

Original Article

GSG2 promotes aerobic glycolysis and colorectal cancer progression via the MDM2-p53 axis

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Abstract: Colorectal cancer (CRC) is the third most common type of cancer worldwide and ranks among the leading causes of cancer-related deaths. During tumor progression, post-translational modifications, mainly the ubiquitin-proteasome system, are critical mediators of cellular metabolic reprogramming, including the metabolic changes of tumor development. Although TP53 mutations occur in approximately half of all CRC cases and could define their genetic makeup, precise therapeutic vulnerabilities remain largely unknown for the subgroup with wild-type p53. This study investigated the significant expression of Germ Cell-Specific Gene 2 (GSG2) in CRC. We demonstrated GSG2 is strongly increased in malignant colorectal tissues compared with adjacent benign tissues, and this high expression strongly predicts poor patient survival. Additionally, the results indicate that removing GSG2 effectively suppresses tumor growth in both in vitro systems and in vivo xenograft models. Mechanistically, GSG2 directly binds to the E3 ligase MDM2, and promoted MDM2-mediated ubiquitination and degradation of p53 in p53 wild-type CRC cells. Consequently, GSG2 enhances the cellular metabolic program, reprogramming glucose use toward aerobic glycolysis to meet the energy needs of uncontrolled growth. Thus, our findings reveal a novel way that p53 is silenced in cancers with wild-type TP53. GSG2 demonstrate control of the MDM2-p53 axis to promote the Warburg effect and drive CRC progression via p53 suppression.

Keywords: GSG2, P53, MDM2, aerobic glycolysis, colorectal cancer

Introduction

Colorectal cancer remains one of the most prevalent and lethal malignancies worldwide [1]. Early-stage patients generally achieve favorable outcomes after surgical resection, reaching a five-year survival rate of over 90% [2]. The situation changes drastically, however, for patients with advanced or metastatic disease [3]. Most systemic options, including chemotherapy, targeted agents, and immunotherapy, show limited long-term efficacy. As a result, the five-year survival rate for advanced CRC drops below 15%, accompanied by frequent relapses [4]. To better guide therapeutic interventions, reliable markers must be identified. Such markers are needed to separate patients by clinical risk and forecast how their disease will progress.

Metabolic reprogramming of colorectal cancer cells, characterized by a shift to aerobic glycolysis, also known as the Warburg effect, is a core driving factor for cancer progression [5-8]. As a tumor suppressor, p53 plays a key negative regulatory role in this glycolytic shift, integrating its classic function in genomic stability with metabolic monitoring activities [9]. Cancer cells during colorectal cancer development are subjected to strong selective pressure and need to block the p53 signaling pathway. Approximately half of colorectal cancer cases achieve this through structural mutations in the TP53 gene [10-13], while a considerable proportion of cases still have wild-type TP53, but the function of p53 is also inhibited. Once the p53 protein is damaged by such structural mutations, it cannot stably trigger apoptosis or block cell cycle progression. Cells with damaged DNA will divide

uncontrollably due to this dysfunction and gradually accumulate more aggressive characteristics [14]. Existing studies have shown that wild-type p53 is often inhibited by abnormal post-translational degradation, which is regulated by upstream E3 ubiquitin ligases such as MDM2. The overactive ubiquitin-proteasome pathway continuously depletes wild-type p53 protein, thereby deregulating the glycolysis pathway to meet the energy demands of malignant tumors. The exact upstream regulators of the abnormally overactivated MDM2-p53 degradation axis in colorectal cancer remain fully undefined, which also represents a key gap in targeted therapeutic interventions.

In the search for such elusive upstream regulators, the germ cell-specific gene Haspin (GSG2) has emerged as a highly valuable candidate for research. Canonically, GSG2 functions as a conserved serine/threonine kinase that phosphorylates histone H3 at threonine 3 (H3T3ph) to secure chromosome alignment during mitosis [15-17]. Recent evidence indicates that GSG2 is abnormally overexpressed in various malignancies and actively participates in cell survival networks, including the AKT pathway [18, 19]. In CRC, preliminary in vitro data suggest that GSG2 depletion restricts cellular proliferation [20]. However, the precise molecular mechanisms, particularly whether GSG2 drives CRC progression strictly through its classical kinase activity or via undefined non-canonical protein-protein interactions, remain completely uncharacterized. In this study, we unveil a novel, non-canonical function of GSG2 in CRC. We demonstrate that GSG2 physically interacts with the E3 ligase MDM2, functioning primarily as a scaffold rather than a kinase, to accelerate the ubiquitination and degradation of wild-type p53.

Materials and methods

Bioinformatics analysis

Transcriptomic profiles and paired clinical metadata for the colorectal cancer cohort were extracted from The Cancer Genome Atlas (TCGA) repository. To quantify the baseline activation of the PI3K-Akt signaling cascade, we implemented a Z-score algorithmic approach. All subsequent statistical graphics and data visualizations were rendered within the R environment (version 4.2.2). First, expression data

(log2-transformed) for all genes within this pathway were extracted. Second, the expression of each gene across all samples was standardized to a Z-score (i.e., subtracting the mean and dividing by the standard deviation) to eliminate scale differences in expression abundance among different genes. Finally, the average of the Z-scores for all genes included in this pathway was calculated for each sample and used as the PI3K-Akt pathway activity score (Pathway Score) for that sample. Potential protein interactors of p53 were predicted using UbiBrowser 2.0 software.

Clinical sample analysis

The CRC specimens used in this study were obtained from patients at Jiujiang First People's Hospital. These patients were postoperatively pathologically diagnosed with CRC and had not received neoadjuvant therapy. This cohort included 30 CRC patients, from whom matched tumor and adjacent normal tissue specimens were obtained. All human sample collection procedures were conducted with written informed consent from the patients. The study protocol was approved by the Clinical Research Ethics Committee of Jiujiang First People's Hospital (JJSDYRMY-YXLL-2025-143).

Cell lines and culture

Human CRC cell lines RKO and HCT116 were purchased from BOSTER Biological Technology Co., Ltd. (Wuhan, China). All cell lines in this study were cultured in a sterile, humidified incubator at 37°C using RPMI-1640 medium (Solarbio, China) supplemented with antibiotics and 10% fetal bovine serum (FBS; Excell, Uruguay).

Western blotting

The following primary antibodies were used in Western blotting experiments: anti-GSG2 (1:1000; Thermo-Fisher, USA), anti-p53 (1:2000; Proteintech, China), anti-Ubiquitin (1:1000; Proteintech, China), and anti-Tubulin (1:10000; Proteintech, China). After electrophoresis and transfer, PVDF membranes were incubated with primary antibodies overnight on a shaker at 4°C. Subsequently, the membranes were washed three times with TBST (Seville, China) and probed with anti-rabbit or anti-mouse secondary antibodies (1:5000; Pro-

Table 1. Sequences of the primers used for RT-qPCR

Name	Sequence
GSG2	Forward (5'-GGAAGGGGTGTTGGCGAAGT-3')
	Reverse (5'-TGAGGAGCAAGGGAGGGTAAG-3')
TP53	Forward (5'-CAGCACATGACGGAGGTTGT-3')
	Reverse (5'-TCATCCAAATACTCCACACGC-3')
GAPDH	Forward (5'-TGACTTCAACAGCGACACCCA-3')
	Reverse (5'-CACCTGTTGCTGTAGCCAAA-3')

teintech, China) for 1 hour. After three TBST washes, the bands were visualized using an enhanced chemiluminescence (ECL) system. Finally, images were captured, and grayscale quantification was performed using ImageJ software (version 1.51).

Immunohistochemistry (IHC)

Tissue samples were embedded in paraffin, and sections of 3 μ m thickness were cut. The sections were incubated with an anti-GSG2 antibody (1:200; Thermo Fisher, USA) overnight at 4°C. The next day, the sections were washed three times with PBST, followed by incubation with an HRP-conjugated secondary antibody. After three PBST washes, DAB staining was performed. Finally, images were captured using a microscope.

Lentiviral transfection and stable cell line construction

Lentiviruses for GSG2 knockdown and overexpression were synthesized by Hanbio Biotechnology Co., Ltd. (Wuhan, China). Stable GSG2 knockdown or overexpression RKO and HCT116 cell lines were obtained through puromycin selection. qRT-PCR and Western blotting confirmed construction efficiency.

