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Yin Yang 1 promotes the Warburg effect and tumorigenesis via glucose transporter GLUT3

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Cancer cells typically shift their metabolism to aerobic glycolysis to fulfill the demand of energy and macromolecules to support their proliferation. Glucose transporter (GLUT) family-mediated glucose transport is the pacesetter of aerobic glycolysis and, thus, is critical for tumor cell metabolism. Yin Yang 1 (YY1) is an oncogene crucial for tumorigenesis; however, its role in tumor cell glucose metabolism remains unclear. Here, we revealed that YY1 activates *GLUT3* transcription by directly binding to its promoter and, concomitantly, enhances tumor cell aerobic glycolysis. This regulatory effect of YY1 on glucose entry into the cells is critical for YY1-induced tumor cell proliferation and tumorigenesis. Intriguingly, YY1 regulation of GLUT3 expression, and, subsequently, of tumor cell aerobic glycolysis and tumorigenesis, occurs p53-independently. Our results also showed that clinical drug oxaliplatin suppresses colon carcinoma cell proliferation by inhibiting the YY1/GLUT3 axis. Together, these results link YY1's tumorigenic potential with the critical first step of aerobic glycolysis. Thus, our novel findings not only provide new insights into the complex role of YY1 in tumorigenesis but also indicate the potential of YY1 as a target for cancer therapy irrespective of the p53 status.

KEYWORDS
glucose transporter 3, glucose transporter family, p53-independent, Warburg effect, Yin Yang 1

1 | INTRODUCTION

Glucose is a major source of cellular energy and new cell mass. It provides not only adenosine three phosphate (ATP) as the free energy needed for various cellular processes but also building blocks for the biosynthesis of macromolecules essential for constructing new cells. Tumor cells are characterized by an uncontrolled, high proliferation rate. To this end, tumor cells reprogram their metabolism to achieve a balance between ATP and biomass production. Although glycolysis is less efficient in producing ATP than mitochondrial respiration, tumor cells prefer glycolysis, even in the condition of sufficient oxygen.[1,2] This metabolic alteration, termed the Warburg effect or aerobic glycolysis, produces not only intermediates that can function as the precursors for chemical constituents used

Abbreviations: ATP, adenosine three phosphate; GLUT3, glucose transporter 3; GLUT, glucose transporter; shRNA, small hairpin RNA; YY1, Yin Yang 1.

for building macromolecules, including ribonucleic acids, proteins and lipids,[2,3] but also nicotinamide adenine dinucleotide phosphate, which is critical for tumor cell antioxidant defense.[4]

Glucose transport across the cell membrane is the pacesetter for glucose metabolism in cells[5] and is mediated by the facilitative SLC2 family of glucose transporters (GLUT).[6] Currently, 14 members of the GLUT family have been identified, each of which likely have specific spatial and temporal distributions, as well as distinct substrate selectivity and transport kinetics.[6-8] Consistent with the excessively high glucose consumption of tumor cells,[1] aberrant GLUT family expression has been found in various cancers, including colorectal cancer,[9] brain cancer,[10] and lung cancer cells.[11] Despite the fact that the GLUT family is critical in tumor cell metabolic reprogramming, the detailed regulatory mechanism has not been fully elucidated.

Yin Yang 1 (YY1) is a GLI-Krüppel class protein with 4 C2H2 zinc finger domains that is evolutionarily well conserved throughout all vertebrate lineages.[12-14] YY1 can activate or inhibit transcriptional activation of its target genes depending on its binding context.[15,16] Accordingly, YY1 has been reported to play crucial roles in various physiological functions, including gestation, embryonic development, cell differentiation and cell cycle.[17-20] YY1 is upregulated in various human cancers, including colon carcinoma, breast carcinoma and prostate cancer.[21-23] We and other groups have reported that YY1 is an oncogene that can promote tumorigenesis by enhancing cell proliferation, tumor angiogenesis and tumor metastasis,[15,19,24] and that these regulatory effects occur in both a p53-dependent and p53-independent manner.[20,24] However, despite its critical roles in tumorigenesis, it remains unclear whether YY1 is involved in tumor cells' glucose metabolism.

In this study, we revealed for the first time that YY1 enhances the transcription of GLUT3, the member of the GLUT family with high affinity for glucose, and, in turn, promotes glucose consumption and lactate production in colorectal tumor cells. Furthermore, we found that this regulation of transcriptional activity occurs most plausibly through a direct binding of YY1 to the *GLUT3* promoter. This metabolic alteration toward glycolysis subsequently supported YY1-induced tumorigenesis. Importantly, we found that the regulatory effect of YY1 on the *GLUT3* promoter, and, concomitantly, the function of YY1/GLUT3 axis in altering tumor cell metabolism and promoting tumorigenesis, occurs in a p53-independent manner. Together, these results reveal an essential function of YY1 that links it to the entry of the tumor cell glucose metabolism and provide a new perspective on the multiple functions of YY1 in tumorigenesis. Furthermore, these findings emphasize the potential of targeting YY1 for cancer therapy, irrespective of the p53 status.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture

HCT116^{WT} and HCT116^{p53null} cells were kindly provided by Dr Bert Vogelstein at The John Hopkins University Medical School[25] and maintained in McCoy's 5A medium (Gibco) with 10% FBS (Biological Industries, Israel) and 1% penicillin-streptomycin. Mycoplasma contamination was routinely tested using the Mycoplasma Detection Kit-QuickTest (Biotool, Houston, TX, USA). Transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol.

