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PSPH promotes colorectal cancer growth by triggering autophagy through AMPK-ULK1 activation and enhancing tumor immune evasion

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Abstract. Phosphoserine phosphatase (PSPH), a key enzyme in the L-serine synthesis pathway, has been found to promote cancer progression through autophagy modulation. Here, we explored the functional role and molecular mechanisms of PSPH in colorectal cancer (CRC). PSPH expression in CRC was evaluated using bioinformatics analysis, RT-qPCR, and Western blot. Functional assays including colony formation, transwell migration/invasion, and flow cytometry were performed. Molecular mechanisms were investigated using Western blot analysis of autophagy-, proliferation-, apoptosis-associated proteins and AMPK-ULK1 signaling components. The biological significance of PSPH was validated using xenograft models and tumor immune microenvironment analysis. We found that PSPH expression was elevated in CRC patients and correlated with poor prognosis. PSPH overexpression promoted CRC cell proliferation, migration, and invasion while suppressing apoptosis and PSPH knockdown produced opposite effects. PSPH overexpression activated AMPK-ULK1 signaling to induce protective autophagy in CRC cells. These oncogenic effects were abrogated by AMPK/ULK1 depletion or chloroquine-mediated autophagy inhibition. *In vivo*, PSPH overexpression accelerated CRC tumor growth and promoted tumor immune evasion by upregulating PD-L1 expression and reducing CD8+ T cell infiltration. Overall, our findings establish PSPH as a critical oncoprotein that drives CRC progression through AMPK-ULK1-mediated autophagy activation and immune evasion.

Key words: Colorectal cancer — Phosphoserine phosphatase — Autophagy — Immune

Introduction

Colorectal cancer (CRC) is a major global health burden, ranking as the third most common malignancy with ~1.9 million new cases annually (Klimeck et al. 2023). While improvements in multimodal therapies including immunotherapy, surgery, and chemotherapy have improved the clinical outcomes of CRC patients (Bürtin et al. 2020), the prognosis for patients with advanced CRC remains dismal, with 5-year survival rates of only 71% and 14% for stages III and IV disease, respectively (Barton 2017). Therefore, it is needed to identify novel molecular drivers of CRC progression that could serve as therapeutic targets.

Phosphoserine phosphatase (PSPH), a key enzyme in the L-serine biosynthesis pathway, has recently emerged as a potential oncoprotein across multiple cancer types (Park et al. 2019; Huang et al. 2022). In gastric cancer, PSPH promotes tumor progression by modulating immune cell infiltration (Huang et al. 2022), while in lung cancer it drives metastatic dissemination through AMPK activation (Liao et al. 2019a). Notably, PSPH has been identified as an oncogenic gene and

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prognostic biomarker in CRC (Sato et al. 2017). However, the functional role and molecular mechanisms of PSPH in this malignancy remain largely unknown.

Autophagy, an evolutionarily conserved lysosomal degradation pathway, plays a complex and context-dependent role in cancer pathogenesis (Levine and Kroemer 2008; Miller and Thorburn 2021). In cancer biology, autophagy exhibits a well-documented duality. On one hand, it can promote tumor cell survival by providing nutrients and energy during stress conditions. On the other hand, it can suppress tumor growth by removing damaged organelles and proteins (Rabinowitz and White 2010; Mizushima and Komatsu 2011; Li et al. 2020). In CRC, many studies have indicated that autophagy activation enhances CRC cell survival following chemotherapy and radiation, while its pharmacological inhibition sensitizes tumors to treatment (Huang and Sinicrope 2010; Zhang et al. 2015; Xie et al. 2020; Pan et al. 2022). The AMPK-ULK1 signaling serves as a central regulator of autophagy induction in response to metabolic stress (Lin et al. 2021; Lu et al. 2023; Song et al. 2023). AMPK, acting as an energy sensor, directly phosphorylates ULK1 at Ser317 and Ser777 to initiate autophagosome formation (Kim et al. 2011; Wang et al. 2020). Activated ULK then phosphorylates Beclin-1, a critical component of the class III PI3K complex, to enhance autophagosome maturation (Russell et al. 2013; Ye et al. 2023). Notably, recent studies have implicated PSPH in modulating this pathway, with demonstrated roles in promoting lung cancer progression through AMPK activation (Liao et al. 2019b), and inducing cytoprotective autophagy in hepatocellular carcinoma (HCC) *via* AMPK-ULK1 signaling (Zhang et al. 2021). These findings suggest a potentially conserved oncogenic mechanism whereby PSPH drives tumor progression through AMPK-ULK1-mediated autophagy activation.

Building on these observations, we hypothesized that PSPH drives CRC progression through AMPK-ULK1-mediated autophagy activation. Our study systematically investigates this molecular axis while exploring its implications for tumor immune evasion, addressing a critical gap in our understanding of CRC pathogenesis and identifying potential novel therapeutic targets.

Materials and Methods

Clinical samples

CRC and adjacent normal tissue samples (n = 49) were obtained from patients aged 35–65 with CRC. All specimens were immediately snap-frozen in liquid nitrogen and stored at −80°C until use. Patients who had received prior anticancer treatments were excluded from the study. The study protocol was approved by the Ethics Committee of The First Affiliated Hospital of Bengbu Medical University, and written informed consent was obtained from all participants.

Cell culture

Human normal colonic epithelial cells (NCM460, Cat No. SNL-519) were obtained from Sunncell, Wuhan, China. Human CRC cell lines including SW620 (Cat No. CL-0225), SW480 (Cat No. CL-0223), LoVo (Cat No. CL-0144), and HCT116 (Cat No. CL-0096) were obtained from Procell, Wuhan, China. These cells were cultured in DMEM (41401ES76, Yeasen, Shanghai, China) supplemented with 10% FBS (A5669701, Gibco, NY, USA) and 1% penicillin/streptomycin (15240096, Gibco, CA, USA) at 37°C in a humidified 5% CO_2 atmosphere.

Plasmid construction and cell transfection

The full-length human PSPH cDNA was inserted into pcDNA3.1 vector (Invitrogen, CA, USA) to generate PSPH overexpression construct. Empty vector acted as control. Small interfering RNAs targeting PSPH (siPSPH#1/2), AMPK (siAMPK), ULK1 (siULK1), and scrambled control (siNC) were synthesized by GENESEED (Guangzhou, China). SW480 and SW620 cells (1×10^5 cells/well in 6-well plates) at 70%~80% confluence were transfected with plasmids or siRNAs using Lipofectamine 3000 (L3000015, Invitrogen, CA, USA) according to the manufacturer's protocol. For rescue experiments, SW480 cells were co-transfected with PSPH plasmid and siAMPK or siULK1. Chloroquine (CQ, 20 μM, 50-63-5, MedChemExpress, Shanghai, China) was used to treat PSPH-overexpressing SW480 cells for autophagy inhibition.