RNA extraction and qRT-PCR

Total RNA was extracted from both cell and tissue samples using an RNA extraction kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol. Subsequent cDNA synthesis was performed utilizing a reverse transcription kit (Beyotime Biotechnology). To preserve enzymatic integrity and minimize nonspecific amplification, all reaction setups were strictly executed on ice. Quantitative real-time PCR (qRT-PCR) was conducted in a final volume of 20 μ L, comprising 10.0 μ L

of TB Green Premix, 0.8 μ L of each forward and reverse primer, 0.4 μ L of ROX Reference Dye II, 2.0 μ L of the cDNA template, and 6.0 μ L of nuclease-free water. Prior to amplification, the sealed 96-well plates were centrifuged at 3000 $\times$ g for 3 min at 4°C. Thermal cycling was performed on a real-time PCR system with an

initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Melt curve analysis was routinely incorporated at the end of the protocol to verify the specificity of the amplified products. All samples were analyzed in technical triplicates, with GAPDH serving as the endogenous control for normalization. The relative mRNA expression levels of GSG2 were calculated employing the $2^{-\Delta\Delta Ct}$ method. Statistical analysis and data visualization were performed using GraphPad Prism software (version 10.1.2). The precise nucleotide sequences of all primers utilized are detailed in **Table 1**.

CCK-8 assay

A total of 5.0×10^3 cells were seeded into each well of 96-well plates. Cell viability was then determined at designated intervals using the Cell Counting Kit-8 (CCK-8; Uelandy, China) as per the manufacturer's protocol. Measurements of absorbance at a wavelength of 450 nm were taken at designated intervals employing a microplate reader.

Wound healing and transwell assays

After seeding cells in a 6-well plate and allowing them to adhere completely, scratches were made using a sterile pipette tip to perform a wound healing assay. Cells were observed periodically under a microscope, and their migration toward the wound area was recorded by photography. In Transwell migration and invasion assays, cells were seeded into the upper chamber of Transwell inserts with a pore size of 8 μ m, which were purchased from Corning Inc., USA. Medium containing 10% fetal bovine serum was added to the lower chamber as a chemoattractant. After incubation for the set duration, the cells that had migrated or invaded to the lower surface of the membrane were fixed, stained, and photographed for record.

Colony formation assay

In the colony formation assay, single-cell suspensions were seeded into 6-well plates at a density of 1000 cells per well and cultured for 14 days. The culture medium was carefully replaced every 72 hours. After incubation, the colonies were fixed with 4% paraformaldehyde for 20 minutes and then stained with 0.1% crystal violet. The stained plates were photographed for record, and the number of colonies was counted using ImageJ software (v1.8).

CHX chase assay

Cycloheximide chase assay was used to detect the half-life of proteins. Stably transduced cells were treated with 25 µg/mL cycloheximide (Cat. No. HY12320, MedChemExpress) to block de novo protein synthesis. Cell pellets were collected at the specified time. To prevent protein degradation during the extraction process, cells were lysed with freshly prepared commercial lysis buffers supplemented with a mixture of protease and phosphatase inhibitors (87787, Thermo Fisher; 78442, Thermo Fisher). The degradation rate changes of the target protein were detected by conventional Western blotting using the centrifuged total protein lysate.

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)

Mitochondrial respiration and glycolytic function were measured with an XF96 Extracellular Flux Analyzer. The XF Cell Mito Stress Test and XF Glycolysis Stress Test kits (Seahorse Bioscience, USA) were used in accordance with the manufacturer's guidelines. Data visualization was carried out using Prism.

Co-immunoprecipitation and ubiquitination assays

Co-immunoprecipitation (Co-IP) experiments were performed as follows: Cells were lysed with pre-cooled RIPA buffer containing protease and phosphatase inhibitors, and the supernatant was collected (4°C, 12000 rpm, 10 min). The supernatant was incubated with 6 µg of primary antibody with gentle shaking overnight at 4°C. The next day, 40 µL of Protein A/G PLUS-agarose beads (Beyotime, China) were added and incubated with shaking at 4°C for 8-12 hours. After incubation, the beads were collected by centrifugation at 2500 rpm for 10 minutes at 4°C, and the precipitate was sub-

jected to Western blot analysis. For ubiquitination assays, GSG2-overexpressing or knockdown cells were pre-treated with the proteasome inhibitor MG132 for 6 hours prior to lysis. Subsequently, immunoprecipitation was performed using an anti-p53 or anti-MDM2 antibody, followed by Western blot analysis using an anti-ubiquitin antibody.

Subcutaneous xenograft tumor model

Five- to six-week-old female BALB/c nude mice were purchased from Hangzhou Ziyuan Laboratory Animal Technology Co., Ltd. and randomly divided into experimental groups (n = 6 per group). After acclimatization for one week under specific pathogen-free (SPF) conditions, each mouse received a subcutaneous injection in the axilla of 1×10^6 CRC cells transduced with lentivirus for GSG2 knockdown or negative control (NC). Tumor formation was monitored regularly after inoculation, and tumor volume was measured every 5 days. After 6 weeks, all mice were deeply anesthetized with isoflurane and then euthanized by cervical dislocation, and tumors were excised for further analysis. The study protocol was approved by the Animal Ethical and Welfare Committee (NCULAE-2026-0325001).

Statistical analysis

Statistical analyses were performed using R (v4.2.2) and GraphPad Prism (v10.1) software. Data are presented as the mean ± standard deviation. Specifically, for comparisons between two groups, two-tailed Student's t-tests (paired or unpaired, as appropriate) were used for normally distributed data, while the Mann-Whitney U test was applied for data that deviated from normality. For data involving repeated measurements over time, such as tumor volume growth, a repeated measures ANOVA was employed. For in vitro time-course assays like CCK-8 proliferation, a two-way ANOVA followed by Sidak's multiple comparisons test was utilized. Differences in survival curves were assessed with the Log-rank test. $P < 0.05$ was considered statistically significant.

Results

GSG2 is upregulated in colorectal cancer and associated with poor prognosis

We performed an analysis based on The Cancer Genome Atlas (TCGA) database, which showed

that the mRNA expression level of GSG2 was significantly higher in colorectal cancer (CRC) tissues compared to normal tissues (**Figure 1A**). To validate this finding in an independent cohort, we examined GSG2 expression in 30 pairs of matched CRC and adjacent normal tissue samples. qRT-PCR analysis confirmed that the mRNA expression level of GSG2 was significantly higher in CRC tissues than in their paired normal counterparts (**Figure 1B, 1C**). Western blotting showed that GSG2 protein levels were higher in CRC tissues than in adjacent normal tissues (**Figure 1D-F**). Immunohistochemical staining then confirmed this upregulation at the tissue morphology level (**Figure 1G, 1H**). To assess the clinical relevance of GSG2 expression, we performed survival analysis using a CRC cohort from Jiujiang First People's Hospital. The level of GSG2 is directly associated with the risk of death in patients, which has been confirmed by long-term follow-up of these patients. The overall survival and disease-free survival curves of the high-expression group show a significant decline (**Figure 1I, 1J**). When such proteins accumulate abnormally, it often directly indicates that the condition will progress to a more severe stage.

GSG2 enhances colorectal cancer cell growth both in vitro and in vivo

Using wild-type TP53 cell lines RKO and HCT116 as core experimental models, the degree of cell dependence on GSG2 was detected (**Figure 2A-D**). After adjusting the expression level of this kinase, the proliferation status of cells changes significantly. The results of CCK-8 and colony formation assays showed that ectopic expression of GSG2 accelerated cell division, while inhibition of GSG2 expression almost completely arrested cell growth, which was consistent with the results shown in **Figure 2E-H**. The motility of cells also depended on the function of GSG2. Wound healing and Transwell migration were severely impaired in GSG2-knockdown cells compared to the robust movement of control groups (**Figure 2I-L**). These in vitro observations were strictly recapitulated in vivo. Following subcutaneous engraftment, GSG2-depleted tumors exhibited profound growth delays, yielding final tumor masses and volumes significantly below those of control xenografts (**Figure 2M-R**). Collectively, the data indicate that CRC cells strictly require GSG2 to sustain aggressive proliferation and tissue invasion.