For gene-silencing experiments, cells were transfected with indicated shRNA expression vectors. Puromycin selection was performed to eliminate untransfected cells 24 h after transfection. For *YY1*-silenced HCT116^{p53null} (HCT116^{p53null}/shYY1), *YY1*-silenced, *GLUT3*-overexpressed HCT116^{p53null} (HCT116^{p53null}/shYY1/GLUT3) or control (HCT116^{p53null}/Con) stable cell lines, cells were transfected with shYY1 or shCon and pGLUT3-Puro or pEF9-Puro vectors before being selected with puromycin. For oxaliplatin treatment, cells transfected with indicated vectors were reseeded and incubated for an additional 24 h prior to being treated with oxaliplatin (Dalian Meilun Biotech, Liaoning, China) for 48 h.

2.2 | Clinical human colon carcinoma specimens

Human colon carcinoma fresh specimens were obtained from patients undergoing surgery at Chongqing University Cancer Hospital (Chongqing, China). Patients did not receive chemotherapy, radiotherapy or other adjuvant therapies prior to the surgery. The specimens were snap-frozen in liquid nitrogen. Prior patients' written informed consent was obtained, and the experiments were approved by the Institutional Research Ethics Committee of Chongqing University Cancer Hospital.

2.3 | Animal experiments

For the xenograft experiment, BALB/c-nu/nu mice (male; body weight, 18-22 g; 6 weeks old) were purchased from the Third Military Medical University (Chongqing, China). Animal studies were carried out at the Third Military Medical University and approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University. All animal experiments conformed to the approved guidelines of the Animal Care and Use Committee of the Third Military Medical University. All efforts to minimize suffering were made. To generate an experimental subcutaneous tumor model, BALB/c-nu/nu mice were randomly divided into 3 groups (n = 5), and each group was injected subcutaneously with 3×10^6 HCT116^{p53null}/Con, HCT116^{p53null}/shYY1 or HCT116^{p53null}/shYY1/GLUT3 cells. Tumor size (V) was evaluated as described previously.[24] The investigator was blinded to the group allocation and during the assessment.

2.4 | Quantitative RT-PCR and western blotting

Detailed methods for quantitative RT-PCR and western blotting are described in Supplementary Data S1. The primers and antibodies used are listed in Supplementary Tables S1 and S2, respectively.

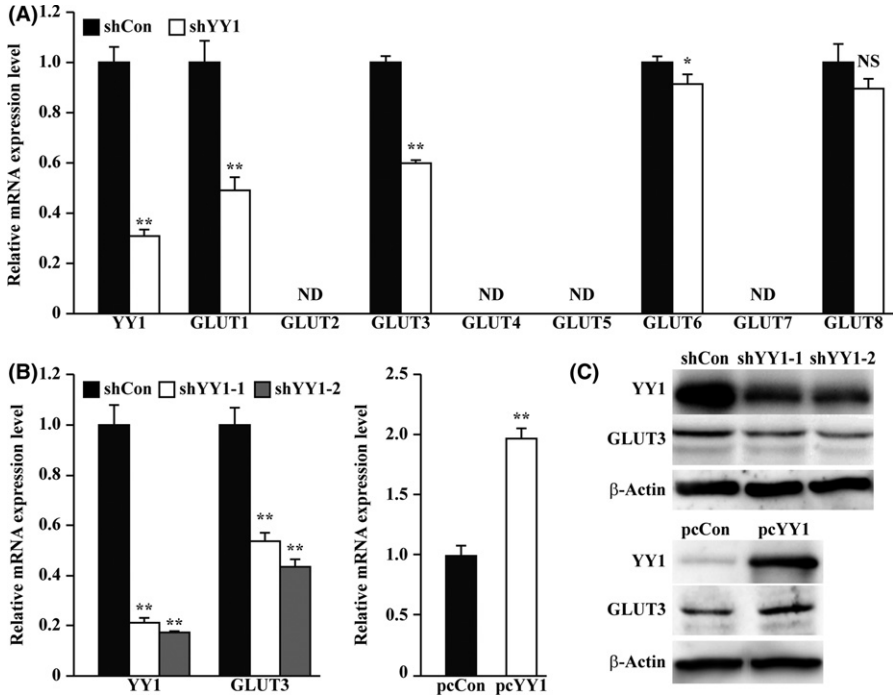

FIGURE 1 Yin Yang 1 (YY1) regulates *GLUT3* expression. A, The mRNA expression levels of *GLUT* family in HCT116^{WT} cells transfected with small hairpin RNA (shRNA) vector against *YY1* were examined using quantitative PCR (qPCR). B, The mRNA expression levels of *GLUT3* in HCT116^{WT} cells transfected with 2 shRNA vectors targeting different sites of *YY1* (left) or *YY1* overexpression vector (right) were examined using qPCR. C, The protein expression levels of GLUT3 in *YY1*-silenced (upper panels) or *YY1*-overexpressed (lower panels) HCT116^{WT} cells were examined using western blotting. β-actin was used for qPCR normalization and western blotting loading control. Cells transfected with shCon or pcCon were used as control. Quantitative data are shown as relative to control and expressed as mean ± SEM from 3 independent experiments. $^{*}P < .05$; $^{**}P < .01$; ND, not detected; NS, not significant; pcCon, pcDNA3.1(+)