RT-qPCR

Total RNAs were extracted using TRIzol Reagent (T0201, GENESEED, Guangzhou, China) and reverse transcribed into cDNA using TonkBio RT Reagent Kit (TB30003A, TonkBio, Shanghai, China) following the manufacturer's instructions. qPCR was performed using SYBR Green I master mix (SR4110, Solarbio, Beijing, China) on a LightCycler 480 Instrument (Roche Laboratories, Basel, Switzerland). GAPDH acted as the endogenous control. Gene expression was calculated utilizing the $2^{-\Delta\Delta Ct}$ method. The qPCR primers were designed using the Primer-BLAST algorithm from NCBI and were chemically synthesized by Sangon Biotech (Shanghai, China). Primer sequences are listed as follows: PSPH forward 5'-CACGGTCATCAGAGAAGAAG-3', reverse 5'-GGTTGCTCTGCTATGAGTCT-3'; GAPDH forward 5'-GGGACCTGACTGACTACCTC-3', reverse 5'-TCATACTCCTGCTTGCTGAT-3'.

Cell viability assay (CCK-8)

CRC cells (1×10^3/well) were seeded in 96-well plates and cultured at 37°C. At each time point (24, 48, 72, and 96 hours), 10 μl CCK-8 solution (G021-1-2, PERFEMIKER, Shanghai, China) was added to the plates and incubated for 2~3 hours. Absorbance was measured at 450 nm using a multifunctional microplate reader (Infinite200 PRO, TECAN, Austria).

Colony formation assay

CRC cells (500 cells/well in 6-well plates) were maintained in a humidified incubator at 37°C with 5% CO_2 for 10~14 days to allow colony formation. The culture medium was refreshed every two days. Colonies of over 50 cells were fixed with 4% paraformaldehyde (XY0427, Xybio, Shanghai, China) for 25 min, stained with 0.1% crystal violet (BP-DL131, Sbjbio, Nanjing, China) for 30 min, and counted under a Nikon Eclipse Ti2-E microscope (Nikon, Japan).

Apoptosis analysis by flow cytometry

CRC cells were harvested, washed with PBS, and stained with Annexin V-FITC/PI Apoptosis Kit (DY20202, DEEYEE, Shanghai, China) according to the manufacturer's protocol. Samples were analyzed on a BD FACS Canto II (BD Biosciences, NJ, USA), and data were processed using FlowJo v10 (FlowJo LLC, OR, USA).

Transwell migration and invasion assays

Cell migration and invasion were assessed using 8-μm pore Transwell inserts (Corning, NY, USA). For migration assays, 5×10^4 cells in 200 μl serum-free medium were seeded in the upper chamber. For invasion assays, inserts were pre-coated with 1 mg/ml Matrigel (Corning, NY, USA). Complete medium (600 μl) containing 10% FBS served as chemoattractant in the lower chamber. After 48 hours of incubation, cells that migrated/invaded through the membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and imaged using a Nikon Eclipse Ti2-E microscope (Nikon, Japan). Five random fields *per* insert were counted for quantification.

Autophagy flux analysis (RFP-LC3 puncta quantification)

CRC cells (1×10^5 cells/well in 12-well plates) were infected with RFP-LC3 adenovirus (HZ1333, HZbscience, Shanghai, China) at a multiplicity of infection (MOI) of 15. After PBS washing and fixation, RFP-LC3 puncta were visualized using a ZEISS LSM 980 Airyscan 2 Confocal Microscope (ZEISS, Germany).

Xenografted tumor model

All animal experiments were approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Bengbu Medical University and conducted in accordance with NIH guidelines. BALB/c-nude mice (5 weeks old) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were randomly divided into four groups: vector, PSPH, shNC, and shPSPH. PSPH gene was cloned into the lentivirus vector Pez-Lv201 (GeneCopoeia, MD, USA). Lentiviral shRNA constructs were provided by Hanbio (Shanghai, China). Suspensions of SW480 cells (5×10^6, 100 μl) infected with PSPH or SW620 cells (5×10^6, 100 μl) infected with shPSPH were subcutaneously injected into the right flank. Tumor volumes were measured every three days over a 20-day period, starting with the first measurement on day 5. Mice were then sacrificed, and tumors were weighed and processed for further analysis.

Flow cytometry analysis of IFN-γ+ CD8+ T cells

To detect IFN-γ+ CD8+ T cells in mouse xenograft tumors, tumor tissues were harvested, dissociated into single-cell suspensions, and stained with fluorescently labeled anti-mouse CD8 antibody (CD8a, Ly-2, 11-0081-81, eBioScience, CA, USA). Cells were then fixed, permeabilized, and intracellularly stained with anti-IFN-γ antibody (Clone Number: XMG 1.2, E-AB-F11101UD, Elabscience, Wuhan, China). Data were acquired on a BD FACS Canto II (BD Biosciences, NJ, USA) and analyzed using FlowJo v10 (FlowJo LLC, OR, USA), with IFN-γ+ percentages calculated within the CD8+ population.

Immunofluorescence

Tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4-μm thickness. After deparaffinization and antigen retrieval (citrate buffer, pH 6.0), sections were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin for 1 hour at room temperature. Sections were incubated overnight at 4°C with anti-PD-L1 antibody (1:1,000, ab237726, Abcam, UK), followed by incubation with Alexa Fluor®-conjugated secondary antibody (1:500, ab150077, Abcam, UK) for 1 hour at room temperature in the dark. Nuclei were counterstained with DAPI for 5 min. PD-L1 fluorescence signal was visualized using a Nikon Eclipse Ni-E fluorescence microscope (Nikon, Japan) and quantified using ImageJ.

Western blot

Total protein was extracted using RIPA buffer (YB-J309, Ybscience, Shanghai, China). Protein concentration was determined

by BCA assay (SNM078, BIOLAB, Beijing, China). Samples (30 μg/lane) were separated by 10% SDS-PAGE (P1050M, LABLEAD, Beijing, China) and transferred onto PVDF membranes. After blocking with non-fat dried milk (5%), membranes were incubated with primary antibodies overnight at 4°C. The antibodies included GAPDH (1:20,000, AF7021, Affinity, USA), PSPH (1:500, DF12711, Affinity, USA), Beclin-1 (1:500, AF5128, Affinity, USA), p62 (1:500, AF5384, Affinity, USA), LC3 (1:500, AF5402, Affinity, USA), AMPK (1:500, AF6423, Affinity, USA), P-AMPK (1:500, AF3423, Affinity, USA), ULK1 (1:3,000, DF7588, Affinity, USA), P-ULK1 (1:500, AF4387, Affinity, USA), PCNA (1:500, AF0239, Affinity, USA), Bcl-2 (1:500, AF6139, Affinity, USA), and cleaved caspase 3 (1:500, AF7022, Affinity, USA). After washing with PBS, samples were incubated with HRP-conjugated secondary antibody (1:500, S0006, Affinity, USA) for 1 hour. Protein bands were visualized using SuperBright Subpico ECL (31059, SUDGEN, Nanjing, China) and analyzed using ImageJ.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 10.2.1 software (GraphPad, CA, USA). All data are presented as mean ± standard deviation. Student's *t*-test and one-way ANOVA with Tukey's *post hoc* analysis were used for group comparisons. $p < 0.05$ was considered statistically significant. *n* = number of biological replicates (e.g., patients, tissues, or animals); N = number of independent experimental repeats.