GSG2 promotes p53 degradation via the proteasome pathway and activates the AKT pathway

To test cell dependency, GSG2 expression was knocked down in a panel of CRC lines (RKO, HCT116, and SW620). Growth was restricted in the RKO and HCT116 cultures. SW620 cells, however, showed little change (**Figure 3A**). Public TCGA datasets were checked to explain this gap. A positive correlation emerged between GSG2 and the AKT signaling pathway (**Figure 3B**). RKO and HCT116 carry wild-type TP53. SW620 carries a mutated version. We hypothesized the p53 background played a role. Protein levels matched this genetic split. In the wild-type TP53 lines, knocking down GSG2 dropped AKT phosphorylation and simultaneously forced an accumulation of p53 (**Figure 3C, 3D**). The SW620 mutant cells reacted differently. AKT signaling still decreased after knockdown, but basal p53 levels did not change at all (**Figure 3E**). To rule out transcriptional regulation, qRT-PCR was performed. Modulating the kinase yielded no significant fluctuations in p53 mRNA transcripts (**Figure 3F-H**), pointing directly to a post-translational mechanism. The protein half-life was detected using the conventional cycloheximide chase assay, and the results showed that the degradation rate of p53 was significantly slowed down after GSG2 knockout (**Figure 3I-L**). After blocking the proteasome function with MG132, the phenomenon that ectopic expression of GSG2 usually accelerates p53 clearance was effectively reversed, corresponding to **Figure 3M, 3N**. To characterize the biochemical properties of this instability, a profile of endogenous ubiquitination was generated. Immunoprecipitation assays showed that inhibition of GSG2 expression significantly reduced the amount of polyubiquitin chains attached to p53 (**Figure 3O, 3P**). The activity pattern of GSG2 shows that it actively promotes the rapid proteasomal degradation of normal p53. This proteolytic process occurs synchronously with the maintenance of AKT, which not only represses the checkpoint function of p53 but also maintains the normal state of survival-related signals.

GSG2 knockdown inhibits aerobic glycolysis in CRC cells

Wild-type p53 can inhibit the Warburg effect by restricting glycolysis [21-23]. Following the pre-

GSG2 drives CRC via MDM2-p53 axis

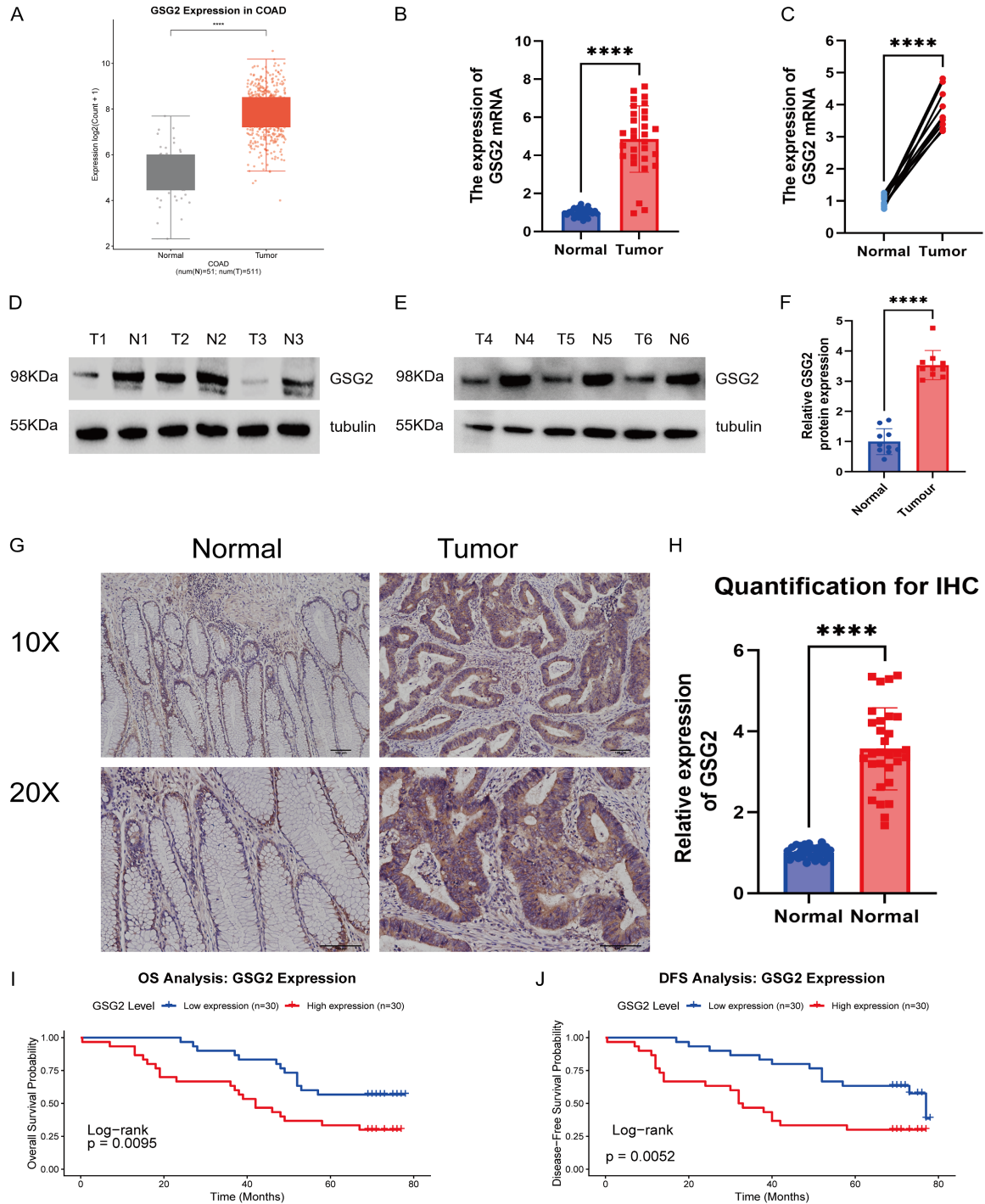
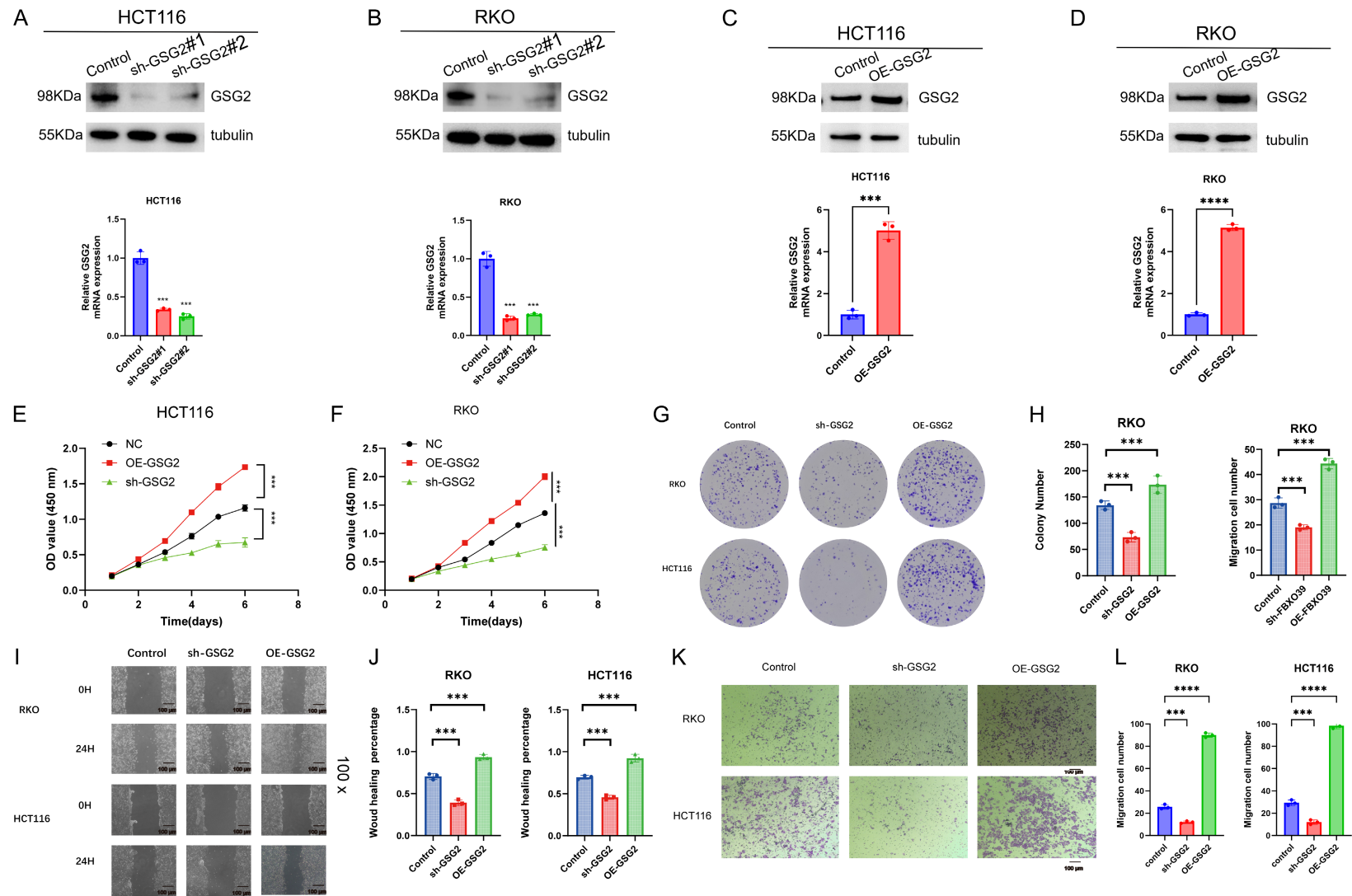


