in the presence of puromycin.

5-Fluorouracil (Cat. No. 343922), L-Serine (Cat. No. S4311), BI-D1870 (Cat. No. 559286) and U0126 (Cat. No. 662009) were obtained from Sigma-Aldrich (St. Louis, MO, USA), and NCT-503(Cat. No. Targetmol4213), was obtained from MedChemExpress (Monmouth Junction, NJ, USA)0.4-Fluoro-L-2-phenylglycin was purchased from Tokyo Chemical Industry (Cat. No. F0862, Japan).

Western blot (WB) analysis

Proteins were extracted from cultured cells and quantified using a Bradford assay. The proteins were separated by SDS-PAGE, transferred onto PVDF membranes (Cat. No. 3010040001, Millipore Corporation, Burlington, MA, USA), and probed with specific antibodies. Antibodies recognizing, PSAT1(Cat. No. PA5-22124, RRID: AB_11153526, Thermofishe, 1:2000), PSPH (Cat. No. A22763, ABclonal, 1:2000), PHGDH (Cat. No. A22129, ABclonal, 1:2000), FOXC1(Cat. No. ab227977, RRID: AB_2916124, Abcam, 1:1000), ERK1/2 (Cat. No. ab184699, RRID: AB_2802136, Abcam, 1:1000), phospho-ERK1/2(Cat. No. ab201015, RRID: AB_2934088, Abcam, 1:1000), RSK1(Cat. No. A4695, RRID: AB_2863326, ABclonal, 1:2000), phospho-RSK1(Cat. No. AP0539, RRID: AB_2771393, ABclonal, 1:2000), ELK1 (Cat. No. A19046, RRID: AB_2862539, ABclonal, 1:2000), phospho-ELK1(Cat. No. AP0033, RRID: AB_2771079, ABclonal, 1:3000), γH2AX (Cat. No. AP0687, RRID: AB_2863808, ABclonal, 1:2000) and β-actin (Cat. No. AC026, RRID: AB_2768234, Abclonal,1:10000).

RT-qPCR assays and bioinformatic analysis

Total RNA was extracted using TRIzol reagent (Cat. No. A33254, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The isolated RNAs were reverse transcribed into complementary DNAs using the PrimeScript RT reagent Kit (Code No. RR036A, Takara Bio, Tokyo, Japan). Quantitative real-time PCR (RT-qPCR) was subsequently performed using SYBR Green I (Cat. No. RR420A, Takara Bio). Primer sequences used are listed in Supplementary Table S1. Expression levels were normalized to β-actin as an internal control.

A comprehensive cancer genomics initiative, The Cancer Genome Atlas (TCGA), has provided molecular characterizations across 33 primary cancer types. For the analysis of FOXC1, ATF4, PHGDH, PSPH, and PSAT1 expression in colorectal cancer (CRC), data were extracted from the TCGA database and analyzed using the UALCAN platform (https://ualcan.path.uab.edu/analysis.html).

For RNA sequencing, SW1116 cells were cultured for 24 h in complete medium (Comp) or in a medium deprived of serine/glycine (Ser(-)). RNA was extracted, and library preparation for RNA-seq was conducted using 1 μg of high-quality RNA. Sequencing was performed on a HiSeq2500 platform (Illumina Inc., San Diego, CA, USA) at Genechem Biotechnology (Shanghai, China).

Serine and glycine measurement

Intracellular serine and glycine concentrations were quantified using the Serine Assay Kit and the Glycine Assay Kit, respectively, sourced from Mlbio (Cat. No. ml077310, Cat. No. Cat. No. ml077310, Shanghai, China). The assays were conducted with 100 μL of cell lysate in reaction buffer, prepared by the kit instructions. Fluorescence was subsequently measured in endpoint mode with a wavelength of 450 nm.

Cell counting Kit-8 (CCK-8) proliferation assay

To evaluate the proliferation of colorectal cancer (CRC) cells, Cell Counting Kit-8 (CCK-8) assays were conducted using kits provided by Dojindo Laboratories (Cat. No. CK04, Kumamoto, Japan). Briefly, CRC cells were seeded at a density of 2×10^3 cells per well in 96-well plates and cultured for durations of either 0–96 h or 0–144 h. Subsequently, each well received 10 μL of CCK-8 reagent mixed with 90 μL of DMEM. Following a one-hour incubation period, the absorbance at 450 nm was measured using a microplate reader. The experiments were performed in triplicate, and data analysis was conducted using GraphPad Prism software.

EdU Staining

The EdU incorporation assay was performed using the EdU Kit (Cat. No. ST067, Beyotime, Jiangsu, China) to assess cell proliferation. Cells were plated at a density of 5×10^5 cells per well in 24-well culture plates 24 h before the assay. After treatment with 50 μM EdU for 2 h, cells were fixed, permeabilized, and subsequently stained following the manufacturer's instructions. The assay was conducted in triplicate with three independent experiments.

Intracellular GSH detection

Intracellular glutathione (GSH) levels were measured using a GSH and GSSG Assay Kit (Cat. No. S0053, Beyotime, Jiangsu, China). Cells were lysed through two cycles

of freezing and thawing, followed by centrifugation to collect the supernatant. The quantification of GSH and GSSG was then conducted according to the manufacturer's protocol.

Measurement of the NADPH/NADP + ratio and ROS levels

The NADPH/NADP + ratio was determined using a NADP/NADPH Quantification Colorimetric Kit (Cat. No. S0179, Beyotime, Jiangsu, China). For this assay, cell lysates were mixed with 800 μL of reaction buffer, prepared according to the kit instructions, and the absorbance was measured at 450 nm at room temperature in a 96-well plate. Additionally, reactive oxygen species (ROS) levels were quantified using a ROS Detection Assay Kit (Cat. No. S0033S, Beyotime, Jiangsu, China), with the procedure conducted as per the manufacturer's guidelines.

Chromatin immunoprecipitation (ChIP) assay

The Chromatin Immunoprecipitation (ChIP) assay was performed using a kit from Upstate Biotechnology, provided by Thermo Fisher Scientific (Waltham, MA, USA). Cells were first cross-linked with 1% formaldehyde and 1.5 mmol/L ethylene glycol-bis at room temperature. The cross-linked chromatin was then sonicated and immunoprecipitated using antibodies specific to various transcription factors. Quantitative real-time PCR (qPCR) was conducted to quantify the amount of DNA bound, employing primers specifically designed to target the FOXC1 binding sites within the promoter regions of the PSAT1, PSPH, and PHGDH genes. Additionally, an experimental group targeting ELK1 binding to FOXC1 was included to explore interaction effects.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was performed as follows: Tissue sections were fixed, embedded, sectioned, and deparaffinized. Blocking was achieved using 3% hydrogen peroxide and 5% bovine serum albumin (BSA). Subsequently, sections were incubated with primary antibodies against Ki-67(Cat. No. A11390, ABclonal, 1:200) and γH2AX(Cat. No. AP0687, ABclonal, 1:200) overnight at 4 °C. After washing with phosphate-buffered saline (PBS), sections were treated with an immunohistochemical secondary antibody for 1 h at room temperature. This was followed by diaminobenzidine (DAB) staining, counterstaining with hematoxylin, and imaging.