Results

PSPH is upregulated in CRC and correlates with poor prognosis

Based on the analysis of GEPIA, PSPH expression is elevated in CRC tumor tissues ($n = 275$) compared to normal controls ($n = 349$) (Fig. 1A). The UALCAN database also indicates a significant increase in PSPH expression in CRC tumors ($n = 286$) compared to that in normal samples ($n = 41$) (Fig. 1B). RT-qPCR analysis further demonstrated high levels of PSPH in clinical CRC specimens ($n = 49$) compared to adjacent normal tissues (Fig. 1C). Kaplan-Meier analysis of these CRC patients revealed that high PSPH expression (above median) correlated with significantly worse overall survival and relapse-free survival (Fig. 1D). Western blot analysis confirmed elevated PSPH protein levels in both tumor tissues (Fig. 1E) and CRC cells compared to normal colonic epithelial cells (NCM460) (Fig. 1F).

PSPH promotes CRC cell growth and suppresses apoptosis

RT-qPCR data revealed overexpression of PSPH in SW480 cells transfected with PSPH vector and knockdown of PSPH in SW620 cells transfected with siPSPH#1/2 (Fig. 2A). CCK-8 revealed that PSPH overexpression increased SW480 cell viability, while PSPH knockdown decreased SW620 cell viability (Fig. 2B). PSPH overexpression increased the number of colonies in SW480 cells. Conversely, depletion of PSPH inhibited colony formation in SW620 cells (Fig. 2C). CRC cell apoptosis was inhibited in the PSPH group and was increased in the PSPH knockdown group (Fig. 2D). Transwell assays demonstrated that PSPH overexpression enhanced migration and invasion of SW480 cells, while PSPH knockdown produced opposite effects in SW620 cells (Fig. 2E).

PSPH promotes CRC cell autophagy by activating AMPK-ULK1 signaling

Upon PSPH overexpression, SW480 cells exhibited a marked increase in Beclin-1 and LC3-II/LC3-I levels and a decrease in p62 protein levels. Conversely, PSPH knockdown in SW620 cells reduced Beclin-1 levels, lowered LC3-II/LC3-I ratio, and increased p62 levels (Fig. 3A,B). Given the established role of AMPK-ULK1 signaling in autophagy induction (Li and Chen 2019; Iorio et al. 2021), we investigated whether PSPH regulates this pathway in CRC cells. Western blot analysis revealed that PSPH overexpression enhanced phosphorylation of AMPK and ULK1 in SW480 cells, while depletion of PSPH suppressed these phosphorylation events in SW620 cells (Fig. 3C,D). Additionally, there was an increase in the number of RFP-LC3 puncta in SW480 cells transfected with PSPH overexpression vector, which was reversed by AMPK or ULK1 depletion (Fig. 3E). Rescue experiments confirmed that AMPK/ULK1 knockdown reversed PSPH-induced autophagy activation, as evidenced by reduced P-AMPK, P-ULK1, and LC3-II/I levels (Fig. 3F).

PSPH drives CRC growth through AMPK-ULK1-mediated autophagy

To establish the functional link between PSPH-induced autophagy and CRC progression, we employed chloroquine (CQ), a well-characterized autophagy inhibitor (Ferreira et al. 2021). Pharmacological autophagy blockade with CQ significantly attenuated PSPH-mediated proliferation enhancement in SW480 cells. Similarly, genetic inhibition of the pathway through AMPK or ULK1 knockdown abolished PSPH's proliferative effects, confirming the dependence on AMPK-ULK1 signaling (Fig. 4A). Flow cytometry analysis revealed that PSPH's anti-apoptotic effects were reversed by either pathway inhibition (siAMPK/siULK1) or CQ treatment (Fig. 4B). Western blot analysis of key regulators further supported these findings: PSPH-induced upregulation of PCNA (proliferation marker) and Bcl-2 (anti-apoptotic protein), along with downregulation of cleaved caspase 3

(apoptosis executor), were all negated by autophagy inhibition (Fig. 4C).

PSPH enhances tumor growth in vivo

Xenograft studies using SW480 cells demonstrated that PSPH overexpression significantly accelerated tumor growth (Fig. 5A–C). Molecular analysis of xenograft tissues recapitulated our *in vitro* findings, showing elevated LC3-II/LC3-I ratios, increased AMPK/ULK1 phosphorylation, and reduced cleaved caspase 3 levels in PSPH-overexpressing tumors (Fig. 5D). Conversely, PSPH knockdown in SW620-derived xenografts yielded opposite effects, with a significant reduction in tumor volume and weight and corresponding modulation of autophagy/apoptosis markers (Fig. 6A–D).