Figure 1. GSG2 is upregulated in colorectal cancer and associated with poor prognosis. A. Analysis of GSG2 mRNA expression in colorectal cancer versus normal tissues based on The Cancer Genome Atlas (TCGA) database. Statistical analysis for public database expression was determined by the two-tailed Unpaired Student's t-test. B, C. qRT-PCR analysis of GSG2 mRNA expression in 10 paired CRC tissues and adjacent normal tissues. Quantitative data for paired clinical mRNA were analyzed using the two-tailed Paired Student's t-test. D-F. Western blot analysis and relative quantification of GSG2 protein levels in paired clinical CRC samples. The protein quantification data were analyzed using the two-tailed Paired Student's t-test. G, H. Immunohistochemistry (IHC) staining and corresponding quantification of GSG2 expression in CRC and normal tissues. The IHC scoring data were evaluated using the two-tailed Paired Student's t-test. I, J. Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) in a retrospective CRC cohort (n = 60) stratified by high or low GSG2 expression, analyzed using the Log-rank test. Data are presented as mean $\pm$ SD. Statistical significance is indicated as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

GSG2 drives CRC via MDM2-p53 axis



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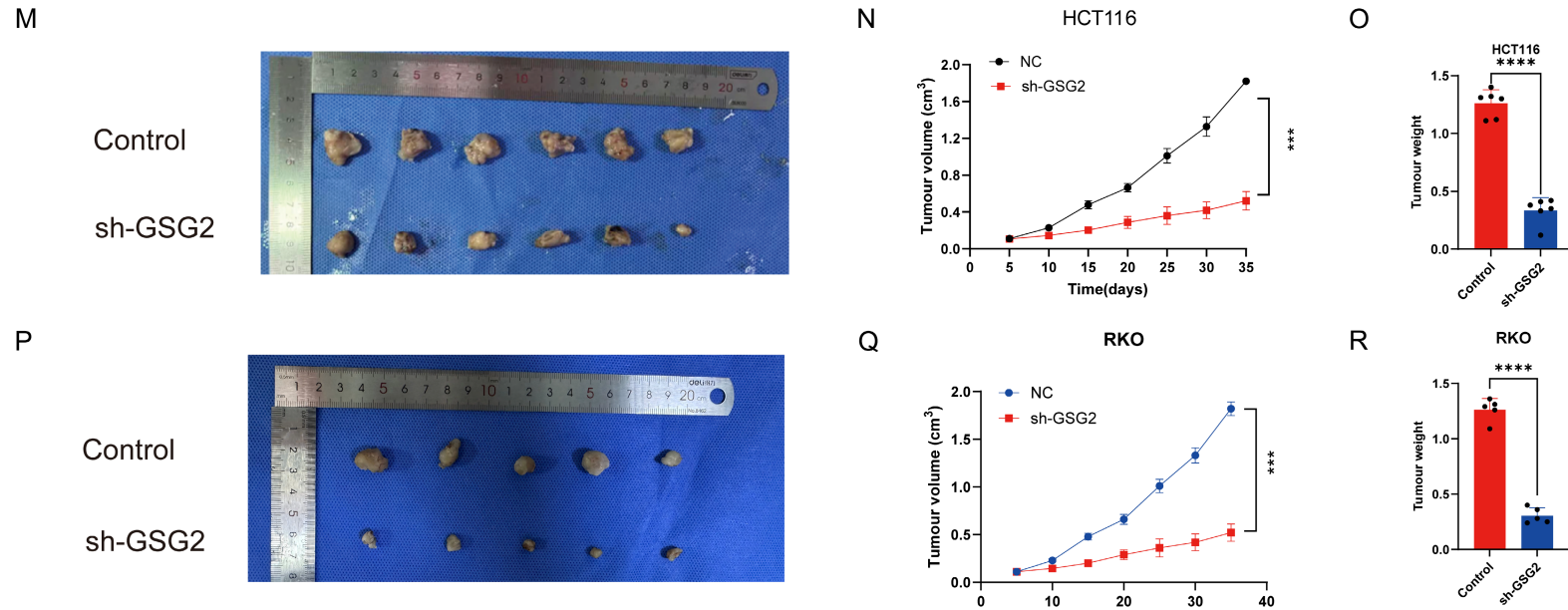
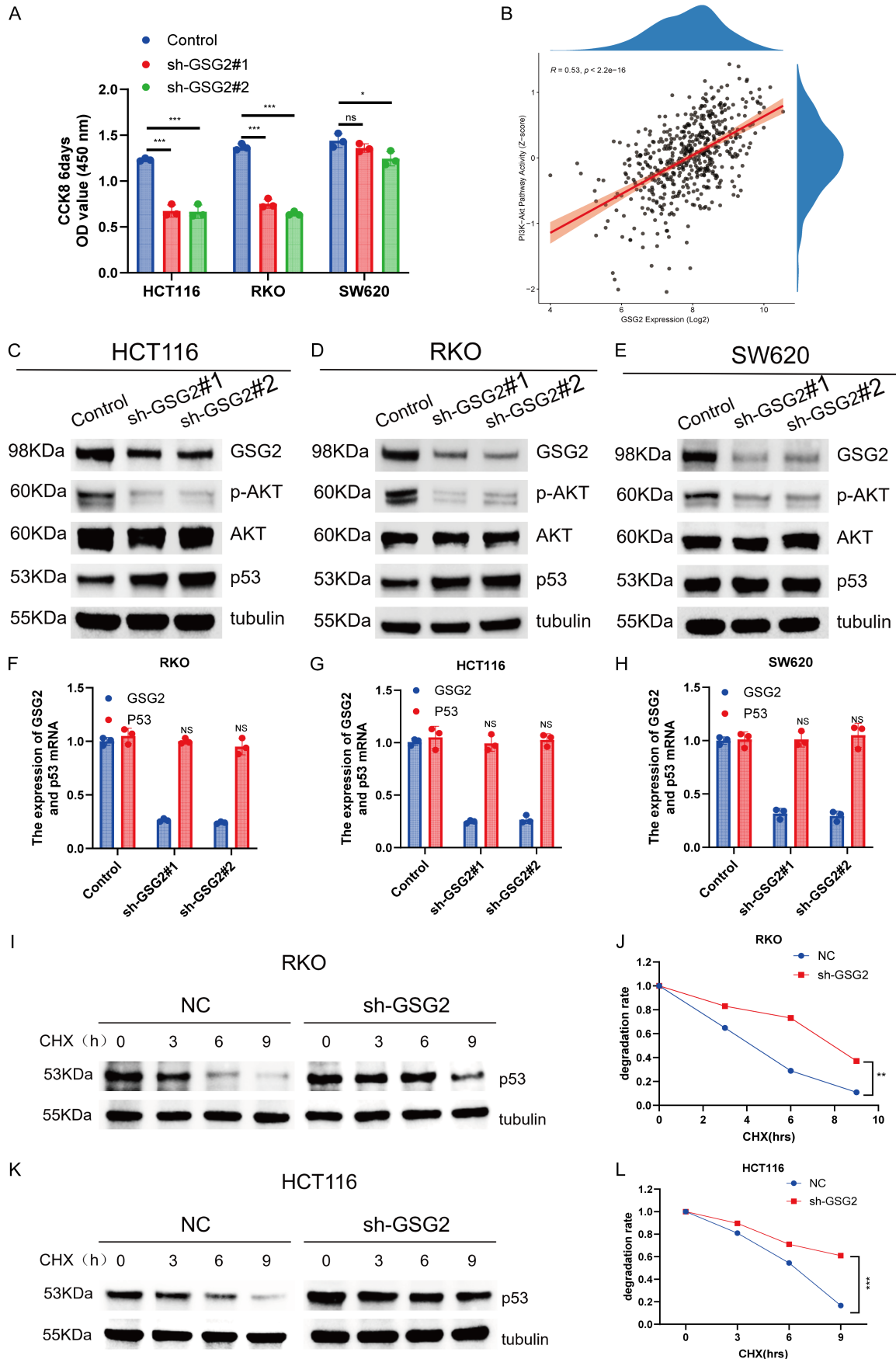