Apoptosis assay

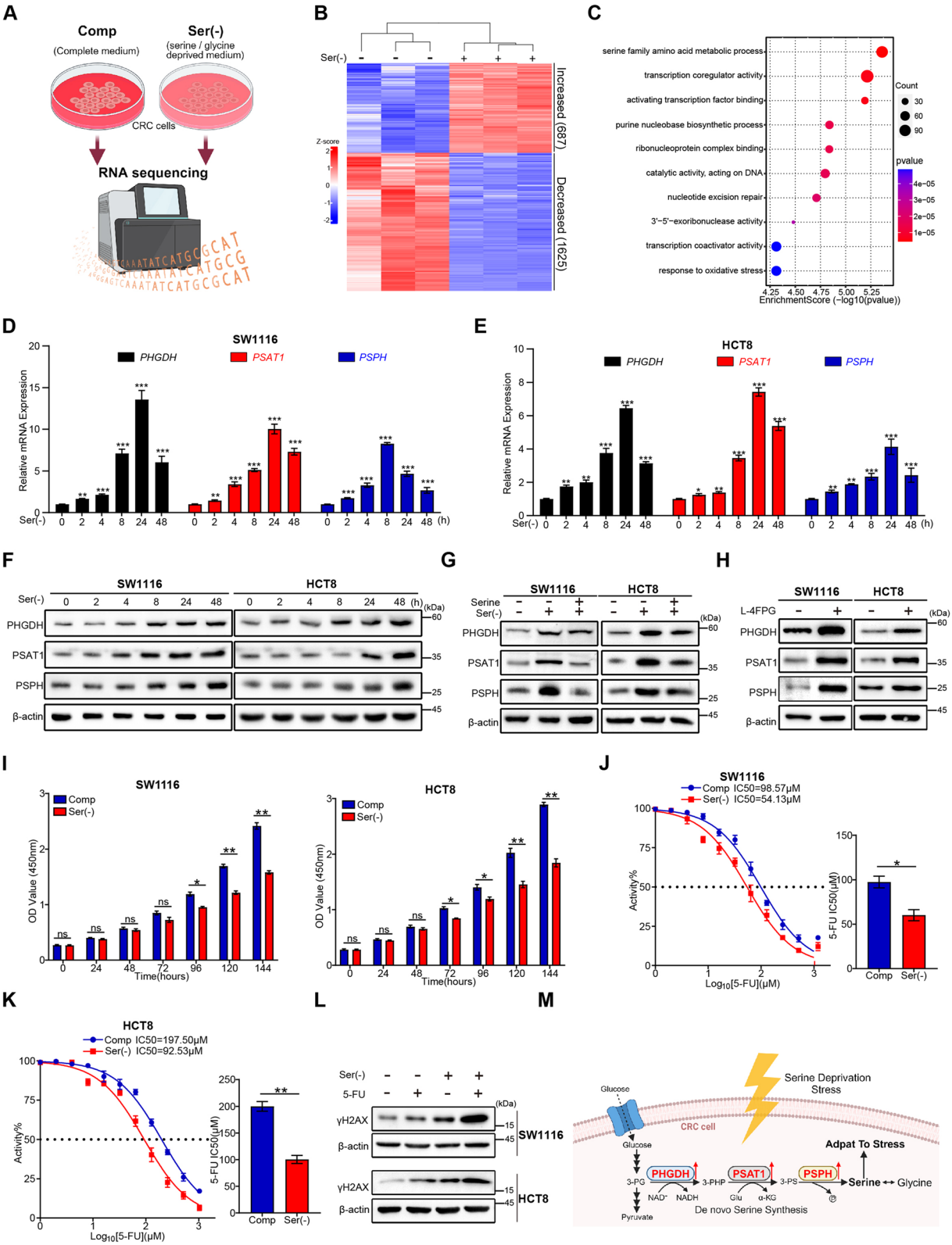
Apoptosis was assessed using Annexin V-FITC/PI staining followed by flow cytometry analysis. Cells were harvested, washed twice with cold phosphate-buffered saline (PBS), and resuspended in 1x binding buffer at a concentration of 1×10^6 cells per mL. Annexin V-FITC (1:100) and propidium iodide (PI) (1:100) from Dojindo (Kumamoto, Japan) were added to the cell suspension, followed by incubation for 15 min at room temperature in the dark. After incubation, 400 μL of 1x binding buffer was added, and apoptosis was analyzed immediately by flow cytometry.The percentage of apoptotic cells was determined by gating for early apoptotic cells (Annexin V-FITC+/PI-) and late apoptotic/necrotic cells (Annexin V-FITC+/PI+). Flow cytometric data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

53BP1 foci formation assay

Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100, and blocked with 5% normal goat serum for 1 h. Cells were then incubated overnight with a primary antibody against 53BP1 (1:500, Abcam, Cat. No. ab175933) at 4 °C. After washing, cells were incubated with an Alexa Fluor 568-conjugated secondary antibody (1:1000, Thermofisher, Cat. No A-11004) for 1 h in the dark, and nuclei were stained with DAPI (1 μg/mL, Beyotime, Cat. No.C1006) for 5 min. Images were captured using a Zeiss Axio Observer fluorescence microscope (Zeiss, Germany), and 53BP1 foci were quantified using ImageJ software.

(See figure on next page.)

Fig. 1 Serine metabolic enzymes are upregulated in response to serine deprivation stress in colorectal cancer cells to enhance 5-FU resistance. **A** SW1116 cells were cultured for 24 h in complete medium (Comp) or serine/glycine-deprived medium (Ser(-)), followed by RNA sequencing. **B** Heatmap showing differentially expressed genes (DEGs) in SW1116 cells under serine/glycine deprivation versus complete culture. **C** Bubble chart of GO enrichment analysis of DEGs under serine/glycine deprivation. **D-E** CRC cell lines cultured in serine/glycine-deprived medium for various durations; expression of SSP genes analyzed by qRT-PCR. **F** Immunoblotting of PHGDH, PSAT1, and PSPH proteins in CRC cell lines at 0, 2, 4, 8, 24, and 48 h of serine/glycine deprivation. **G** CRC cell lines cultured in serine/glycine-deprived medium for 24 h, with or without exogenous serine (200μM), followed by immunoblotting. **H** CRC cell lines cultured in complete medium for 24 h, with or without serine transporter inhibitor (4-Fluoro-L-2-phenylglycine; 50μM), followed by immunoblotting. **I** CCK8 assay evaluating daily viability of CRC cell lines over 6 days in serine/glycine-deprived or complete medium. **J-K** CCK8 results showing cell viability of CRC cell lines in serine/glycine-deprived or complete medium exposed to a gradient of 5-FU concentrations, with IC50 values after 24 h treatment. **L** CRC cell lines cultured for 24 h in serine/glycine-deprived or complete medium, treated with or without 5-FU, followed by immunoblotting. **M** Diagram illustrating activation of de novo serine biosynthesis pathway from glycolysis in response to serine deprivation. 3-PG, 3-phosphoglycerate; 3-PHP, 3-phosphohydroxypyruvate; 3-PS, phosphoserine. Data are mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$



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Phospho-kinase antibody array

The Human Phospho-Kinase Array Kit (Cat. No. ARY003B) from R&D Systems (Minneapolis, Minnesota, USA) was utilized to identify potential kinase activities. This array includes 43 kinase phosphorylation sites and two related total proteins. Cell lysate samples were diluted according to the manufacturer's instructions and incubated with the array membranes overnight at 4 °C. Following incubation, the membranes were washed to remove unbound proteins and then incubated with a cocktail of biotinylated detection antibodies for 2 h at room temperature. Streptavidin-horseradish peroxidase was subsequently added. The detection of kinase activity was performed using chemiluminescent detection reagents and imaged with an ImageQuant LAS 4000 analyzer (GE Healthcare, Pittsburgh, Pennsylvania, USA).

Xenograft assay

The Animal Protection and Use Committee of Tongji University approved all protocols for the animal experiments described in this study. Forty female NOD scid gamma (NSG) mice, aged 4 weeks, were used. These mice were subcutaneously injected in the right abdomen with SW1116 cells that had been engineered to express either control shRNA (shCtrl), shRNA targeting FOXC1 (shFOXC1), or control shRNA with U0126 treatment (shCtrl + U0126), provided by Changzhou Cavens Model Animal Co., Ltd. The U0126 was administered intraperitoneally at a dose of 10.5 mg/kg daily. Once tumors were established, the mice were divided into two dietary groups: one receiving a normal control diet and the other a serine/glycine-deprived diet (Ser(-) diet). Three days after dietary modification, treatments commenced. Mice received either no drug treatment or were treated with 5-FU (40 mg/kg) following a cyclic regimen of three consecutive daily injections and four days of recovery. After 4 weeks, mice were euthanized, and tumors were excised for measurement and weighing. A portion of each tumor was fixed in 10% paraformaldehyde, paraffin-embedded for immunohistochemical analysis, and the remainder was used for protein extraction and serine level assessment.

Statistical analysis

Differences between groups were calculated using Student's t-test, one-way analysis of variance, Chi-squared test, or Fisher's exact test. GraphPad Prism version 8.0 was used for all statistical analyses. Data are presented as mean ± SD. NS: not significant. Significance is defined as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