2.5 | Statistical analysis

All values of the experimental results are presented as mean ± SD of triplicates. Statistical analysis was performed using Student's *t* test. For clinical samples and xenograft experiments, statistical analysis was performed using one-way ANOVA. A value of $^{*}P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Yin Yang 1 regulates GLUT3 expression in tumor cells

Glucose metabolism is critical for the highly proliferative tumor cells, and the GLUT family is the pacesetter of glucose intake. To investigate whether YY1 affects the expression of members of the GLUT family, we examined the effect of an shRNA vector targeting YY1 (shYY1-1) on the expression of GLUT family members in human colon carcinoma HCT116^{WT} cells. As shown in Figure 1A, *YY1* significantly affected *GLUT1* and *GLUT3* expressions, while it only slightly affected *GLUT6* expression and did not affect *GLUT8* expression. In contrast, GLUT2, GLUT4, GLUT5 and GLUT7 could not be detected in colon carcinoma cells.

Among the GLUT family affected by *YY1* silencing, GLUT3 has the highest affinity to glucose.[8,11] To further confirm the regulatory effect of YY1 on GLUT3, we transfected 2 shRNAs targeting *YY1* at different sites, as well as *YY1* overexpression vector (Supplementary Figure S1), and investigated their effects on *GLUT3* expression. As shown in Figure 1B, *YY1* silencing robustly reduced *GLUT3* mRNA expression (left) in colon carcinoma cells, while *YY1* overexpression clearly induced it (right). A similar tendency was observed for protein expression (Figure 1C). Thus, our results showed that YY1 might regulate GLUT3 at the transcriptional level.

3.2 | Glucose transporter 3 is involved in Yin Yang 1-induced tumor cell metabolic shift and proliferation

Given that GLUT3 is critical for glucose transport into the cells, we next examined the glucose consumption in *YY1*-silenced HCT116^{WT} cells. Manipulation of *YY1* expression significantly altered glucose consumption by tumor cells: *YY1* silencing reduced the glucose consumption (Figure 2A, left), while *YY1* overexpression robustly increased it (Figure 2A, right), suggesting that YY1 might enhance tumor cell glucose metabolism.

The shift toward glycolysis in tumor cells is accompanied by an increase in lactate production.[2,26] Therefore, we next examined the lactate production in *YY1*-silenced and *YY1*-overexpressing HCT116^{WT} cells. The results also showed that *YY1* suppression robustly decreased the lactate production, while *YY1* overexpression increased it (Figure 2B).