Figure 2. GSG2 promotes CRC cell proliferation, migration, and tumor growth. A-D. Western blot analysis and qRT-PCR validation of stable GSG2 knockdown and overexpression in p53 wild-type CRC cell lines HCT116 and RKO. Statistical significance for knockdown multi-group comparisons was determined by One-way ANOVA with Dunnett's post hoc test, and for overexpression two-group comparisons by the two-tailed Unpaired Student's t-test. E, F. Cell proliferation assessed by CCK-8 assay in RKO and HCT116 cells over a 6-day period. Time-course data were analyzed using Two-way ANOVA followed by Sidak's post hoc test. G, H. Representative images and quantification of colony numbers evaluating long-term clonogenic ability. Data were evaluated using One-way ANOVA followed by Tukey's multiple comparisons test. I, J. Cell migration capability analyzed by wound healing and Transwell assays. Statistical analysis was performed using One-way ANOVA followed by Tukey's multiple comparisons test. K, L. Cell migration capability analyzed by wound healing and Transwell assays. Statistical analysis was performed using One-way ANOVA followed by Tukey's multiple comparisons test. M-O. In vivo tumor growth evaluated in a subcutaneous xenograft model using nude mice, showing tumor volume growth curves, final tumor images, and tumor weight. The longitudinal tumor volume growth curves were strictly analyzed via Repeated-measures Two-way ANOVA, while final tumor weights were compared using the two-tailed Unpaired Student's t-test. P-R. Subcutaneous xenograft tumor model in nude mice using RKO cells. Representative images of dissected tumors, tumor volume growth curves, and final tumor weights from the Control and sh-GSG2 groups. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

GSG2 drives CRC via MDM2-p53 axis



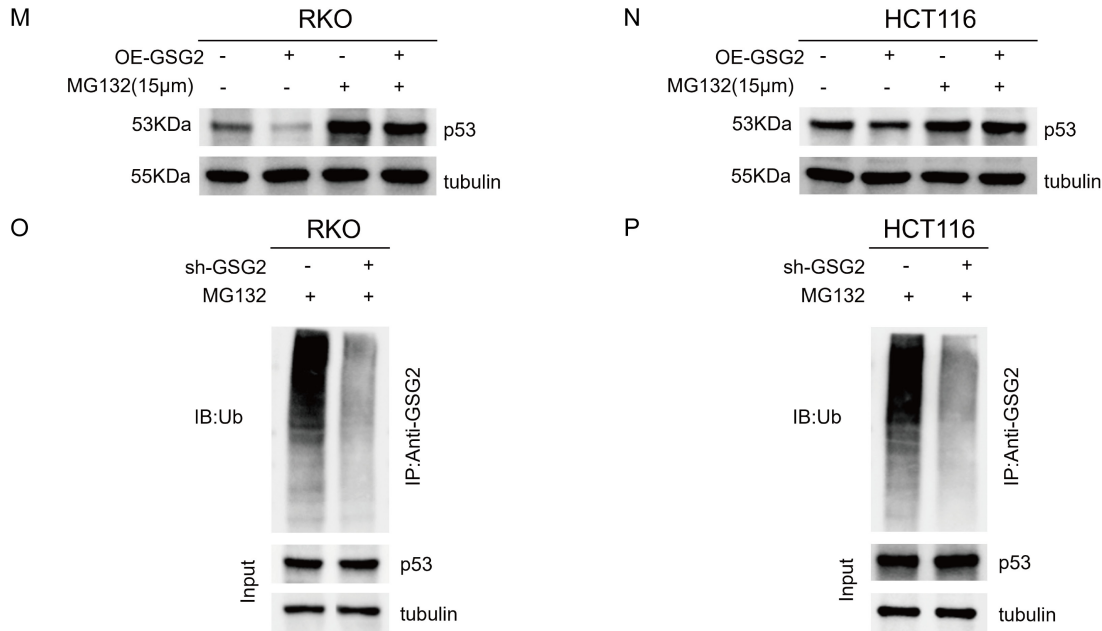


Figure 3. GSG2 promotes p53 degradation via the ubiquitin-proteasome pathway and activates AKT signaling. A. Cell proliferation measured by CCK-8 assay in RKO, HCT116 (p53-WT), and SW620 (p53-Mut) cells upon GSG2 knockdown. Statistical significance was determined using One-way ANOVA followed by Dunnett's post hoc test. B. Correlation analysis showing the positive association between GSG2 expression and PI3K-Akt pathway activity (Z-score) in CRC patient cohorts. The correlation was evaluated using the Pearson correlation coefficient. C-E. Western blot analysis of AKT pathway markers (p-AKT) and p53 protein levels after GSG2 knockdown. F-H. qRT-PCR analysis of p53 mRNA levels after GSG2 manipulation in RKO, HCT116, and SW620 cells. Data were analyzed using One-way ANOVA followed by Dunnett's post hoc test. I-L. p53 protein stability assessed by cycloheximide (CHX) chase assay. The time-course degradation rates were statistically analyzed using Two-way ANOVA accompanied by Sidak's post hoc test. M, N. Effect of the proteasome inhibitor MG132 (15 μM, 6 h) on p53 levels in GSG2-overexpressing cells. O, P. In vivo ubiquitination assay detecting polyubiquitinated p53 levels after GSG2 knockdown in the presence of MG132. Data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ns: not significant.

vious observation of the GSG2-p53 degradation pathway, we tracked the basal metabolic markers in TP53 wild-type cell lines RKO and HCT116. After GSG2 is knocked down, both glucose uptake and intracellular glucose-6-phosphate accumulation decrease directly. Subsequently, substrate availability decreases, and lactate secretion and total ATP production are also significantly reduced. Knockout of GSG2 in cells effectively blocks the glycolytic metabolic process (Figure 4A, 4B). Subsequently, the extracellular acidification rate was recorded in real time to dynamically track such metabolic changes. Standard inhibitors such as glucose, oligomycin and 2-DG were added sequentially. The results showed that GSG2 deficiency reduces the level of basal glycolysis. The maximum glycolytic capacity of these cells is also limited (Figure 4C, 4D, 4G, 4H). Mitochondrial function was subsequently examined. The oxygen consumption rate was monitored. Compensatory changes occurred after the sequential

addition of oligomycin, carbonyl cyanide m-chlorophenylhydrazone, and rotenone/antimycin A. Cells exhibit greater dependence on mitochondrial metabolism in the absence of GSG2. Significant increases are observed in both basal respiration and maximal respiration (Figure 4E, 4F, 4I, 4J). After the removal of GSG2, the Warburg phenotype basically disappears, and cancer cells no longer rely on aerobic glycolysis. They switch to mitochondrial oxidative phosphorylation to meet their own energy demands.

GSG2 promotes p53 ubiquitination and degradation by interacting with MDM2

The specific E3 ligase that acts upstream of GSG2 remains to be identified. A search of UbiBrowser 2.0 allows for the prediction of proteins that may interact with p53. From this screening, MDM2 emerged as a key candidate (Figure 5A). This specific ligase has been previ-