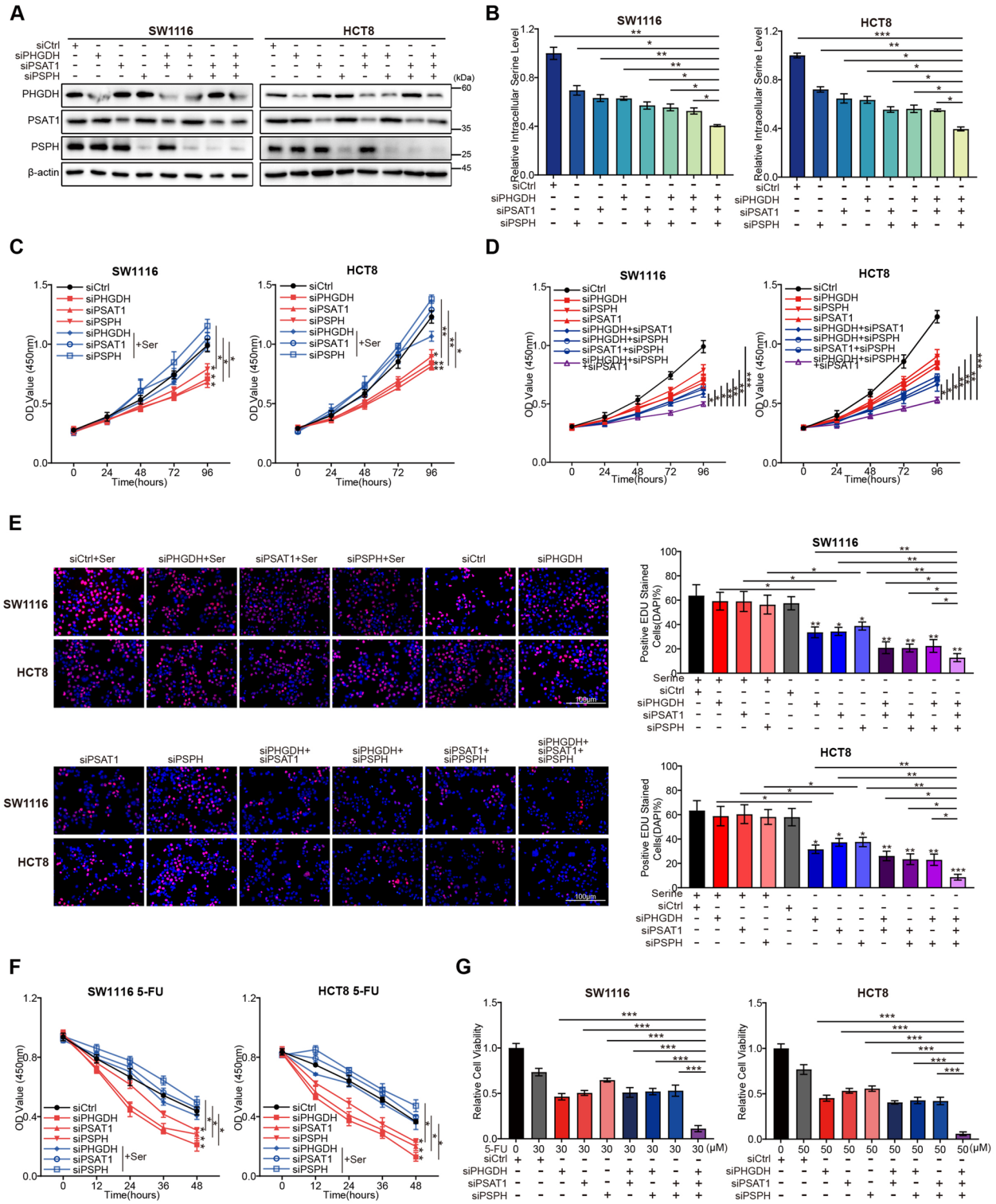
Results

Serine metabolic enzymes are upregulated in response to serine deprivation stress in colorectal cancer cells to enhance 5-FU resistance

Serine is a crucial amino acid in tumors, and serine metabolism is vital for tumor growth [15–19]. To investigate whether CRC cells exhibit altered gene expression profiles under limited serine and glycine conditions, we subjected complete medium and serine/glycine medium deprived SW1116 CRC cells to high-throughput RNA sequencing (Fig. 1A). A total of 23,924 genes were detected, with 2,312 differentially expressed genes. Among these, 687 genes were upregulated, and 1,625 were downregulated in response to serine deprivation (Fig. 1B). GO analysis revealed significant enrichment in processes such as serine family amino acid metabolism, transcription coregulator activity, and response to oxidative stress. DEGs were notably associated with purine metabolism and nucleotide excision repair pathways (Fig. 1C). In our experiments, we noted that SSP genes (*PHGDH*, *PSAT1*, *PSPH*) were highly expressed in CRC cell lines, with expression levels further increasing under conditions of serine/glycine deprivation (Fig. S1A-D). The SW1116 and HCT8 cell lines showed the most significant increases in expression, leading to their use in further studies (Fig. S1B-D). Thus, we further explored that serine/glycine deprivation markedly enhances both mRNA and protein expression levels of SSP genes, with mRNA expression peaking at 24 h, and protein levels reaching their maximum at 48 h (Fig. 1D-F, Fig. S1E and

(See figure on next page.)

Fig. 2 SSP gene influences CRC cell growth and 5-FU resistance under serine-deprived conditions. **A** Immunoblotting was conducted to verify the efficiency of individual SSP genes (PHGDH, PSAT1, PSPH), dual SSP gene combinations, and triple SSP gene knockdown in CRC cell lines. **B** Analysis of relative intracellular serine levels in CRC cell lines following knockdown of individual SSP genes (PHGDH, PSAT1, PSPH), dual combinations, complete depletion, and control, when cultured in serine/glycine-deprived medium. **C** CCK8 assay assessing cell proliferation in CRC cell lines with SSP gene depletion, SSP gene depletion plus serine supplementation (+ Ser), and control, all cultured in serine/glycine-deprived medium. **D** and **E** CCK8 (D) and Edu (**E**) assays assessing cell proliferation in CRC cell lines following SSP gene knockdown in serine/glycine-deprived medium. The merged image shows EdU + cells in magenta, indicating proliferating cells, with nuclei stained in blue (DAPI). **F** CCK8 assays evaluating the viability of CRC cell lines with SSP gene depletion, SSP genes-depleted plus serine supplementation (+ Ser), and controls, cultured in serine/glycine-deprived medium with treatment with 5-FU. **G** Cell viability was evaluated in CRC cells with knockdown of individual SSP genes (PHGDH, PSAT1, PSPH), dual combinations, complete depletion, and control, all cultured in serine/glycine-deprived medium with and without treatment with 5-FU for 24 h. Data are presented as mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$



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F). This elevation was reversible upon the re-addition of serine (400μM) [41, 42] to the serine/glycine -deprived CRC cells (Fig. 1G). Additionally, inhibition of serine uptake through treatment with 4-fluoro-L-2-phenylglycine (L-4FPG, a serine transport inhibitor) also enhanced the protein expression levels of SSP enzymes in CRC cells (Fig. 1H).

Previous studies have shown that serine/glycine deprivation inhibits CRC cell growth [20]. However, we were surprised to find that short-term deprivation (within 48 h) does not significantly affect CRC cell proliferation, while prolonged deprivation (beyond 72 h) substantially reduces it (Fig. 1I). Serine biosynthesis is known to influence purine synthesis and DNA repair, thereby affecting chemoresistance to 5-FU [6, 20, 24]. RNA sequencing results have also shown that serine/glycine deprivation impacts these processes (Fig. 1C). In our experiments, we observed that serine/glycine deprivation in CRC cells leads to an increase in γH2AX (phosphorylated histone 2AX, a marker of double-stranded DNA breaks [43]) (Fig. 1L) indicating DNA damage accumulation. We also performed gradient experiments and found that γH2AX levels continue to increase over time under serine/glycine deprivation (Fig. S1G). Serine deprivation also significantly decreases the IC50 for 5-FU (Fig. 1J and K). Collectively, these results demonstrate that serine deprivation initiates SSP enzymes such as PHGDH, PSAT1, and PSPH, which in turn enhances 5-FU resistance and proliferation in CRC cells (Fig. 1M).

SSP gene influences CRC cell 5-FU resistance and growth under serine-deprived conditions

To explore the influence of SSP enzymes on the 5-FU resistance and proliferation of CRC cells, we utilized targeted silencing of these genes using specific small interfering RNA in CRC cells (Fig. S2A-D and Fig. 2A) Silencing individual SSP genes, as well as simultaneous knockdown of two or three SSP genes, led to a notable decrease in intracellular serine levels under serine/glycine deprivation, with the greatest reduction observed upon silencing all three genes (Fig. 2B). Following CCK8 and EdU assays demonstrated that silencing each SSP gene individually decreased CRC cell proliferation under serine/glycine-deprived conditions, and serine supplementation restored proliferation (Fig. 2C-E). Notably, concurrent silencing of all three SSP genes produced the most substantial reduction in cell proliferation compared to silencing one or two genes alone (Fig. 2D and E). Further, CRC cells treated with 5-FU at half the IC50 for 24 h exhibited significantly reduced viability upon individual SSP gene silencing under serine/glycine deprivation. This reduction in drug-induced cell viability was reversed by serine supplementation (Fig. 2F). Silencing all three SSP genes under serine/glycine deprivation and 5-FU treatment (half IC50 for 24 h) further decreased cell viability compared to single or dual gene silencing (Fig. 2G).