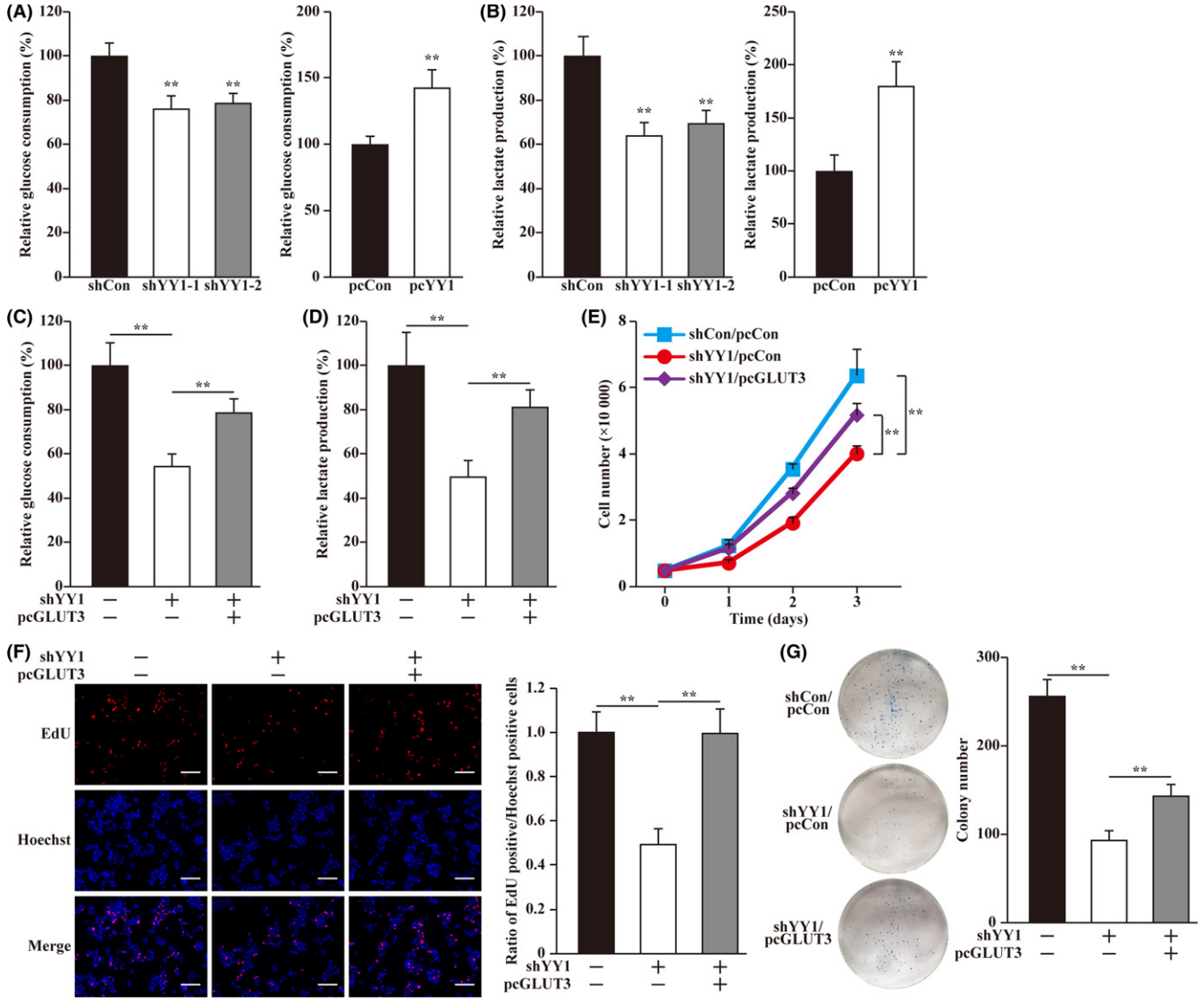

FIGURE 2 Yin Yang 1 (YY1) regulates tumor cells glucose metabolism. A, Relative glucose consumption in *YY1*-silenced (left) and *YY1*-overexpressed (right) HCT116^{WT} cells. B, Relative lactate production in *YY1*-silenced (left) and *YY1*-overexpressed (right) HCT116^{WT} cells. C, D, Relative glucose consumption (C) and lactate production (D) in *YY1*-silenced, *GLUT3*-overexpressed HCT116^{WT} cells. E, Total cell number of *YY1*-silenced, *GLUT3*-overexpressed HCT116^{WT} cells at indicated time points. F, Percentage of proliferative cells was examined using EdU-incorporation assay. Representative images (left) and the quantitative results (right) are shown. Hoechst was used to stain the nuclei. The ratio of proliferative cells is shown as relative to control. G, Colony formation potential of *YY1*-silenced, *GLUT3*-overexpressed HCT116^{WT} cells was determined in vitro. Representative images (left) and the quantification results (right) are shown. Cells transfected with shCon or pcCon were used as control. Total protein was used for normalization for glucose consumption and lactate production. Quantitative data are expressed as mean ± SEM from 3 independent experiments. Scale bars: 200 μm. $^{**}P < .01$; pcCon: pcDNA3.1(+)

Next, we investigated whether GLUT3 is involved in the YY1-mediated regulation of the metabolic shift. We cotransfected both shYY1 and *GLUT3* overexpression vectors (pcGLUT3, Supplementary Figure S2A) into HCT116^{WT} cells and investigated their glucose consumption and lactate production. *GLUT3* overexpression rescued the glucose consumption and lactate production suppressed by *YY1* silencing (Figure 2C,D). Together, these results clearly showed that YY1 regulates the tumor cell metabolic shift toward glycolysis via glucose transporter GLUT3.

Yin Yang 1 induces tumorigenesis by promoting cell proliferation.[20,24] Meanwhile, glycolysis supports the high proliferation rate of tumor cells.[2,27] Thus, we next tested whether GLUT3 is involved in YY1-induced tumor cell proliferation. We found that *YY1* silencing conspicuously suppressed the total cell number, while *GLUT3* overexpression restored it (Figure 2E and Supplementary Figure S2B). Furthermore, EdU-incorporation assay also showed that the number of proliferative cells suppressed by *YY1* silencing increased robustly with *GLUT3* overexpression (Figure 2F). Similarly, *GLUT3* overexpression restored the colony formation potential repressed by *YY1* silencing (Figure 2G). Together, these results revealed that the YY1/GLUT3 pathway is essential for tumor cell growth and colony formation.

3.3 | Yin Yang 1 enhances tumor cell glycolysis in a p53-independent manner

A previous study reported that YY1 negatively regulates p53,[20] while p53 suppresses GLUT3 expression.[28] However, we previously revealed that YY1 regulates tumor cell proliferation even in the absence of p53.[24] These facts led us to question whether YY1 could enhance GLUT3 expression and induce tumor cell metabolic shift in a p53-independent pathway. To this end, we examined the effect of *YY1* silencing on GLUT3 expression and found that despite the absence of p53, *YY1* silencing could still suppress GLUT3 mRNA expression in HCT116^{p53null} cells (Figure 3A, left), while *YY1* overexpression significantly induced it (Figure 3A, right). A similar tendency was observed in GLUT3 protein expression: YY1 positively regulates GLUT3 protein expression in HCT116^{p53null} cells (Figure 3B). Indeed, we found that despite the absence of p53, *YY1* positively regulates glucose consumption (Figure 3C). Similarly, the lactate production in the HCT116^{p53null} cells showed a positive correlation with the YY1 expression level (Figure 3D). These results clearly indicated that YY1 induces the tumor cell glucose metabolic shift by regulating GLUT3 in a p53-independent manner.