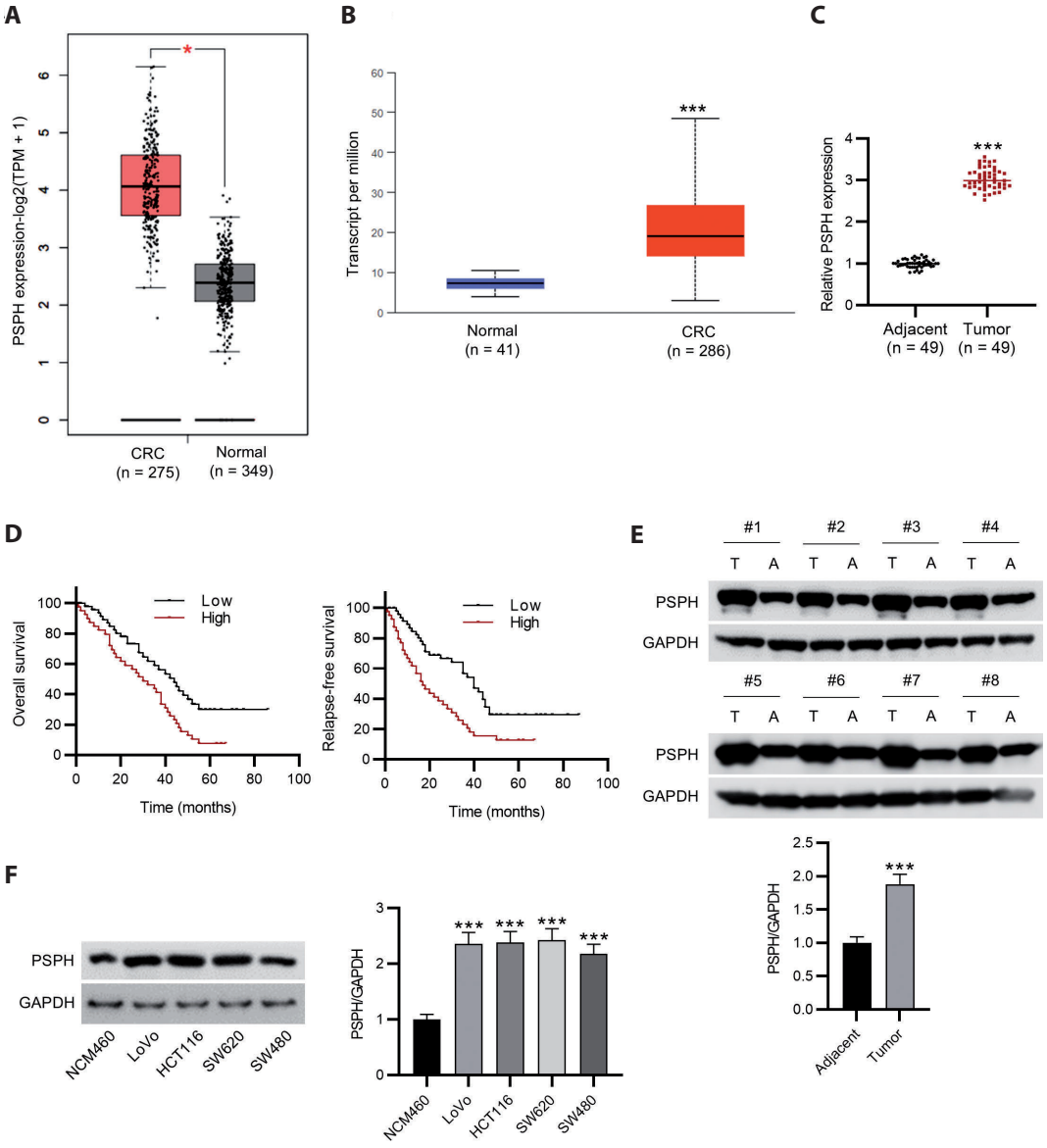

Figure 1. PSPH is upregulated in CRC and correlates with poor prognosis. **A.** Analysis of PSPH mRNA expression in CRC (n = 275) *versus* normal colon tissues (n = 349) from the GEPIA database. **B.** PSPH expression levels in CRC (n = 286) and normal samples (n = 41) from UALCAN. **C.** RT-qPCR quantification of PSPH mRNA in matched tumor and adjacent normal tissues from CRC patients (n = 49). **D.** Kaplan-Meier survival analysis of overall survival (left) and relapse-free survival (right) in CRC patients stratified by PSPH expression (high *vs.* low, n = 49). **E.** Representative Western blot and quantification of PSPH protein levels in paired tumor (T) and adjacent normal (A) tissues (n = 8). **F.** PSPH protein expression in CRC cell lines compared to normal colonic epithelial cells (NCM460). N = 3; * $p < 0.05$, *** $p < 0.001$.

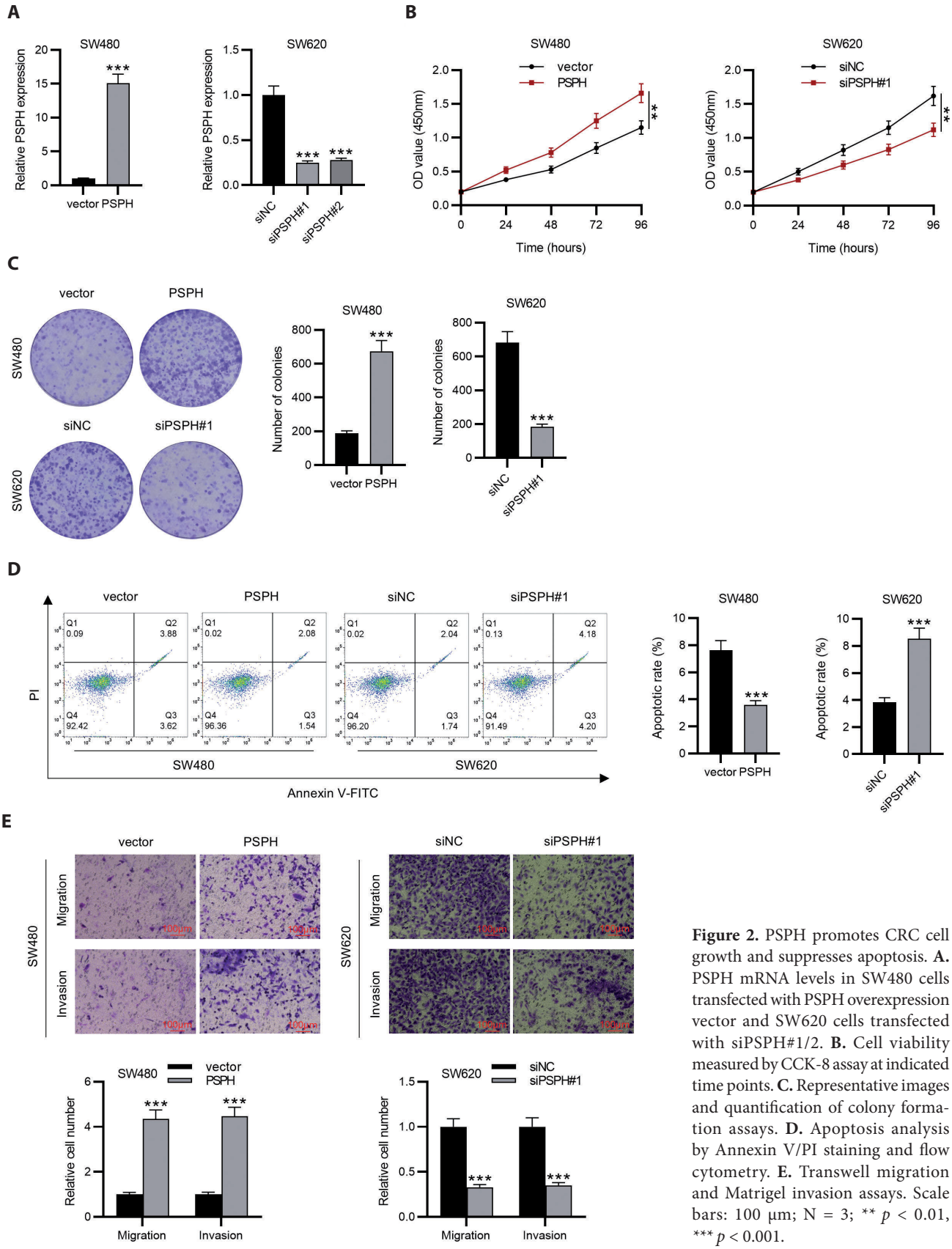

Figure 2. PSPH promotes CRC cell growth and suppresses apoptosis. **A.** PSPH mRNA levels in SW480 cells transfected with PSPH overexpression vector and SW620 cells transfected with siPSPH#1/2. **B.** Cell viability measured by CCK-8 assay at indicated time points. **C.** Representative images and quantification of colony formation assays. **D.** Apoptosis analysis by Annexin V/PI staining and flow cytometry. **E.** Transwell migration and Matrigel invasion assays. Scale bars: 100 μm; N = 3; ** $p < 0.01$, *** $p < 0.001$.