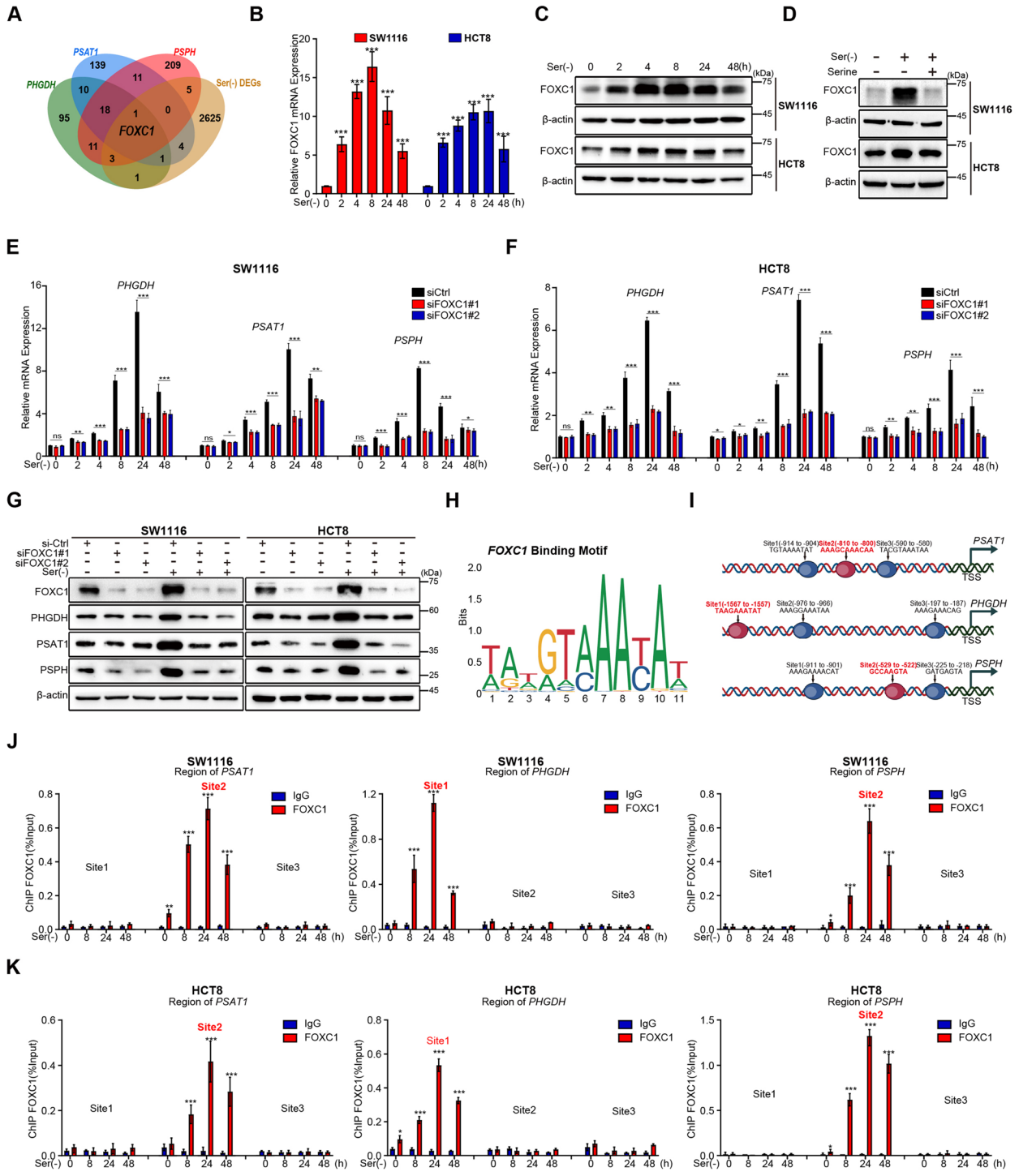
NCT-503, a commonly used inhibitor of serine synthase activity [44], resulted in a significant reduction in the intracellular serine levels of CRC cells through decreased enzymatic activity of PHGDH during serine/glycine deprivation (Fig. S2E), without significant changes in the expression levels of PHGDH, PSAT1, or PSPH (Fig. S2F). CCK8 assays demonstrated that treatment with 5 mM NCT-503 significantly reduced CRC cell proliferation under serine/glycine-deprived conditions, with serine supplementation restoring proliferation (Fig. S2G). Additionally, treatment with 5-FU at half the IC50 concentration for 24 h significantly reduced cell viability in CRC cells treated with NCT-503, compared to the control group under serine/glycine deprivation. When serine was replenished, the differences in drug-induced cell viability between the NCT-503-treated and control groups disappeared (Fig. S2H).

FOXC1 activates the transcription of SSP genes in response to serine deprivation

To elucidate the mechanism driving SSP gene upregulation in response to serine deprivation, GO analysis identified multiple transcription factor activity-related entries among the top ten enriched categories under serine/

(See figure on next page.)

Fig. 3 FOXC1 activates the transcription of SSP genes in response to serine deprivation. **A** Venn diagram depicting the intersection between potential transcription factors predicted to regulate SSP genes and differentially expressed genes (DEGs) in CRC cell lines under conditions of either serine/glycine deprivation or complete culture. **B** and **C** CRC cell lines were cultured in a serine/glycine-deprived medium for varying durations. Quantitative real-time PCR (B) and Immunoblotting (C) were utilized to analyze the expression of FOXC1 using specific primers and targeted antibodies. **D** CRC cell lines were cultured in serine/glycine-deprived medium for 24 h, with or without the addition of exogenous serine (200 M) followed by Western blot analysis. **E-G** Indicated cells with or without the depletion of FOXC1 were cultured with serine/glycine-deprived or complete medium. Quantitative realtime PCR (E-F) and immunoblotting (G) analyses were performed with the indicated primers and antibodies respectively. **H** The predicted FOXC1 binding motif according to JASPAR. **I** Schematic diagram of potential FOXC1 binding sites in the promoter region of SSP genes. **J** and **K** CRC cell lines cultured in the serine/glycine-deprived medium for varying durations were subjected to ChIP assays using immunoglobulin G (IgG) or the FOXC1 antibody. The amounts of precipitated DNA were quantified by qPCR using primers amplifying the regions indicated in (I). Data are presented as mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$



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glycine-deprived conditions (Fig. 1B). Using the JASPAR transcription factor database and intersecting with differentially expressed genes, we identified FOXC1 as a transcription factor of interest (Fig. 3A). FOXC1 is well-documented for its role in various cellular processes, including metabolic reprogramming [45]. Consistently, serine/glycine deprivation caused a dramatic increase in both the protein and the mRNA level of FOXC1, suggesting that serine deprivation induced FOXC1 expression at the transcription level (Fig. 3B and C). Notably, FOXC1 protein expression was reversed after 24 h of serine/glycine deprivation followed by 48 h of serine repletion (Fig. 3D), and similar upregulation was observed with L-4FPG treatment in CRC cells (Fig. S3A).

To assess FOXC1's regulatory impact on serine/glycine-deprivation-activated genes, we employed FOXC1-specific siRNA, which led to a significant reduction in SSP gene mRNA levels, particularly at 24 h post-serine deprivation (Fig. 3E and F). Furthermore, silencing FOXC1 decreased, while overexpressing FOXC1 increased SSP enzyme protein levels under serine/glycine deprivation, with this regulatory effect being much more pronounced compared to complete medium conditions. (Fig. 3G and Fig. S3A). To establish FOXC1's direct regulatory role on SSP genes, JASPAR analysis predicted FOXC1 binding sites within *PHGDH*, *PSAT1*, and *PSPH* promoter regions (Fig. 3H). Focusing on the three highest-scoring sites (Fig. 3I), we confirmed their existence within the promoter regions of *PHGDH*, *PSAT1*, and *PSPH*. Chromatin immunoprecipitation (ChIP) assays using an anti-FOXC1 antibody indicated that serine deprivation significantly increases FOXC1's binding to these sites in the promoter regions of SSP genes in CRC cells (Fig. 3J and K).

Regarding the transcription factor ATF4, it has been previously reported as capable of regulating the transcription of SSP genes [34, 35]. In our experiments, we observed that under serine/glycine deprivation, ATF4 expression is significantly upregulated at both the RNA and protein levels (Fig. S3C-D). Additionally, ATF4 appears to play a regulatory role in the expression of serine metabolic enzymes under serine deprivation (Fig. S3E-F). However, our analysis of data from TCGA and GEO reveals that the expression level of ATF4 is not associated with CRC (Fig. S3G and H) incidence or prognosis. (Fig. S3I) Moreover, under conditions of serine/glycine deprivation, we determined through real-time PCR and immunoblotting that there is no regulatory relationship between FOXC1 and ATF4(Fig. S3J-M).

FOXC1 influences CRC cell 5-FU resistance and growth under serine-deprived conditions

To elucidate the role of FOXC1 in modulating 5-FU resistance and cell proliferation in CRC cells, we employed FOXC1 downregulation using targeted siRNA and upregulation through a FOXC1 overexpression plasmid (Fig. 4A and B). FOXC1 silencing decreased intracellular serine levels, while FOXC1 overexpression significantly enhanced serine levels under serine/glycine deprivation (Fig. 4D). We observed an elevation in γH2AX, upon silencing FOXC1 (Fig. 4C). To further investigate the DNA damage response, we performed a 53BP1 recruitment assay and found significantly increased 53BP1 foci in FOXC1-silenced cells under serine/glycine deprivation (Fig. S4A). To explore the impact of FOXC1 silencing on cell death, we conducted apoptosis assays using Annexin V-FITC/PI staining, followed by flow cytometry analysis. These results (Fig. S4E) showed significantly enhanced apoptosis in FOXC1-silenced cells under serine/glycine deprivation, particularly when combined with 5-FU treatment. Furthermore, treatment with 5-FU at half the IC50 concentration for 24 h showed a marked reduction in cell viability in FOXC1-silenced cells under serine/glycine deprivation, with serine repletion mitigating this effect (Fig. 4E). Following this, we developed a shRNA construct for the stable silencing of FOXC1 expression (Fig. S4B-C). Employing