To investigate whether p53-independent YY1 regulation of tumor cell metabolism, and subsequently tumor cell growth, occurs via its regulatory effect on GLUT3, we overexpressed *GLUT3* in *YY1*-silenced HCT116^{p53null} cells. We found that both the glucose consumption and the lactate production, which was repressed by *YY1* silencing, were conspicuously restored by *GLUT3* overexpression (Figure 3E,F). Given that tumor cells prefer to use aerobic glycolysis for producing ATP, we investigated the role of the YY1/GLUT3 axis in intracellular ATP production and found that in p53-null cells, *YY1* silencing robustly suppressed intracellular ATP level, while *GLUT3* overexpression significantly restored it (Figure 3G).

Next, we examined whether p53-independent YY1 regulation of GLUT3 affects tumor cell proliferation and colony formation potential. As shown by the total cell numbers and the ratio of EdU-positive cells, as well as the crystal violet staining, *GLUT3* overexpression rescued HCT116^{p53null} cell proliferation suppressed by *YY1* silencing (Figure 4A,B, and Supplementary Figure S3). Moreover, *GLUT3* overexpression significantly enhanced the colony formation potential of *YY1*-silenced HCT116^{p53null} cells (Figure 4C). Altogether, these results clearly indicated that YY1 enhances the tumor cell glycolytic shift, and, subsequently, tumor cell growth and colony formation, by positively regulating GLUT3, irrespective of p53-status.

Oxaliplatin, which is commonly used for treating colon carcinoma, had been reported to affect tumor cell glucose metabolism.[29,30] We next examined the effect of oxaliplatin on YY1 and GLUT3 expression. As shown in Figure 4D, oxaliplatin grossly suppressed the expression levels of both YY1 and GLUT3 in HCT116^{p53null} cells in a dose-dependent manner. To confirm that the suppressive effect of oxaliplatin on GLUT3, and subsequently on tumor cell proliferation, occurs via YY1, we treated HCT116^{p53null} cells overexpressing *YY1* with oxaliplatin. *YY1* overexpression obviously restored *GLUT3* expression and tumor cell proliferation suppressed by oxaliplatin (Figure 4E,F). These results suggested that oxaliplatin suppresses tumor cell proliferation by, at least partly, inhibiting the YY1/GLUT3 pathway-mediated glucose metabolism.

3.4 | Yin Yang 1 directly affects glucose transporter 3 transcriptional activity

Next, we questioned whether YY1 could directly regulate GLUT3 transcription. To this end, we first examined the effect of YY1 on a firefly reporter vector bearing the −1004 to +258 region of the *GLUT3* promoter (GLUT3-Luc, Supplementary Figure S4A). The activity of GLUT3-Luc was suppressed in both *YY1*-silenced HCT116^{WT} and HCT116^{p53null} cells (Figure 5A). Concomitantly, GLUT3-Luc activity was enhanced in both cells when YY1 was overexpressed (Figure 5B), indicating that YY1 positively regulates *GLUT3* promoter activity, irrespective of p53 status. Using the UCSC Genome Browser,[31] we found a predicted putative YY1 binding site (GACATTTT)[32] at the +158 to +165 region of the *GLUT3* promoter. Through ChIP assay, we demonstrated that YY1 could directly bind to the +81 to +251 region of the *GLUT3* promoter (Figure 5C). We then constructed another *GLUT3* reporter vector bearing the −1004 to +170 region of the *GLUT3* promoter (GLUT3(s)-Luc), and a *GLUT3* reporter vector without the predicted YY1 binding site (−1004 to +137 region of the *GLUT3* promoter, GLUT3(del)-Luc), as shown in Supplementary Figure S4B. In both wild-type and p53-null cells, *YY1* silencing and overexpression significantly altered the activities of GLUT3-Luc and GLUT3(s)-Luc at a similar level, without affecting GLUT3(del)-Luc (Figure 5D,E).

To confirm the importance of the predicted binding site, we further constructed a mutated GLUT3 reporter vector (GLUT3(mut)-Luc) by inducing 3 mutations into the putative YY1 binding sequence (Supplementary Figure S4B). As shown in Figure 5F,G, *YY1* silencing and overexpression failed to affect the luciferase activity of mutant GLUT3 reporter vector in both wild-type and p53-null cells, indicating that binding to GLUT3 promoter is essential for YY1 regulation on *GLUT3* promoter activity.

Mutation of threonine to arginine at 372 positions of YY1 had been shown to inhibit DNA binding of YY1.[33] Thus, we further examined *GLUT3* transcriptional activity in HCT116^{p53null} cells overexpressing mutant *YY1*^{T372R} (Supplementary Figure S5A) We found that *YY1*^{T372R} overexpression could not significantly affect the luciferase activity of GLUT3 reporter (Figure 5H), as well as its mRNA expression level (Figure 5I), indicating that YY1 directly regulates *GLUT3* transcription. Concomitantly, *YY1*^{T372R} could not upregulate glucose consumption and lactate production (Supplementary Figure S5B,C). Together, these results clearly indicated that YY1 directly regulates *GLUT3* transcriptional activity, and subsequently, glucose metabolism, by binding to *GLUT3* promoter, and acts as a transcription factor.