GSG2 drives CRC via MDM2-p53 axis

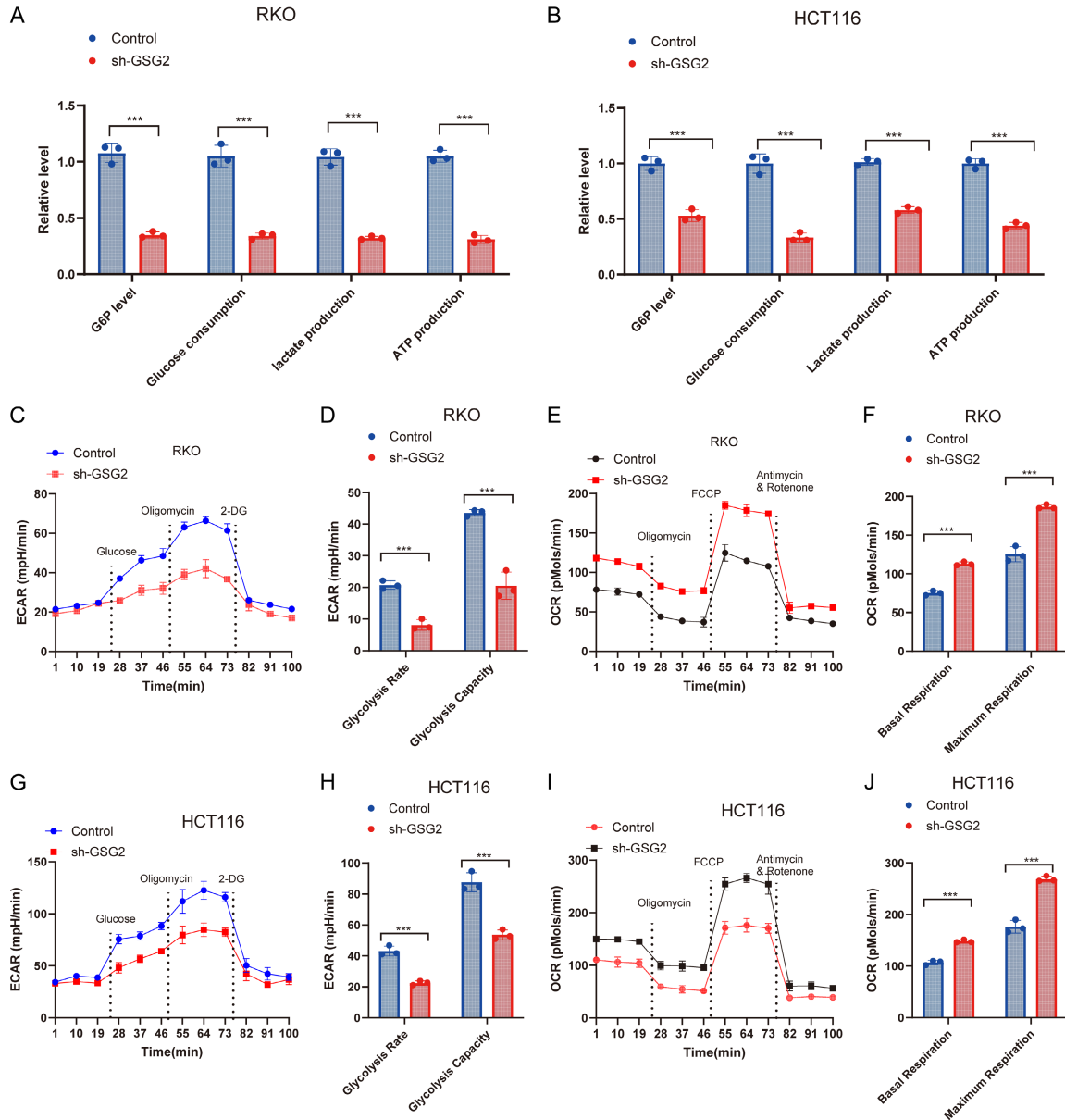


Figure 4. GSG2 knockdown inhibits aerobic glycolysis in CRC cells. (A, B) Relative levels of glucose-6-phosphate (G6P), glucose consumption, lactate production, and ATP generation in control and GSG2-knockdown RKO and HCT116 cells. Statistical significance was determined using the two-tailed Unpaired Student's t-test. (C, G) Real-time extracellular acidification rate (ECAR) measured to assess glycolytic flux in RKO and HCT116 cells. Vertical dashed lines indicate the sequential injection of glucose (10 mM), oligomycin (1 μ M), and 2-deoxyglucose (2-DG, 50 mM). Time-resolved kinetic traces were evaluated via Two-way ANOVA. (D, H) Quantification of Glycolysis Rate and Glycolysis Capacity derived from the ECAR assays. Statistical differences between the two groups were calculated using the two-tailed Unpaired Student's t-test. (E, I) Real-time oxygen consumption rate (OCR) measured to assess mitochondrial respiration in RKO (E) and HCT116 (I) cells. Vertical dashed lines indicate the sequential injection of oligomycin (1 μ M), FCCP (1 μ M), and antimycin A & rotenone (0.5 μ M). Time-resolved kinetic traces were evaluated via Two-way ANOVA. (F, J) Quantification of Basal Respiration and Maximum Respiration derived from the OCR assays. Statistical differences between the two groups were calculated using the two-tailed Unpaired Student's t-test. Data are presented as mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001.

ously found to ubiquitinate and degrade p53 [24, 25]. The basic activity of MDM2 was first checked in our cell models. Cycloheximide

(CHX) chases were run. Adding extra MDM2 caused p53 to degrade much faster (Figure 5B, 5C). The proteasome inhibitor MG132 was then

added to the cultures. The loss of p53 driven by MDM2 was completely blocked by this drug (**Figure 5D, 5E**). The ubiquitin-proteasome system is clearly responsible for this clearance. A physical link between GSG2 and MDM2 was examined next. Endogenous co-immunoprecipitation (Co-IP) confirmed a direct association. GSG2 and MDM2 form a stable complex inside the cells (**Figure 5F, 5G**). Molecular docking simulations were also performed. These models pointed to a highly favorable binding interface between the two proteins (**Figure 5J, 5K**). The biological effect of this binding was then tested. MDM2-p53 affinity was measured while altering GSG2 levels. Total MDM2 protein amounts stayed exactly the same. Yet, the interaction between MDM2 and p53 shifted drastically. Co-IP outputs showed that excess GSG2 forces more p53 to bind with MDM2. The level of free p53 in the lysis buffer decreased almost to zero, and the binding between MDM2 and p53 was disrupted after GSG2 was knocked down. Intracellular p53 begins to accumulate again (**Figure 5H, 5I**), and GSG2 acts as a physical scaffold in this mechanism. It binds directly to MDM2. This binding is likely to induce allosteric changes, allowing the RING domain of the E3 ubiquitin ligase to open. MDM2 significantly increases the ubiquitination rate of p53, thereby rapidly degrading p53.

GSG2 regulates CRC cell proliferation and glycolytic metabolism via the MDM2-p53 axis

A rescue assay was established to fully map the signaling pathway, and the role of p53 was examined first. Dual knockdown experiments of GSG2 and p53 were performed in HCT116 cells (**Figure 6A**). Cell proliferation was detected using the CCK-8 assay. Growth arrest typically occurs when GSG2 is absent, and this phenomenon can be reversed by complete knockout of p53 (**Figure 6B**). Obviously, GSG2 requires the downregulation of p53 to promote cell proliferation. This study turned to MDM2. The researchers reintroduced the expression of MDM2 into the cell culture system in which GSG2 was knocked out. Western blot results showed that the p53 protein content returned to the original level after reintroduction of the relevant substance (**Figure 6C**). The cell proliferation rate accelerated again accordingly (**Figure 6D**). The responses of tumor metabolism are completely consistent. After the initial knockdown of GSG2, glucose uptake, glucose-6-phosphate accumu-

lation, lactate release, and ATP production are all inhibited. After restoration of MDM2, all metabolic abnormalities were corrected (**Figure 6E**). The results of dynamic flux analysis were consistent with the static detection data. In the absence of GSG2, the extracellular acidification rate decreases, and after the addition of MDM2, this rate returns to its original level (**Figure 6F**). The compensatory increase in oxygen consumption has also reversed (**Figure 6G**). GSG2 eliminates the p53 checkpoint via MDM2. After the elimination of this tumor suppressor, the glycolysis process restarts, providing the necessary conditions for the proliferation of colorectal cancer cells.

Discussion

Metabolic reprogramming, characterized by a fundamental shift toward aerobic glycolysis, is a core driver of colorectal cancer malignant progression and a major hurdle for clinical intervention [26]. While the tumor suppressor p53 canonically restricts this glycolytic shift, the specific mechanisms by which CRC cells with wild-type TP53 evade this metabolic checkpoint remain incompletely defined. To break through the current therapeutic bottleneck, it is essential to clarify the specific molecular pathways driving disease progression and identify new actionable vulnerabilities [27, 28]. This study identifies GSG2 as a previously underappreciated therapeutic target. Instead of acting through a single linear pathway, this kinase drives tumorigenesis via a dual-node regulatory axis.