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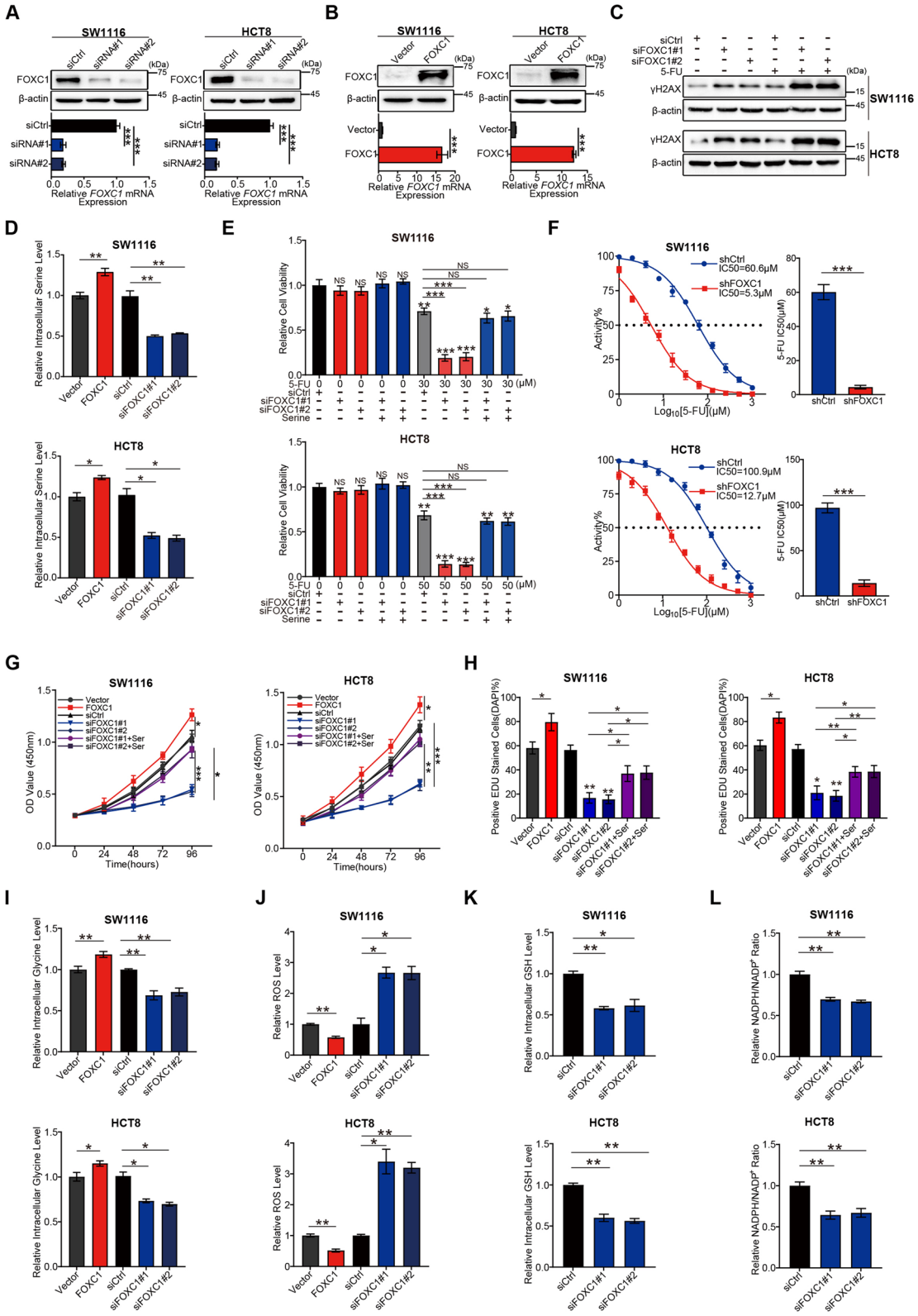
Fig. 4 FOXC1 influences CRC cell growth and 5-FU resistance under serine-deprived conditions. **A** and **B** Efficiency of FOXC1 silencing (A) and overexpression (B) in CRC cell lines was confirmed via quantitative real-time PCR and Western blotting using specific primers and antibodies. **C** CRC cell lines with FOXC1 knockdown and control were cultured under serine/glycine-deprived conditions, followed by treatment with or without 5-FU followed by Western blot analysis. **D** Analysis of relative intracellular serine levels in CRC cell lines following FOXC1 knockdown and overexpression, when cultured in serine/glycine-deprived medium. **E** Cell viability was evaluated in CRC cells with or without FOXC1 knockdown, and with or without serine supplementation, under treatment with 5-FU in serine/glycine-deprived medium. **F** CCK8 results demonstrating the effect on cell viability of CRC cell lines with shFOXC1 and shCtrl, cultured in either serine/glycine-deprived medium when exposed to a gradient of 5-FU concentrations. Data are reported as half maximal inhibitory concentration (IC50) values after 24 h of treatment. **G-H** CCK8 (G) and Edu (H) assays assessing cell proliferation and viability in CRC cell lines with FOXC1 knockdown, FOXC1 overexpression, FOXC1 knockdown plus serine supplementation (+Ser), and control, all cultured in serine/glycine-deprived medium. **I-L** CRC cell lines were cultured in serine/glycine-deprived media and subjected to FOXC1 overexpression or knockdown. Intracellular glycine levels (I), ROS levels (J), GSH level (K), and NADPH/NADP + levels (L) were measured. Data are presented as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$



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this FOXC1-targeted shRNA, we documented a pronounced decrease in the IC50 value of 5-FU under serine/glycine deprivation conditions within CRC cell lines (Fig. 4F), indicating increased sensitivity to 5-FU chemotherapy. These findings emphasize FOXC1's role in promoting DNA damage repair, thereby promoting 5-FU resistance in CRC cells.

Furthermore, under serine/glycine deprivation, FOXC1 overexpression promoted CRC cell proliferation, while FOXC1 knockdown reduced it, with serine repletion restoring proliferation (Fig. 4G-H and Fig. S4D). Catabolism of serine and glycine provides one-carbon units for one-carbon metabolism, essential for the support of tumor cell growth and DNA damage repair, as well as for maintaining redox balance through NADPH production-mediated detoxification of reactive oxygen species (ROS) [15–19, 22]. In our study, within CRC cells, FOXC1 overexpression led to an increase in intracellular glycine levels (Fig. 4I) and a decrease in ROS (Fig. 4J) under serine/glycine deprivation. Conversely, silencing FOXC1 resulted in reduced glycine levels (Fig. 4I), elevated ROS (Fig. 4J), and a decrease in the glutathione (GSH) (Fig. 4K) and NADPH/NADP + ratio (Fig. 4L) under serine/glycine deprivation. These findings suggest that FOXC1 modulates de novo serine synthesis, thereby increasing serine availability, which in turn influences one-carbon unit metabolism and ROS, ultimately impacting the growth of CRC tumors.

Serine deprivation enhances FOXC1 expression through the ERK1/2-p-ELK1 pathway

Given the observed increase in FOXC1 mRNA and protein levels, we further investigated the underlying mechanisms responsible for the elevated FOXC1 expression under serine/glycine deprivation conditions. Serine metabolism supports essential one-carbon units crucial for de novo purine synthesis and redox balance through NADPH-mediated ROS detoxification [16, 17].Our experiments identified elevated ROS levels in CRC cells under serine/glycine deprivation (Fig. 5A), which are known to activate various signaling pathways. Using an RTK phosphokinase array, we observed significant phosphorylation changes in RSK, ERK1/2, p38, and p53 under serine/glycine -deprived and normal conditions (Fig. 5B). We further employed RSK and ERK1/2 phosphorylation inhibitors, BI-D1870 and U0126, respectively [46, 47]. Our results demonstrated that only the inhibition of ERK1/2 phosphorylation by U0126 suppressed FOXC1 expression (Fig. 5C). ChIP experiments revealed that FOXC1 binding to the promoter regions of *PHGDH*, *PSAT1*, and *PSPH* was inhibited exclusively by U0126 treatment (Fig. 5D). Previous studies have shown that ROS induces FOXC1 overexpression via the ERK1/2-p-ELK1 signaling pathway [48]. L-Buthionine-sulfoximine (BSO) treatment notably elevated ROS levels in SW1116 cells compared to the DMSO control (Fig. S5A). Subsequently, western blot analysis showed that BSO significantly increased ERK1/2 phosphorylation and upregulated FOXC1 expression. However, treatment with U0126 reduced both ERK1/2 phosphorylation and FOXC1 expression, indicating that ERK1/2 is essential for FOXC1 regulation under oxidative stress (Fig. S5B). It is well known that ELK1 is a classical downstream target of ERK. Moreover, BSO enhanced ELK1 phosphorylation while leaving total ELK1 levels unchanged. Furthermore, ELK1 silencing via shRNA led to a reduction in FOXC1 expression, supporting the role of phosphorylated ELK1 in regulating FOXC1 (Fig. S5C). Additionally, ChIP assays confirmed that ELK1 binding to the FOXC1 promoter region was increased following BSO treatment, but was significantly reduced by U0126, further reinforcing the involvement of the ERK1/2-p-ELK1 pathway in FOXC1 regulation under oxidative stress (Fig. S5D). Additional ChIP experiments confirmed that U0126 inhibited ELK1 binding to the FOXC1 promoter region