3.5 | The p53-independent Yin Yang 1/glucose transporter 3 pathway is critical for tumor cell growth

To examine the function of the p53-independent YY1/GLUT3 pathway in tumorigenesis in vivo, we performed a xenograft experiment.

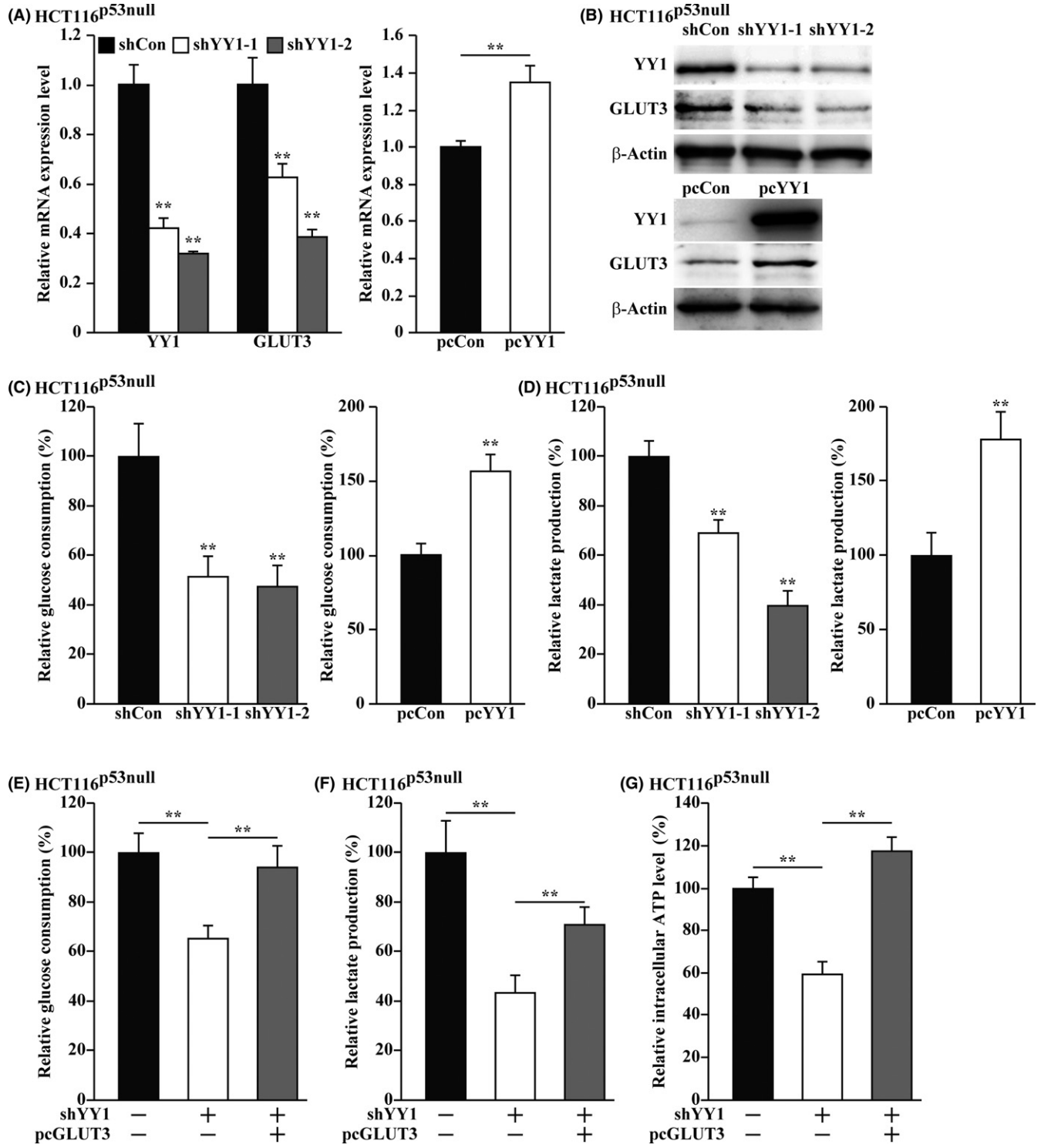

FIGURE 3 Yin Yang 1 (YY1) regulates glucose transporter 3 (GLUT3) expression p53-independently. A, The mRNA expression level of *GLUT3* in *YY1*-silenced (left) and *YY1*-overexpressed (right) HCT116^{p53null} cells was determined using qPCR. B, The protein expression level of GLUT3 in *YY1*-silenced (upper panels) and *YY1*-overexpressed (lower panels) HCT116^{p53null} cells was determined using western blotting. C, D, Relative glucose consumption (C) and lactate production (D) in *YY1*-silenced (left) and *YY1*-overexpressed (right) HCT116^{p53null} cells. E, F, Relative glucose consumption (E) and lactate production (F) in *YY1*-silenced, *GLUT3*-overexpressed HCT116^{p53null} cells. G, Relative intracellular ATP level in *YY1*-silenced, *GLUT3*-overexpressed HCT116^{p53null} cells. Cells transfected with shCon or pcCon were used as control. β-actin was used for qPCR normalization and western blotting loading control. Total protein was used for normalization for glucose consumption, lactate production and intracellular ATP level. Quantitative data were expressed as mean ± SEM from 3 independent experiments. $^{**}P < .01$; pcCon, pcDNA3.1(+)