PSPH modulates tumor immune evasion in vivo

We also investigated the effect of PSPH on immune response *in vivo*. Immunofluorescence revealed that PSPH-overexpressing tumors exhibited significantly elevated PD-L1 expression compared to control tumors (Fig. 5E). Conversely, PSPH-knockdown tumors showed markedly reduced PD-L1 levels (Fig. 6E). Flow cytometry analysis of tumors demonstrated that PSPH overexpression correlated with a diminished proportion of IFN-γ+ CD8+ T cells (Fig. 5F). In contrast, PSPH-knockdown tumors displayed enhanced infiltration of functional IFN-γ+ CD8+ T cells (Fig. 6F).

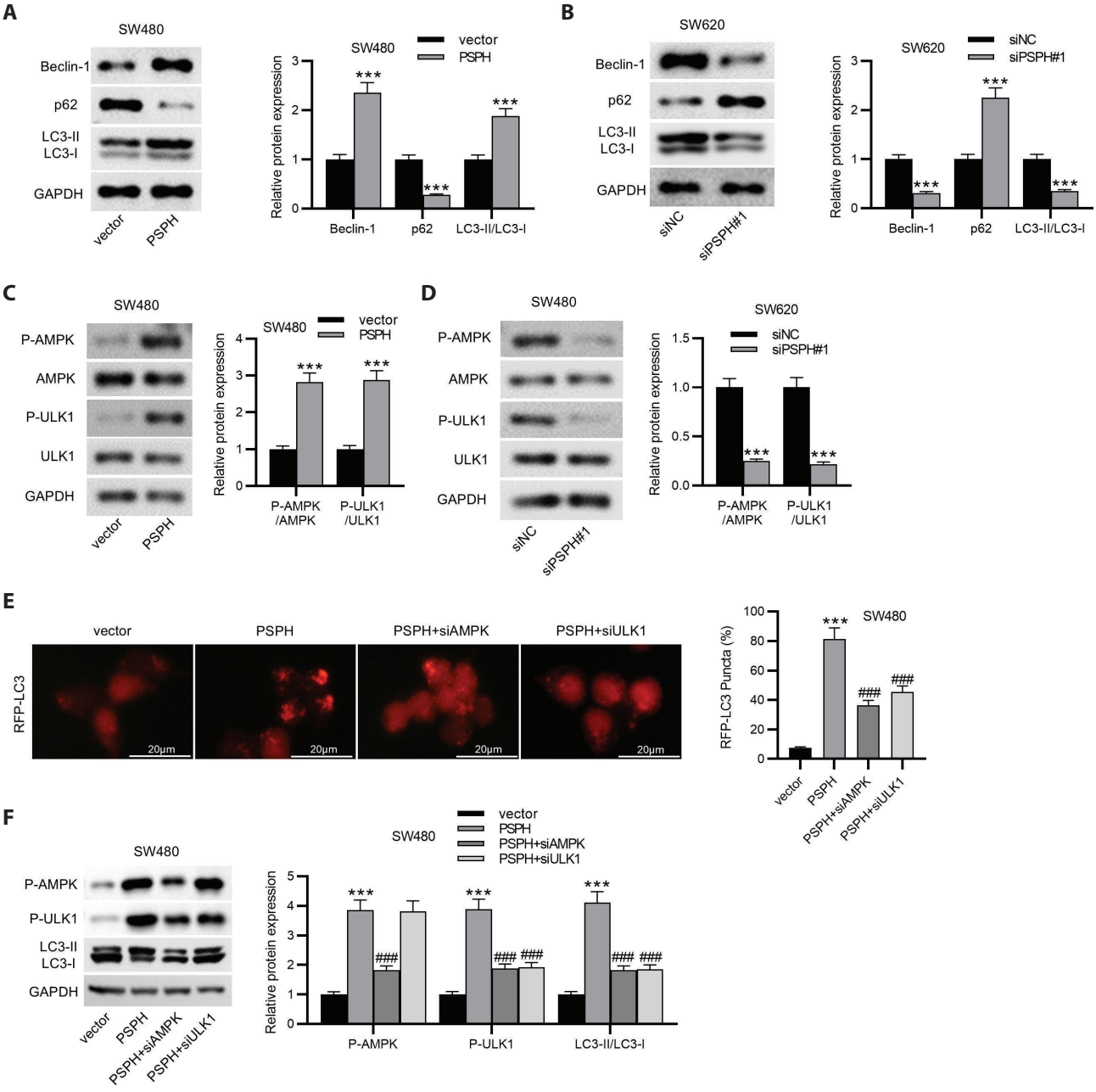

Figure 3. PSPH promotes CRC cell autophagy by activating AMPK-ULK1 signaling. Western blot analysis of autophagy markers (Beclin-1, p62, LC3-II/I) in PSPH-overexpressing SW480 cells (**A**) and PSPH-knockdown SW620 cells (**B**). Phosphorylation status of AMPK and ULK1 in SW480 cells (**C**) and SW620 cells (**D**). **E.** Representative confocal images and quantification of RFP-LC3 puncta (red) in SW480 cells. Scale bar: 20 μm. **F.** Rescue experiments showing protein levels in PSPH-overexpressing SW480 cells with AMPK or ULK1 knockdown. N = 3; *** $p < 0.001$, ### $p < 0.001$. (For color Figure see online version).