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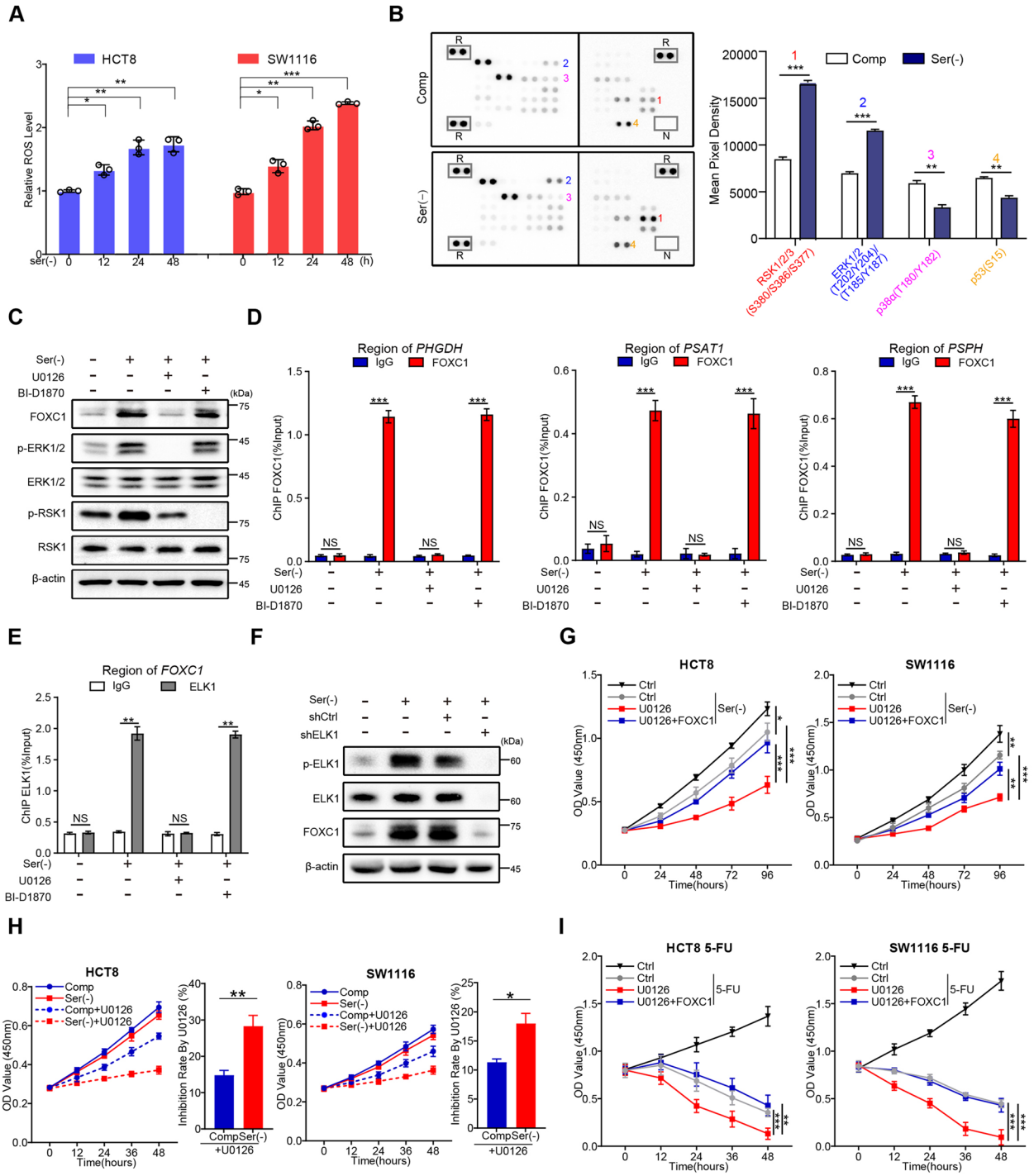
Fig. 5 Serine deprivation enhances FOXC1 expression through the ERK1/2-p-ELK1 pathway. **A** Relative ROS levels in CRC cells line upon serine/glycine deprivation (Ser (-)) at different time points. **B** SW1116 cells under serine/glycine-deprived or complete conditions were applied to the phosphokinase antibody array. Pixel densities of indicated proteins are shown. N, negative control; R, reference spot. **C** Western blot analysis was performed to assess FOXC1 expression and the activation of ERK1/2, RSK1 pathways by phosphorylation in response to serine/glycine deprivation and kinase inhibition (U0126, BI-D1870) in SW1116 cells. **D** ChIP-qPCR analysis evaluated FOXC1 binding to the promoters of PHGDH, PSAT1, and PSPH genes under serine/glycine deprivation and kinase inhibitor treatments. **E** ChIP-qPCR analysis determined ELK1 binding to the FOXC1 promoter region under various treatment conditions including serine/glycine deprivation and kinase inhibition. **F** Protein expression of FOXC1 and phosphorylation states of ELK1 were analyzed by Western blot under different experimental conditions in SW1116 cells. **G** CCK8 assays assessing cell proliferation in CRC cell line under serine/glycine deprivation with or without the addition of U0126 or FOXC1 overexpression. **H** CCK8 assay evaluating the viability of CRC cell lines treated with U0126 and control, cultured in either serine/glycine-deprived or complete medium. **I** Viability of HCT8 and SW1116 cells treated with 5-FU under serine/glycine deprivation with or without U0126 or FOXC1 overexpression were assessed to explore the impact of kinase inhibition on chemosensitivity. Data are presented as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$



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(Fig. 5E), and Western blot showed increased ELK1 phosphorylation with serine/glycine deprivation. Silencing ELK1 via lentivirus reduced FOXC1 expression, implicating ELK1 phosphorylation in FOXC1 regulation under serine deprivation (Fig. 5F). CCK-8 revealed that the addition of U0126 significantly inhibited CRC cell proliferation under serine/glycine deprivation, an effect reversed by FOXC1 overexpression (Fig. 5G and E). Our observations indicated that short-term serine/glycine deprivation (within 48 h) did not significantly decrease CRC cell proliferation (Fig. 1I). However, the introduction of U0126 during this period significantly amplified the impact on proliferation under serine/glycine deprivation compared to normal culture conditions (Fig. 5H). Additionally, treatment with 5-FU at half the IC50 concentration for 24 h significantly reduced cell viability in CRC cells with the addition of U0126, compared to the control group under serine/glycine deprivation. Overexpression of FOXC1 restored cell viability in the presence of U0126, reducing the enhanced sensitivity to 5-FU caused by U0126 and mitigating the loss of cell viability (Fig. 5I).

FOXC1 promotes CRC proliferation and 5-FU resistance in vivo under serine-deprived conditions

To elucidate the role of FOXC1 in promoting CRC proliferation and resistance to 5-FU under serine-deprived conditions in vivo, we utilized NSG mice, known for their heightened sensitivity to dietary and pharmacological interventions [49]. These mice were subcutaneously injected in the right abdomen with SW1116 cells engineered to express either control shRNA (shCtrl), shRNA targeting FOXC1 (shFOXC1), or control shRNA with U0126 treatment (U0126) (Fig. 6A, Fig. S4B and C). The U0126 was administered intraperitoneally at a dose of 10.5 mg/kg daily. Once tumors were established, the mice were divided into two dietary groups: one receiving a normal control diet and the other a serine/glycine-deprived diet (Ser(-) diet). Three days after dietary modification, treatments commenced with either no drug treatment or 5-FU (40 mg/kg) delivered in a cyclic regimen of three consecutive daily injections followed by four recovery days (Fig. 6A).

We measured FOXC1 expression levels in xenograft tumors and observed a significant decrease in the shFOXC1 group, confirming the effectiveness of lentiviral knockdown (Fig. S6G). Additionally, FOXC1 expression was higher in the shCtrl group of mice on a serine/glycine-deprived diet compared to the shCtrl group on a normal diet (Fig. S6H). Additionally, we measured serine levels in mouse plasma (Fig. 6E, Fig.S6A), tail tissue (Fig. S6C and D), and tumor tissue (Fig. 6F, Fig.S6B). Our findings revealed that serine levels significantly decreased in the plasma, tail tissue, and tumor tissue of mice fed a serine/glycine-deprived diet. Notably, in mice on the serine/glycine-deprived diet, the plasma serine levels in the U0126-treated group were significantly lower than those in the control group, suggesting that U0126 can inhibit serine metabolism in vivo under serine/glycine-deprived conditions (Fig. 6E, Fig.S6A). Similarly, within the tumor tissue, serine levels in the shFOXC1 group and the U0126 group were significantly lower than those in the shCtrl group under serine/glycine deprivation conditions (Fig. 6F, Fig. S6B).