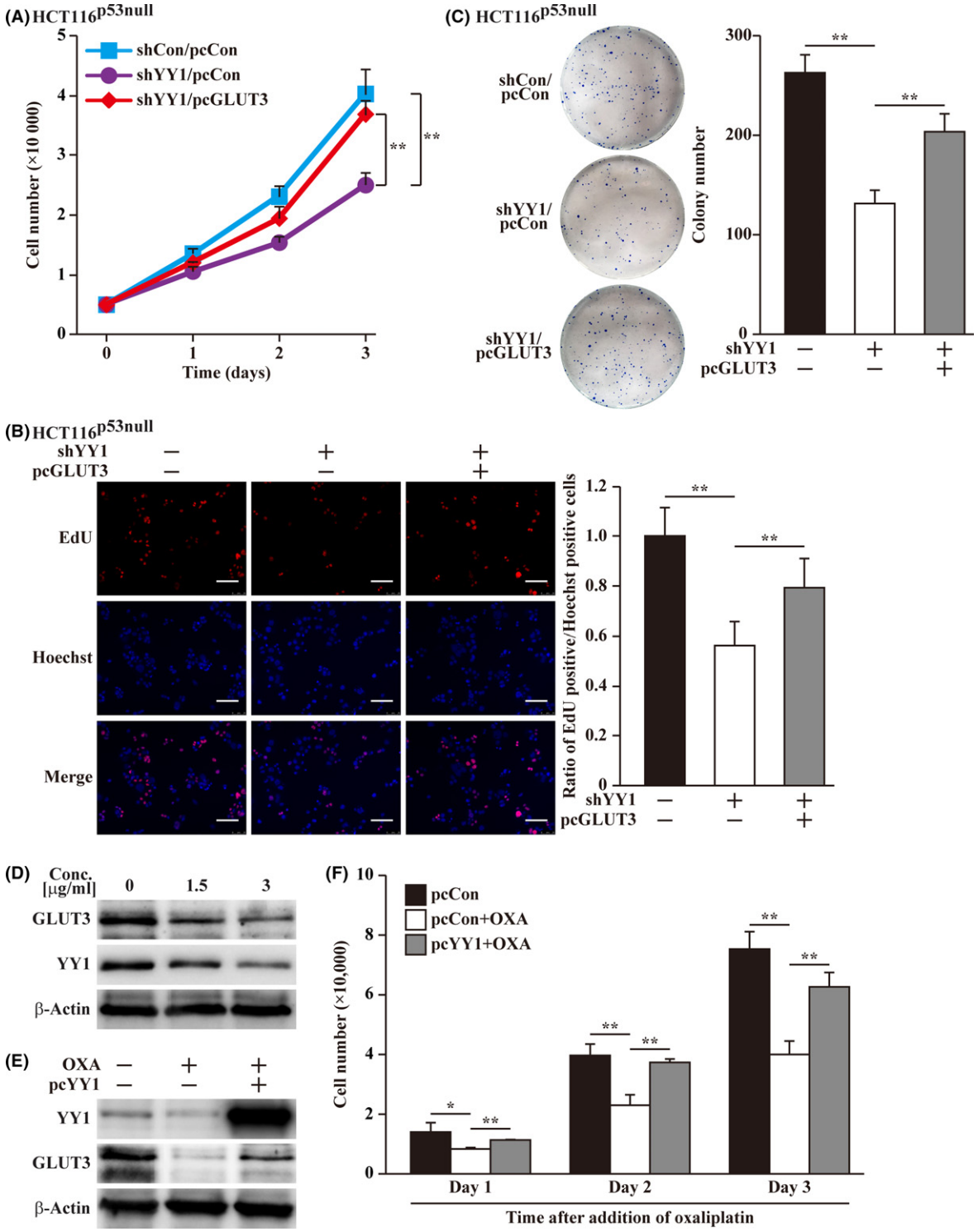

FIGURE 4 Yin Yang 1 (YY1) positively regulates p53-null tumor cells proliferation through glucose transporter 3 (GLUT3). A, Total cell number of *YY1*-silenced, *GLUT3*-overexpressed HCT116^{p53null} cells at indicated time points. B, Percentage of proliferative cells was examined using EdU-incorporation assay. Representative images (left) and the quantitative results (right) are shown. Hoechst was used to stain the nuclei. The ratio of proliferative cells is shown as relative to control. C, Colony formation potential of *YY1*-silenced, *GLUT3*-overexpressed HCT116^{p53null} cells was determined in vitro. Representative images (left) and the quantitative results (right) are shown. D, The protein expression levels of YY1 and GLUT3 in the cells treated with indicated concentration of oxaliplatin were determined using western blotting. E, The protein expression levels of YY1 and GLUT3 in *YY1*-overexpressed HCT116^{p53null} cells treated with oxaliplatin (final concentration: 3 μg/mL) were determined using western blotting. F, Total cell number of *YY1*-overexpressed HCT116^{p53null} cells at indicated time points after oxaliplatin treatment (final concentration: 3 μg/mL). β-actin was used for western blotting loading control. Scale bars: 200 μm. $^{*}P < .05$; $^{**}P < .01$; OXA, oxaliplatin; pcCon, pcDNA3.1(+)