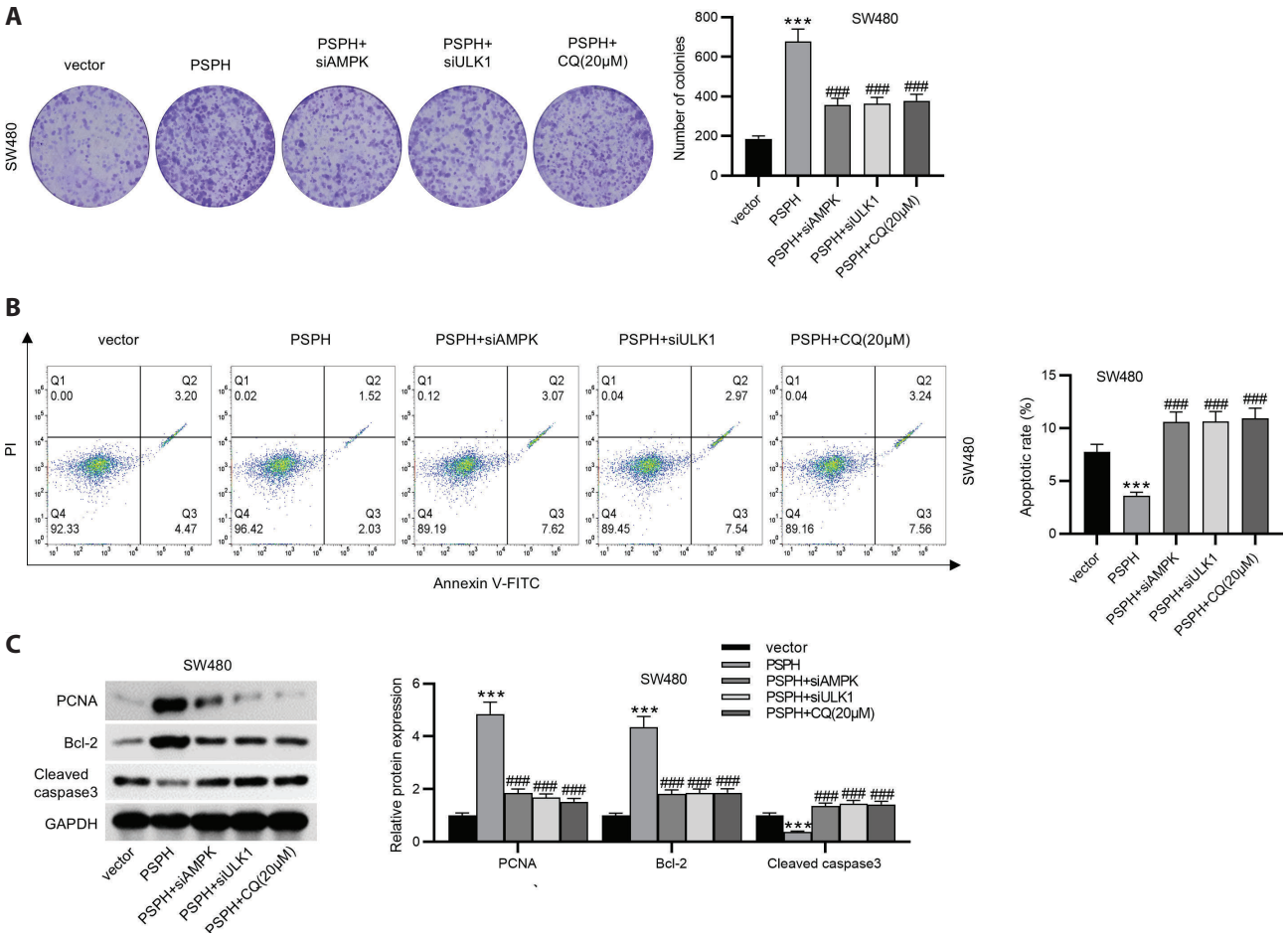

Figure 4. PSPH drives CRC cell growth by inducing autophagy *via* AMPK-ULK1 signaling. **A.** Cell proliferation assessed by CCK-8 assay in PSPH-overexpressing SW480 cells with AMPK/ULK1 knockdown or chloroquine (CQ, 20 μM) treatment. **B.** Corresponding apoptosis rates by flow cytometry. **C.** Western blot analysis of proliferation (PCNA) and apoptosis (Bcl-2, cleaved caspase3) markers. N = 3; $^{***}p < 0.001$, $^{###}p < 0.001$.

Discussion

Previous studies have identified elevated PSPH expression in various tumors, where it serves as a predictor of poor patient survival (Ross et al. 2017; Svoboda et al. 2018). PSPH also exhibits high levels in CRC and has been established as a prognostic marker for unfavorable outcomes (Sato et al. 2017). Consistent with these findings, our study demonstrated significantly higher PSPH levels in CRC cells and patient-derived tissues. Kaplan-Meier survival analysis further corroborated that elevated PSPH expression correlates with reduced survival rates, reinforcing its potential as a prognostic biomarker in CRC. The oncogenic role of PSPH has been well-documented. For instance, PSPH upregulation has been shown to drive tumor growth and metastasis in melanoma, contributing to disease progression (Rawat et al. 2021). Li et al. reported that suppression of PSPH enhances the antitumor efficacy of 5-fluorouracil in CRC *in vivo* (Li et al. 2016). Our *in vivo* and *in vitro* experiments revealed that PSPH overexpression promotes CRC cell proliferation, migration, and invasion while inhibiting apoptosis. Conversely, PSPH knockdown using siRNA produced opposing effects, significantly attenuating CRC tumor growth *in vivo*. Importantly, PSPH overexpression can promote tumor immune evasion by upregulating PD-L1 expression and reducing CD8+ T cell infiltration in CRC models. These results collectively suggest that PSPH may represent a promising therapeutic target for CRC.

Autophagy is a self-digestion cellular process that degrades damaged proteins or organelles, which is responsible for maintaining the homeostasis of intracellular environment (Glick et al. 2010). It plays a dual role in cancer by enabling tumor cells to adapt to environmental stress (Aguilar-Gallardo et al. 2022). In CRC, enhanced autophagy has been

linked to metastatic potential and therapeutic resistance. For example, sphingosine kinase 1-driven autophagy was shown to facilitate CRC metastasis (Wu et al. 2021), while autophagy inhibition suppressed tumor growth by inducing apoptosis (Liu et al. 2019). These findings underscore the importance of elucidating the molecular mechanisms governing autophagy in CRC progression. Our investigation revealed that PSPH overexpression upregulates autophagy-related proteins (Beclin-1 and LC3-II/LC3-I) while downregulating p62 in CRC cells. These effects were reversed by CQ, an autophagy