GSG2 is classically recognized as a mitotic kinase essential for cell division [17, 29]. GSG2 is increasingly recognized for its oncogenic roles, its overexpression reliably predicts poor prognosis and drives disease progression in several solid tumors [30-33]. While earlier work has mapped its ability to co-opt AKT signaling in endocrine and gynecological models [18, 19], its potential impact on the gastrointestinal tract had not been explored. We establish here that this regulatory dynamic firmly extends to CRC. Clinical CRC specimens exhibited widespread GSG2 upregulation. More importantly, silencing this kinase led to severe growth arrest in both in vitro cultures and in vivo xenografts. Rather than pushing a single downstream pathway, GSG2 acts as a dual-node signaling hub, concurrently fueling AKT activity while accel-

GSG2 drives CRC via MDM2-p53 axis

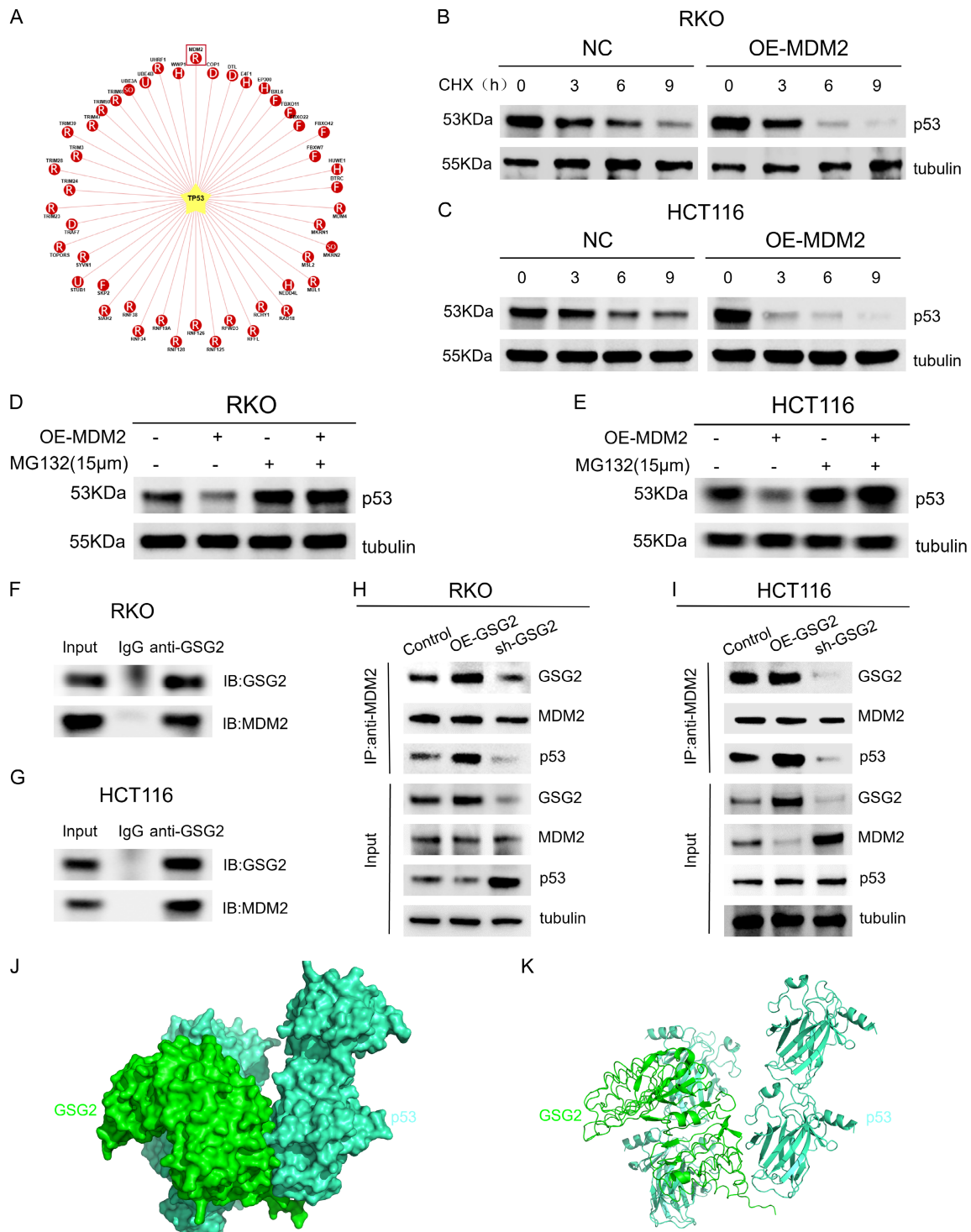


Figure 5. GSG2 interacts with MDM2 to enhance p53 ubiquitination and degradation. A. Predicted interaction network between p53 and its E3 ligases from UbiBrowser 2.0 analysis. B, C. p53 protein stability assessed by CHX chase assay in MDM2-overexpressing RKO and HCT116 cells. D, E. Effect of MG132 (15 μM) on p53 levels in MDM2-overexpressing cells. F, G. Co-immunoprecipitation (Co-IP) assay demonstrating the interaction between endogenous GSG2 and MDM2. H, I. Co-IP using an anti-MDM2 antibody to assess MDM2-p53 binding under conditions of GSG2 knockdown or overexpression; total cell lysates (Input) show corresponding p53 levels. J, K. Molecular docking simulation model of the GSG2-MDM2 interaction interface.

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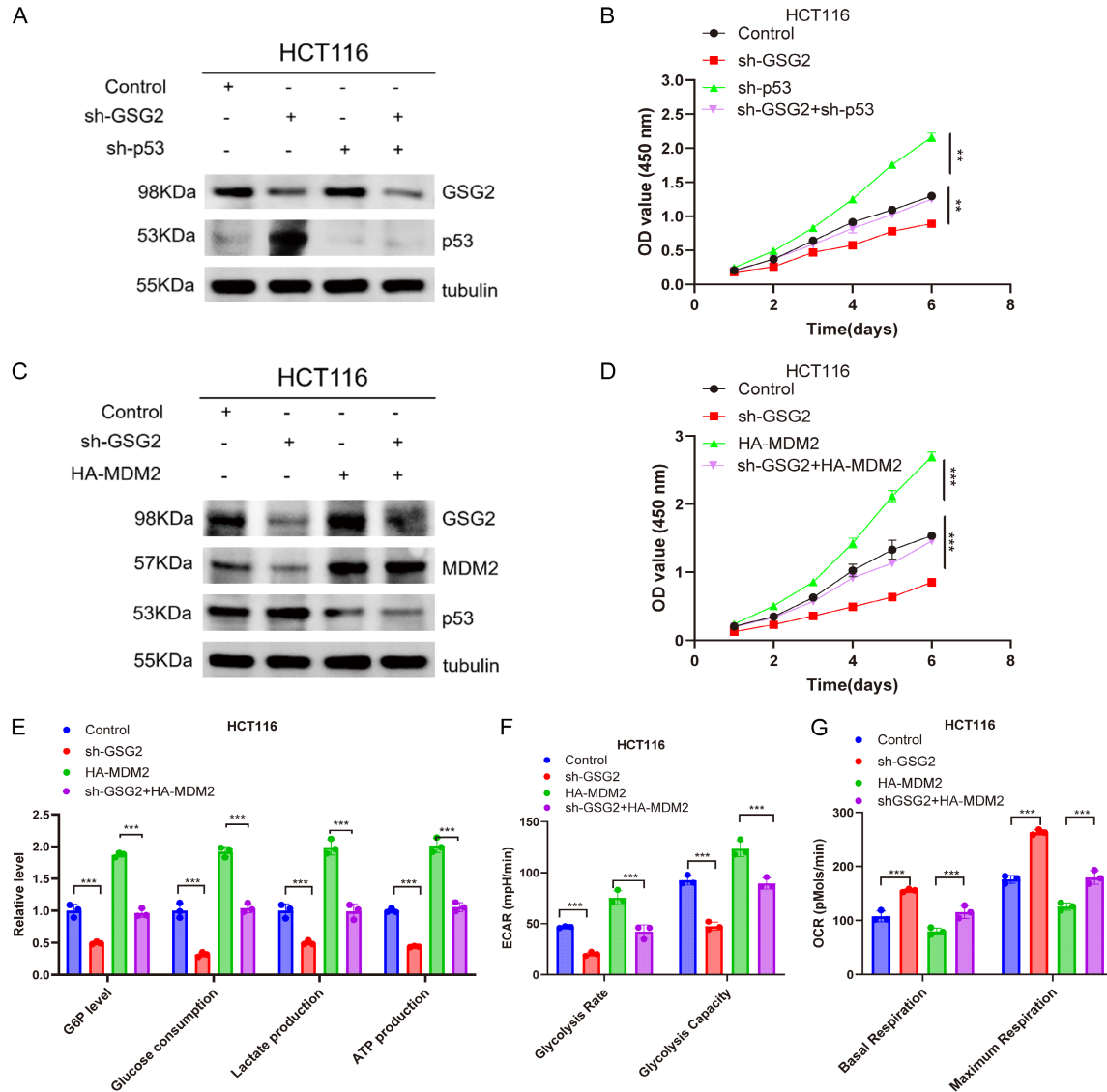