As previously reported [31], dietary restriction of serine significantly inhibited both the growth rate and the weight of SW1116 xenografts (Fig. 6B, D, G and H) while showing no overt toxicity, as evidenced by stable body weights in mice (Fig. S6E and S6F). Importantly, while the shFOXC1 group and the U0126 group did not impact the growth or weight of tumors in mice on a complete diet compared to the control group, the shFOXC1 group and the U0126 group exhibited substantially slower growth rates (Fig. 6B, D and H) and reduced tumor weights (Fig. 6G) under serine/glycine-deprived diet conditions. Immunohistochemical analysis of tumor sections for proliferation markers Ki67 indicated that serine/glycine deprivation reduced the proliferation rate of the cancer cells. This reduction was more pronounced in the absence of the shFOXC1 group and the U0126 group (Fig. 6J). Additionally, in mice on a serine/glycine-deprived diet, tumor growth rates (Fig. 6C, D and H) and weights (Fig. 6G) were significantly lower, and the inhibition rate by 5-FU (Fig. 6J) was higher compared to the complete diet group

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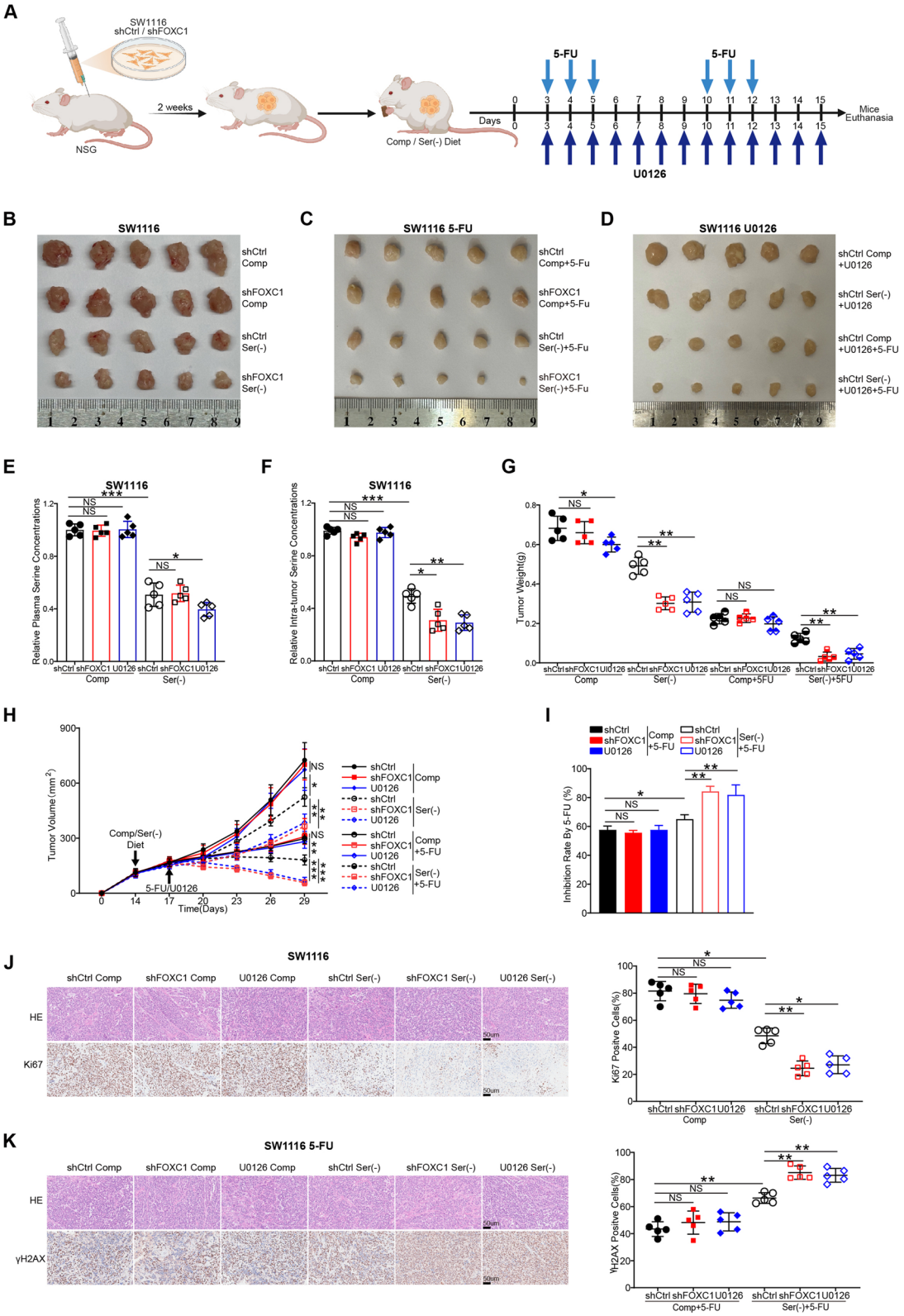
Fig. 6 FOXC1 promotes CRC proliferation and 5-FU resistance in vivo under serine-deprived conditions. **A** Workflow for the in vivo assessment of cell proliferation and 5-FU sensitivity under serine/glycine-deprived conditions. **B-D** Representative images of tumors harvested from different groups of mice ($n=5$) are displayed **E** and **F** Quantification of serine levels in mice: relative plasma serine concentrations (E) and intra-tumor serine concentrations (F). Each dot represents a tissue sample from an individual mouse. **G** Weight of subcutaneous tumors in each group. **H** and **I** Growth curves of subcutaneous tumor volume in each group. **J** The inhibition rates of 5-FU were measured according to (H and I). **K** and **L** Representative images of Ki67 (K) and γH2AX (L) expression in subcutaneous tumors detected through IHC. Data are presented as mean ± SD. *$P<0.05$; **$P<0.01$; ***$P<0.001$



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in mice treated with 5-FU. In the serine/glycine-deprived diet, the shFOXC1 group and U0126 group showed even greater reductions in tumor growth (Fig. 6C, D and H) and weight (Fig. 6G), as well as markedly higher inhibition rates by 5-FU (Fig. 6I) compared to the control group. Immunohistochemical analysis using γH2AX to measure DNA damage (Fig. 6K) revealed greater damage in the serine/glycine-deprived diet group compared to the complete diet group in mice treated with 5-FU. Additionally, within the serine/glycine-deprived diet group, the shFOXC1 and U0126 groups exhibited more DNA damage compared to the control group, corroborating the enhanced response to 5-FU. These results highlight ERK1/2-FOXC1 crucial role in modulating tumor response to dietary serine availability and chemotherapeutic agents such as 5-FU, underscoring the potential therapeutic benefit of targeting serine metabolism in CRC treatment.

FOXC1-mediated serine metabolism reprogramming is elevated in CRC and associated with poor prognosis

SSP genes and FOXC1, as revealed by analysis using data from various cancers and the TCGA database, are notably elevated in multiple tumor types, particularly in CRC (Fig. 7A). This pattern of high expression was consistent across several CRC databases (Fig. 7B) Additionally, Kaplan-Meier survival analysis using this cohort and GEO database data demonstrated that higher expression levels of these genes were associated with poorer survival outcomes (Fig. 7C). ROC curve analysis using TCGA data further substantiated these findings (Fig.S7A). In CRC, GSEA of differentially expressed genes highlights significant enrichment in amino acid metabolism, biosynthesis, and specifically in the biosynthesis of serine family amino acids (Fig. 7D). These results collectively underscore the critical role of the ERK1/2-P-ELK1-FOXC1 Mediated Serine Metabolism Reprogramming in CRC progression.

Discussion

Development and drug resistance are primary factors that compromise the effectiveness of current CRC treatments [5]. In the treatment of advanced CRC, 5-Fluorouracil, a widely used chemotherapeutic agent for treating advanced colorectal cancer, inhibits thymidylate synthase, disrupting nucleotide biosynthesis and DNA repair [7, 8]. The metabolic plasticity of cancer cells plays a pivotal role in the development of chemotherapy resistance [13, 14]. Several factors, including alterations in tumor metabolism, contribute to resistance to 5-FU [6, 50]. Serine metabolism, a critical aspect of metabolic reprogramming, significantly influences tumor growth and chemotherapy resistance [15–19].

Our experimental results and previous literature indicate that depriving CRC cells of exogenous serine may inhibit tumor growth and decrease resistance to 5-FU [20, 24]. However, the clinical effectiveness of solely restricting dietary serine in treating CRC is deemed suboptimal, primarily because external deprivation induces a compensatory activation of endogenous serine synthesis pathways [29, 32, 51]. Surprisingly, we observed that short-term serine deprivation (less than 48 h) does not significantly impact CRC cell proliferation. We speculate that the insignificant impact of short-term deprivation (within 48 h) on the growth of colorectal cancer cells may be related to the compensatory upregulation of serine metabolic enzymes and the activation of de novo serine biosynthesis. Sequencing analysis confirmed that endogenous de novo serine biosynthesis is highly enriched in CRC cells after 24 h of serine deprivation. Additionally, our experiments revealed an increase in the expression of SSP enzymes under serine deprivation, which may explain the transient lack of impact on proliferation, suggesting an adaptive response of CRC cells to the absence of exogenous serine. Previous studies using U-13 C-glucose tracing have shown that serine deprivation upregulates the SSP, enhancing nucleotide biosynthesis and other metabolic pathways crucial for cancer cell survival and adaptation [24, 42]. Thus, our study further investigates the effects of SSP enzymes on CRC 5-FU resistance and growth during serine deprivation. We observed that these enzymes significantly influence serine production, which in turn impacts one-carbon metabolism and oxidative stress balance, affecting tumor growth and proliferation [15–19]. Additionally, serine contributes to purine metabolism and DNA damage repair, thus affecting resistance to 5-FU [6, 20, 24]. Consequently,