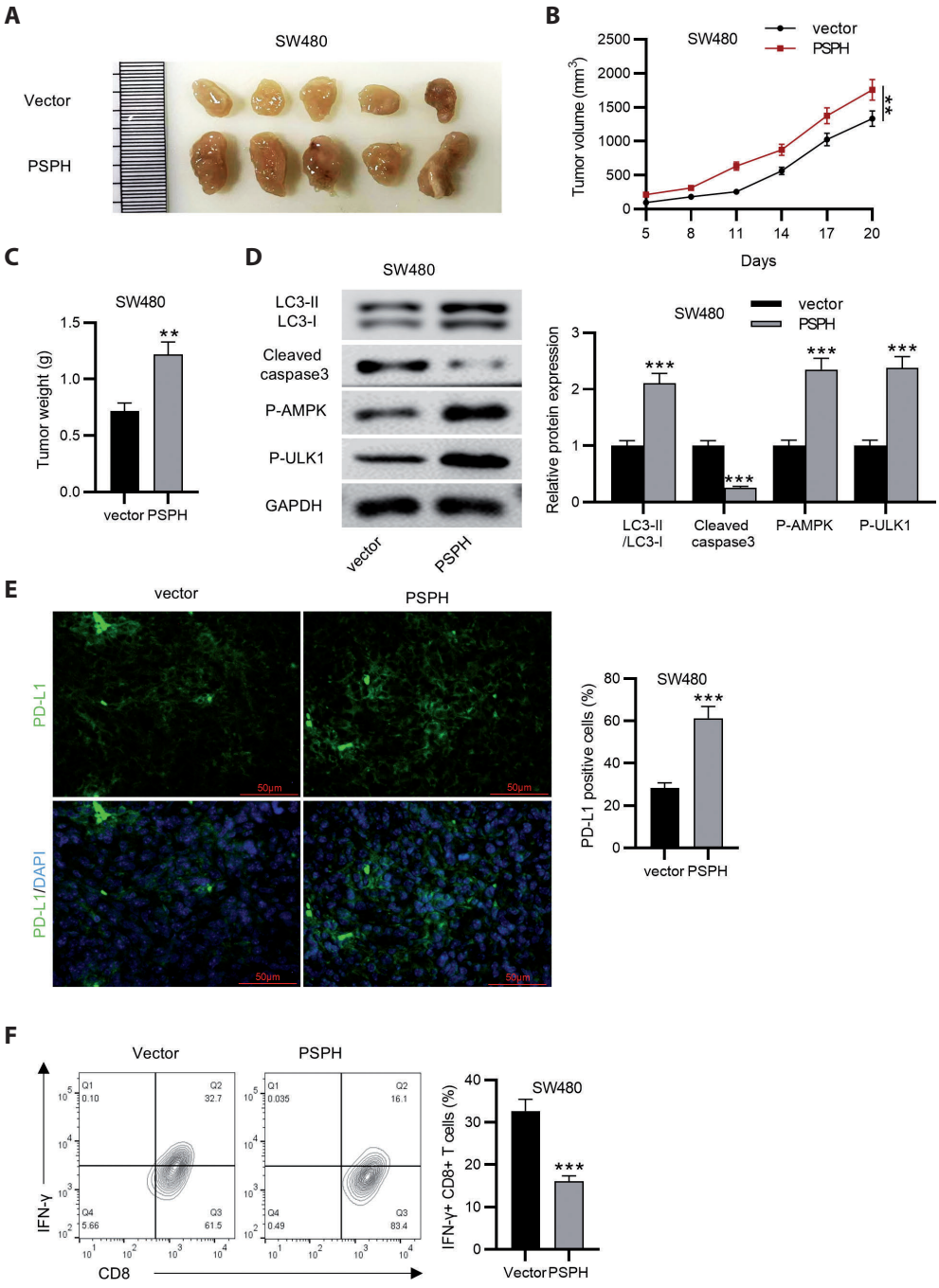

Figure 5. PSPH overexpression enhances tumor growth and modulates immunity *in vivo*. Representative images (**A**), tumor growth curves (**B**), and weights (**C**) of SW480 xenografts. **D.** Western blot analysis of autophagy and apoptosis markers in excised tumors. **E.** Representative immunofluorescence images and quantification of PD-L1 expression. Scale bar: 50 μm. **F.** Flow cytometry analysis of tumor-infiltrating IFN-γ+ CD8+ T cells. $n = 3$ mice/group; ** $p < 0.01$, *** $p < 0.001$.

inhibitor. Notably, CQ also abolished the pro-survival and proliferative effects of PSPH, confirming that PSPH-induced autophagy supports CRC cell viability. These observations align with prior research linking PSPH-mediated autophagy to HCC progression (Zhang et al. 2021).

Autophagy regulation involves multiple signaling pathways, with the AMPK/ULK1 axis playing a central role (Chen et al. 2019). Under metabolic stress, AMPK activates ULK1 through phosphorylation at Ser317 and Ser777, initiating autophagy (Kim et al. 2011; Hardie 2015). Activation

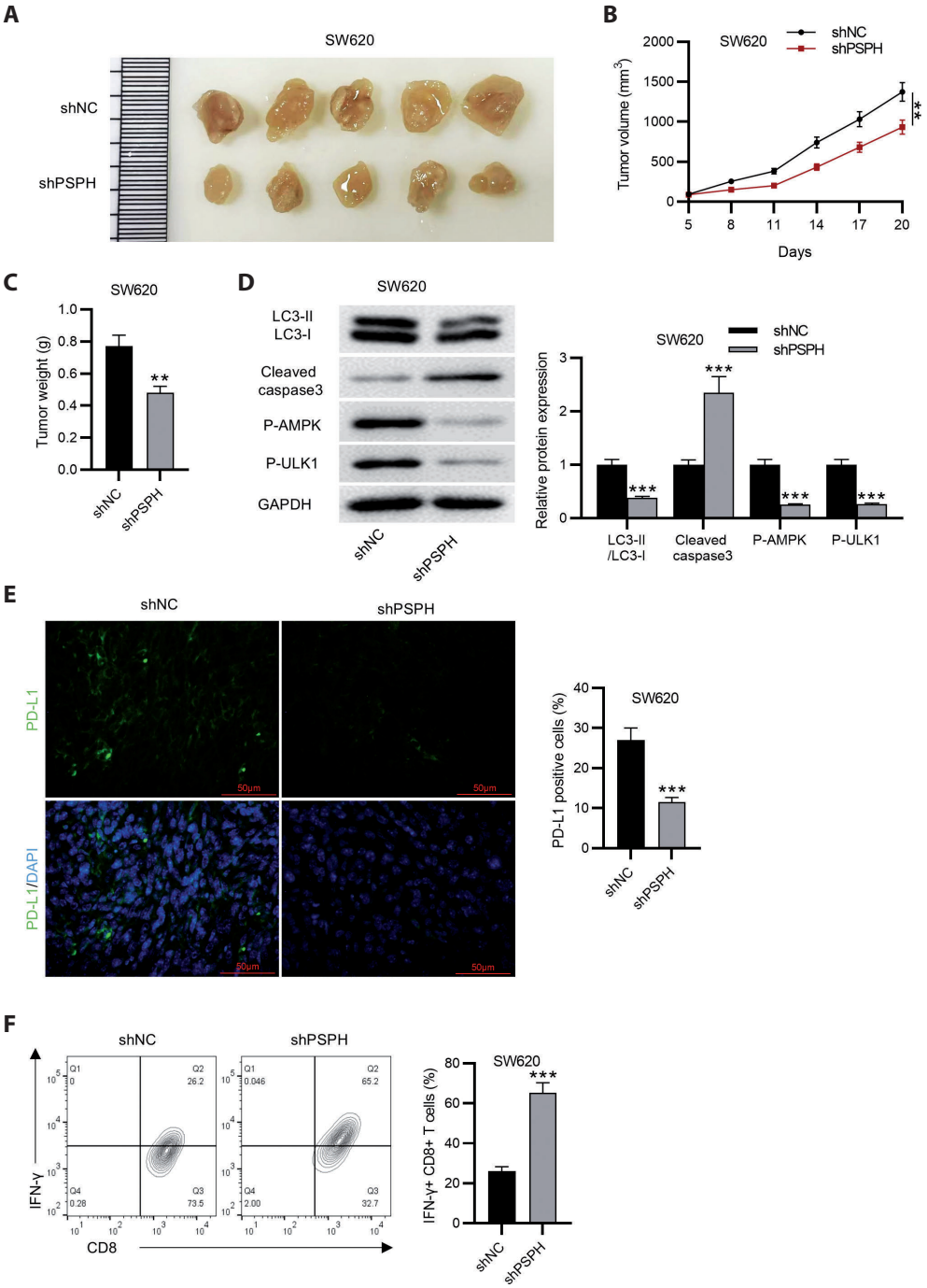

Figure 6. PSPH knockdown suppresses tumor growth and enhances anti-tumor immunity. Representative images (**A**), tumor growth curves (**B**), and weights (**C**) of SW620 xenografts. **D.** Western blot analysis of tumor lysates. **E.** PD-L1 immunofluorescence staining and quantification. Scale bar: 50 μm. **F.** Percentage of IFN-γ+ CD8+ T cells in tumors. $n = 3$ mice/group; ** $p < 0.01$, *** $p < 0.001$.