Figure 6. GSG2 regulates cell proliferation and glycolysis via the MDM2-p53 axis. A, B. Western blot validation of p53 and GSG2 co-knockdown in HCT116 cells and the corresponding cell proliferation assessed by CCK-8 assay over a 6-day period. Time-course proliferation data were analyzed using Two-way ANOVA followed by Tukey's post hoc test. C, D. Western blot analysis confirming the restoration of MDM2 (HA-MDM2) in GSG2-knockdown HCT116 cells and the resulting cell proliferation assessed by CCK-8 assay. Statistical significance for the growth curves was determined using Two-way ANOVA followed by Tukey's post hoc test. E. Relative levels of G6P, glucose consumption, lactate production, and ATP generation under indicated conditions. Data were evaluated using One-way ANOVA followed by Tukey's multiple comparisons test. F, G. Quantification of glycolytic function (Glycolysis Rate and Capacity) assessed by ECAR measurement, and mitochondrial respiration (Basal and Maximum Respiration) assessed by OCR measurement. Statistical differences among the four experimental groups were calculated using One-way ANOVA followed by Tukey's multiple comparisons test. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ating the breakdown of p53. Notably, cancer cells typically require multiple distinct genetic alterations to achieve concurrent activation of pro-survival signaling and inactivation of tumor suppressor pathways, whereas GSG2 accomplishes both via a single molecule. Specifically,

GSG2 acts as a catalytic scaffold for MDM2 to mediate the ubiquitination and degradation of wild-type p53, while concurrently sustaining the activation of the AKT signaling cascade to drive metabolic flux. This dual regulatory mechanism provides a clear molecular basis for the

potent oncogenic role of GSG2 in CRC, and establishes GSG2 as a high-value therapeutic target for CRC treatment. Although our research findings provide a fundamental understanding of GSG2 mediated metabolic reprogramming in colorectal cancer, there are still several key limitations that need to be addressed in future related studies. The results we measured showed that after knocking down GSG2, the protein level of p53 would increase, while the phosphorylation level of AKT would decrease. However, it is not yet fully understood how these two signals are related. It is still uncertain whether GSG2 affects the activation of AKT by regulating the degradation of p53, such as preventing p53 from blocking downstream pathways, or whether GSG2 takes a completely unrelated path to regulate AKT. Future investigations utilizing TP53 knockout models in combination with targeted PI3K/AKT inhibitors are warranted to elucidate this potential signal crosstalk.

Aerobic glycolysis is a core metabolic hallmark of CRC, and acts as the main metabolic engine fueling CRC malignant progression [34, 35]. Accumulating studies have uncovered multiple epigenetic and post-translational regulatory mechanisms involved in CRC metabolic reprogramming, including METTL3-mediated mRNA stabilization [36], PRMT1-driven methylation modification [37], and UBD1-dependent c-Myc accumulation [38]. However, the upstream kinase regulatory networks that govern this metabolic reprogramming in CRC remain incompletely understood. Given that both AKT and p53 are well-established master regulators of cellular energy metabolism, we further investigated the regulatory role of GSG2 in CRC metabolic reprogramming. Our data demonstrate that GSG2 functions as a master regulator of aerobic glycolysis in CRC. Genetic depletion of GSG2 markedly blocked aerobic glycolysis in CRC cells, and redirected cellular energy metabolism towards mitochondrial oxidative phosphorylation. These findings reveal that GSG2 is essential for maintaining the tumor-preferred metabolic state in CRC, and that this metabolic dependency driven by GSG2 represents a critical therapeutic vulnerability in CRC.

Focusing on the p53 tumor suppressor gene, a core regulator of cellular metabolism, this study aims to further clarify the molecular mechanism by which GSG2 regulates aerobic glycoly-

sis. The ability of p53 to inhibit tumor growth is largely dependent on the restriction of aerobic glycolysis [39, 40]. However, such metabolic regulation is not static and is susceptible to post-translational modifications. The CAD enzyme directly deamidates p53 at specific asparagine residues N235 and N239, which significantly impairs its transcriptional activity [41]. As an E3 ligase, TRIM33 targets p53 for K48-linked polyubiquitination. Proteasomal degradation through this specific pathway can effectively relieve glycolysis arrest and remove obstacles for tumorigenesis [42]. In addition to enzymes, metabolic byproducts can also play regulatory roles. Local lactic acid accumulation triggers AARS1-dependent lactylation modification of p53. This structural change impairs DNA binding and weakens the inhibitory function of p53 [43]. In clinical TP53 wild-type colorectal cancer, despite these known modification mechanisms, p53 protein is frequently lost, which is a major paradox [44]. This unresolved situation directly drives subsequent experimental studies. Knockdown of GSG2 significantly elevated p53 protein levels in wild-type p53 CRC cells. Mechanistically, GSG2 facilitates the ubiquitination and degradation of p53. Functional assays further confirmed that GSG2 promotes aerobic glycolysis and suppresses mitochondrial oxidative phosphorylation by downregulating p53 in CRC cells. Collectively, our results demonstrate that GSG2 enhances aerobic glycolysis and malignant proliferation of CRC cells by accelerating the ubiquitination-dependent degradation of wild-type p53. The targeted degradation of p53 is predominantly mediated by the E3 ubiquitin ligase MDM2, which is the primary negative regulator of the p53 signaling network [45, 46]. Under physiological conditions, MDM2 and wild-type p53 form a classic autoregulatory feedback loop to maintain cellular homeostasis [47], while mutant p53 can evade MDM2-mediated recognition and degradation [48]. Our data establish that, in CRC cells with wild-type TP53, GSG2 physically interacts with MDM2 to accelerate MDM2-mediated p53 ubiquitination and degradation, rather than merely upregulating MDM2 expression. We further confirmed that GSG2 acts as a critical upstream scaffold for the MDM2-p53 axis: restoration of MDM2 in GSG2-depleted CRC cells fully reversed the metabolic reprogramming and growth inhibition induced by GSG2 knockdown, and re-established aero-

bic glycolysis and rapid tumor growth. This functional dependency demonstrates that GSG2 locks CRC cells into an aggressive, glycolytic phenotype via the MDM2-p53 axis, which has important therapeutic implications. We have indeed found direct binding between GSG2 and E3 ubiquitin ligase MDM2 through endogenous co immunoprecipitation experiments and molecular docking simulations. However, the specific biochemical details behind the binding of these two proteins still need to be further studied. After GSG2 binding, it may serve as a scaffold to induce conformational changes, such as opening up the RING domain of MDM2. However, there is currently no clear structural data to confirm this change in the three-level structure. In addition, GSG2 may have an impact on the subcellular localization of MDM2, especially the nuclear cytoplasmic shuttle process, but as a spatial regulatory mechanism for regulating p53 degradation, this aspect has not been well studied. In the experimental samples, it can be seen that the ubiquitination level of p53 has increased, but it is still unclear whether GSG2 directly enhances the E3 ligase catalytic rate of MDM2 itself or whether the enhanced ubiquitination is simply due to the addition of more physical scaffolds. If we want to separate the scaffold effect from the intrinsic catalytic activity and thoroughly study specific conformational changes, we will need to use structural biology techniques in the future. For example, X-ray crystallography or cryo electron microscopy, as well as the use of purified recombinant proteins, combined with cell-free in vitro ubiquitination assays. The mechanism details that have not been fully understood are actually a crucial frontier direction for future research.

Conclusions

This study shows GSG2 as a core driver of CRC. GSG2 is highly upregulated in malignant tissues. Patient poor survival is dictated by its overexpression. GSG2 acts as a direct catalytic scaffold. Physical interaction of GSG2 with the E3 ubiquitin ligase MDM2 hyperactivated MDM2, forced its rapid polyubiquitination and proteasomal degradation of wild-type p53. Destruction of this p53 checkpoint triggered a massive reprogramming of cancer metabolism. Cancer cells let go of mitochondrial respiration for aerobic glycolysis, the metabolic fuel for

their rapid expansion. Depletion of GSG2 abrogated this Warburg phenotype. It blocked tumor glucose metabolism, forced a return to oxidative phosphorylation and arrested in vivo tumor growth.

We identified a novel, non-mutational mechanism for p53 silencing. GSG2 can bypass the need for TP53 genetic mutations by hijacking the MDM2-p53 axis. This GSG2-MDM2 interaction represents a precise target for CRC patients with wild-type p53.

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Disclosure of conflict of interest

None.

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