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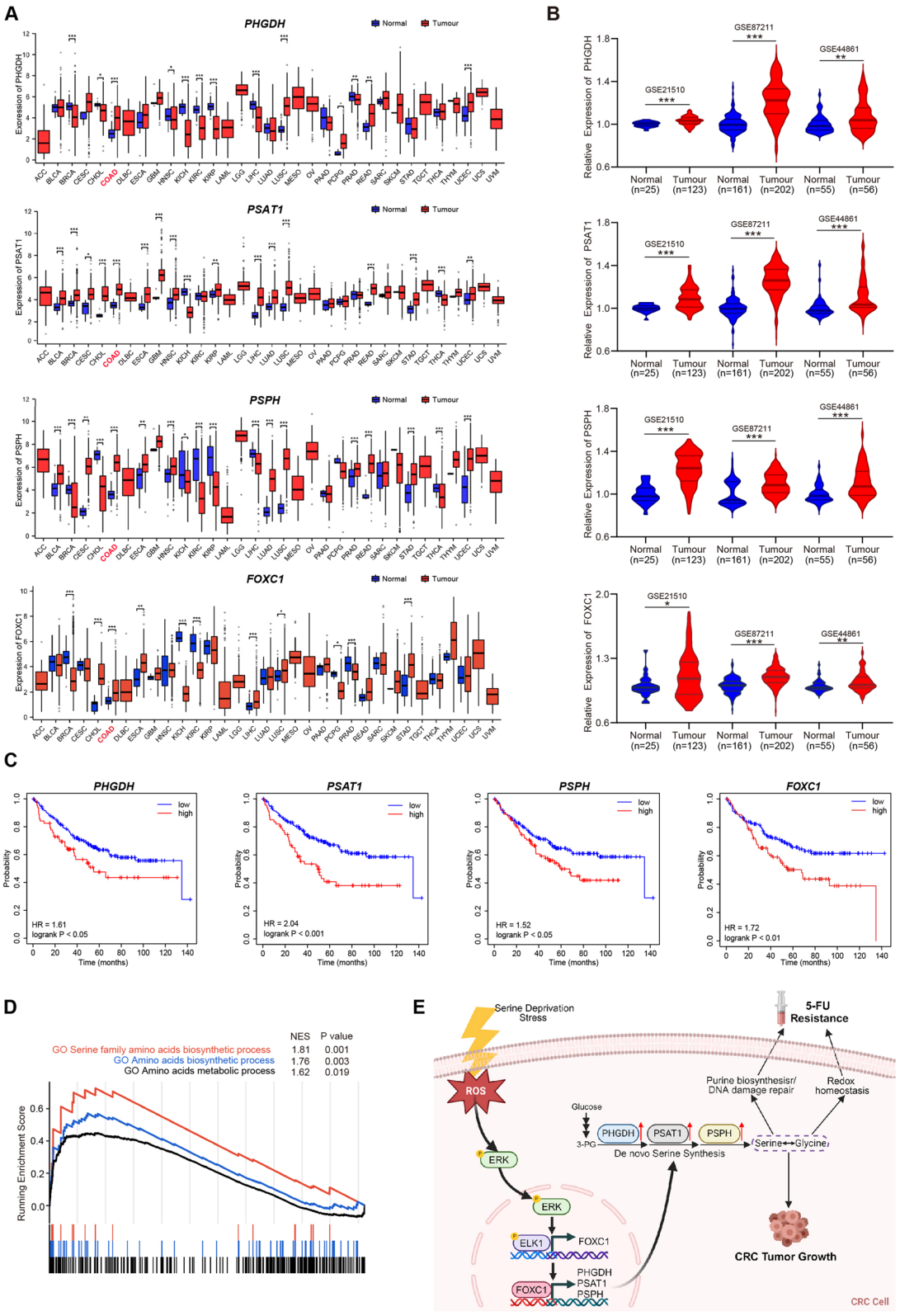
Fig. 7 Serine synthesis pathway genes are Elevated in CRC and associated with poor prognosis of CRC patients. **A** Expression of PHGDH, PSAT1, PSPH, and FOXC1 across pan-cancer tumors versus normal tissues in TCGA. **B** The relative expression of PHGDH, PSAT1, PSPH, and FOXC1 across CRC tumors versus normal tissues in GSE21510, GSE87211, and GSE44861. **C** Kaplan-Meier plots depicting the correlation between the expression of PHGDH, PSAT1, PSPH, FOXC1, and overall survival in CRC patients. **D** GSEA profiles showing CRC differential genes were significantly enriched in amino acid metabolism, amino acid metabolism biosynthetic, and the serine family amino acids biosynthesis. **E** Illustration of how FOXC1 facilitates CRC proliferation and 5-FU resistance by regulating serine metabolism reprogramming under serine-deprived conditions. Data are presented as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$



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the knockdown of each serine synthesis enzyme during serine deprivation significantly suppresses CRC cell 5-FU and growth resistance. Additionally, reports suggest interactions among these three enzymes, potentially leading to synergistic effects [52]. When all three enzymes are concurrently knocked down, the suppression of cancer growth and 5-FU resistance is significantly greater than the inhibition of any single enzyme alone. This highlights the importance of exploring the mechanisms behind the simultaneous upregulation of these enzymes during serine deprivation.

The compensatory activation of endogenous serine synthesis under serine deprivation is associated with signaling pathways and transcription factors [34–40]. Studies suggest that serine deprivation can activate the transcription factor ATF4's regulation of SSP enzymes [34, 35]. However, targeting ATF4 may damage normal growth and development in humans, making it unsuitable as a therapeutic target [53]. In our experiments, however, we found no significant correlation between ATF4 expression and CRC or its prognosis, and no regulatory relationship between FOXC1 and ATF4 was observed. Our study uncovers a novel mechanism by which serine synthesis is activated by exogenous serine deprivation in CRC. Through high-throughput sequencing and predictive analysis, we identified the transcription factor FOXC1, which is known for its role in various cellular processes, including metabolic reprogramming [45]. Under conditions of serine deprivation, FOXC1 expression is elevated, which in turn activates the transcription of serine synthesis enzymes. Under serine deprivation conditions, FOXC1 significantly impacts serine production and one-carbon metabolism, which influences CRC growth by affecting oxidative stress responses. Additionally, by modulating one-carbon metabolism, FOXC1 promotes DNA damage repair, thereby promoting the resistance of CRC to 5-FU chemotherapy.

The metabolism of serine generates crucial one-carbon units necessary for the synthesis of purines de novo and for preserving redox balance through the detoxification of reactive oxygen species [16, 17]. Our study found that elevated reactive oxygen species levels in CRC cells under serine-deprived conditions activate the ERK1/2 pathway, which phosphorylates ELK1, leading to increased FOXC1 expression. During serine deprivation, inhibition of ERK1/2 phosphorylation with U0126 significantly reduces FOXC1 levels in CRC cells, along with the expression of downstream SSP enzymes. Although we observed that U0126 decreases plasma serine concentrations in animal models, which could have potential side effects, targeting of FOXC1 or U0126 has shown excellent tumor-suppressive effects in vivo. Furthermore, targeting FOXC1 or using U0126 under serine deprivation conditions markedly inhibits colorectal cancer growth and enhances sensitivity to 5-FU chemotherapy, underscoring the therapeutic potential of targeting the ERK1/2-pELK1-FOXC1 axis. Moreover, while dietary deprivation of serine alone is not the most effective therapy, the simultaneous inhibition of both endogenous and exogenous serine sources appears to be more beneficial [51]. Our findings suggest that combining dietary serine restriction with targeted therapy against ERK1/2-pELK1-FOXC1 axis could be a highly effective strategy for treating colorectal cancer, thereby enhancing the efficacy of 5-FU.

While our study provides valuable insights into the role of FOXC1-mediated serine metabolism reprogramming enhances colorectal cancer growth and 5-FU resistance, several limitations should be noted. First, while we have shown increased expression of SSP enzymes under serine deprivation, further metabolic experiments, such as U-13 C-glucose tracing, are needed to confirm the functional activation of the SSP. Additionally, our in vitro findings regarding the transient compensatory mechanism in CRC cells may not fully reflect the more complex and sustained effects of serine deprivation in vivo, where the tumor microenvironment and systemic factors may influence the outcome. Lastly, while our apoptosis assays and 5-FU sensitivity experiments support the role of FOXC1 in promoting chemoresistance, the impact of short-term serine deprivation in animal models remains to be further explored.

Conclusions

This study investigates that serine deprivation activate the ERK1/2-p-ELK1 signaling axis, leading to upregulated FOXC1 expression in CRC cells. Elevated FOXC1 promoted the transcription of serine metabolism enzymes PHGDH, PSAT1, and PSPH, which in turn facilitated serine production, supporting CRC growth. Additionally, through serine metabolism, FOXC1 promoted purine metabolism and DNA damage repair, thereby increasing 5-FU resistance. Combining dietary serine restriction with targeted therapy against the ERK1/2-pELK1-FOXC1 axis could be a highly effective strategy for treating colorectal cancer, enhancing the efficacy of 5-FU.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
Z.K.C. and T.C. wrote the manuscript. Z.K.C. and Z.Y.L. carried out the experimental work. Z.K.C., K.F., J.C.X., Y.C., and Z.X.L. conducted animal experiments. ZYL, K.F, H.Y.J., J.C.X. revised the manuscript. Z.K.C., S.H.Z., A.Q.F., Z.H.Z., Z.Y.Z., M.C.S and H.Y.J. do cell experiments. T.C., M.D.X., L.N.H and L.C.Y. designed the experiments and supervised the project. All authors have approved the final version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Shanghai East Hospital, School of Medicine, Tongji University.

Competing interests
The authors declare no competing interests.

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