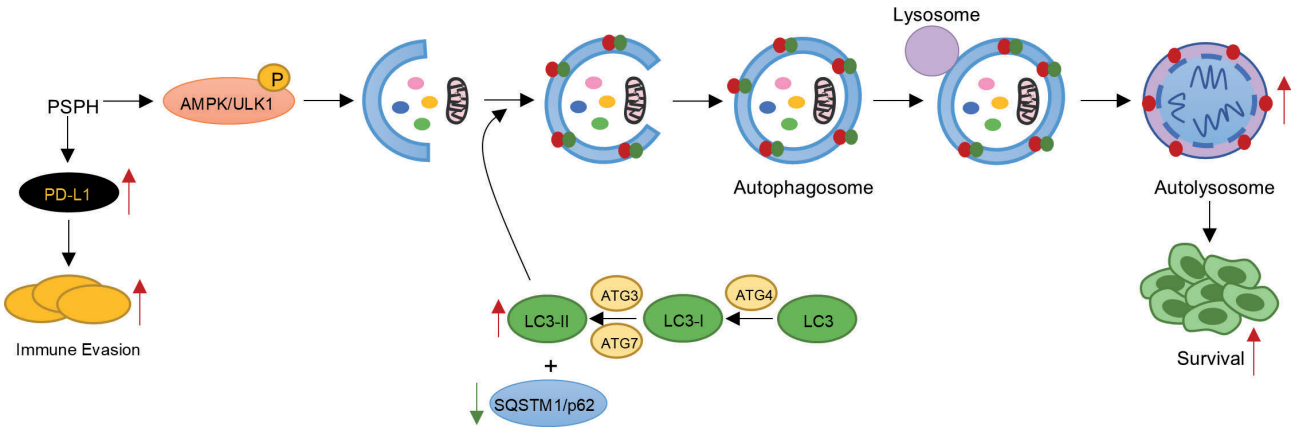

Figure 7. Schematic model of PSPH's oncogenic mechanism in CRC. SPH promotes CRC progression through two synergistic pathways. (1) PSPH activates AMPK phosphorylation, which triggers phosphorylation of ULK1, initiating autophagy. This process is characterized by increased LC3-II/LC3-I ratio, degradation of p62, and enhanced autophagic flux, enabling CRC cells to sustain proliferation. (2) PSPH upregulates PD-L1 expression on CRC cells to promote PD-L1-dependent immune evasion. Together, these mechanisms drive tumor growth by coupling stress-adaptation (autophagy) with immune escape, as demonstrated in xenograft models.

of ULK1 subsequently upregulates autophagy-associated effectors including Beclin-1 and LC3 (Peña-Martinez et al. 2022). Disruption of AMPK/ULK1 signaling has been shown to inhibit CRC growth and enhance apoptosis (Min et al. 2014; Xie et al. 2014). In our study, overexpression of PSPH increased the levels of P-AMPK, P-ULK1, Beclin-1, and LC3-II/LC3-I while reducing p62. Crucially, AMPK or ULK1 depletion reversed the pro-autophagic and tumor-promoting effects of PSPH, indicating that PSPH drives cytoprotective autophagy in CRC *via* AMPK-ULK1 signaling.

Our study reveals novel insights into PSPH's role in shaping the immunosuppressive tumor microenvironment in CRC. The significant upregulation of PD-L1 in PSPH-overexpressing tumors aligns with emerging evidence that metabolic reprogramming can influence immune checkpoint expression (Xia et al. 2021). This finding is particularly relevant given the established role of PD-L1 in mediating T cell exhaustion and immune evasion in CRC (Gao et al. 2021; Lin et al. 2023). The concurrent reduction in tumor-infiltrating IFN-γ+ CD8+ T cells suggests PSPH may create an immune-privileged niche, potentially through both PD-L1-dependent and independent mechanisms. These findings align with previous observations in gastric cancer showing that PSPH promotes tumor progression by modulating immune cell infiltration (Huang et al. 2022), and provide mechanistic support for clinical studies showing poor immunotherapy response in tumors with activated serine metabolism. Notably, our findings complement recent work demonstrating that autophagy modulation can alter PD-L1 expression and T cell function (Cui et al. 2023), suggesting PSPH may coordinate a feed-forward loop between metabolic reprogramming and immune suppression. Our findings suggest that PSPH may serve as a novel regulator of immune checkpoint signaling, potentially contributing to resistance against immunotherapy. Further investigations are warranted to explore whether targeting PSPH could synergize with immune checkpoint inhibitors to enhance antitumor immunity in CRC.

Building on these findings, several critical research directions emerge. First, the precise molecular mechanisms linking PSPH to both AMPK-ULK1-mediated autophagy and PD-L1 regulation require further elucidation. Second, studies combining PSPH inhibition with immune checkpoint blockers (e.g., anti-PD-1/PD-L1) in CRC models could validate its therapeutic potential to overcome immunotherapy resistance. Third, the clinical relevance of PSPH expression as a predictive biomarker for immunotherapy response warrants validation in larger patient cohorts. These investigations could pave the way for novel therapeutic strategies simultaneously targeting metabolic, autophagic and immune pathways in CRC.

In conclusion, our study establishes PSPH as a critical oncogenic driver in CRC through its dual regulation of AMPK-ULK1-mediated autophagy and immune evasion (Fig. 7). We demonstrate that PSPH overexpression activates a cytoprotective autophagy program that promotes tumor cell survival and proliferation, while simultaneously creating an immunosuppressive microenvironment through PD-L1 upregulation and CD8+ T cell exclusion. These findings not only expand our understanding of metabolic-immune crosstalk in CRC progression but also identify PSPH as a potential therapeutic target and predictive biomarker.

Ethical approval. The study was approved by the Ethics Committee of The First Affiliated Hospital of Bengbu Medical University.

Conflict of interest. The authors declare that there are no competing interests in this study.

Authors' contributions. Jiajia Guan was the main designer of this study. Jiajia Guan, Jie Ji, Bisheng Sun, Jun Fu, Jie Luo and Bing Zhu performed the experiments and analyzed the data. Jiajia Guan and Bing Zhu drafted the manuscript. All authors read and approved the final manuscript.

Data availability statement. All data generated or analyzed during the current study are available from the corresponding author on reasonable request.

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