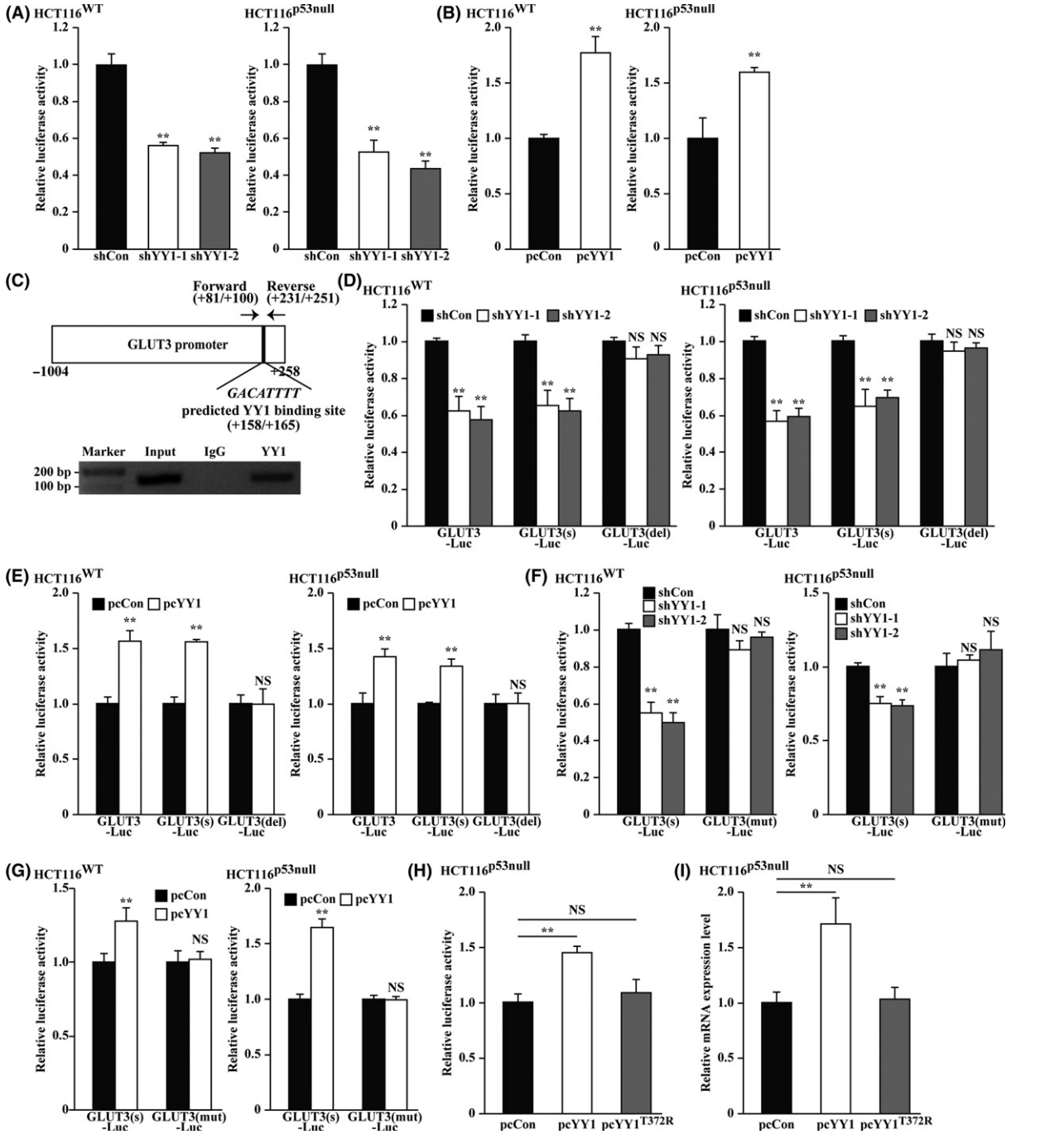

FIGURE 5 Yin Yang 1 (YY1) directly activates glucose transporter 3 (GLUT3) transcription by binding to its promoter. A, The activity of *GLUT3* promoter reporter vector (GLUT3-Luc) in *YY1*-silenced HCT116^{WT} (left) or HCT116^{p53null} (right) cells. B, The activity of GLUT3-Luc in *YY1*-overexpressed HCT116^{WT} (left) or HCT116^{p53null} (right) cells. C, Binding of YY1 to the *GLUT3* promoter region was examined using chromatin immunoprecipitation assay with anti-YY1 antibody followed by PCR in HCT116^{WT} cells. The predicted YY1 binding site on the *GLUT3* promoter and the location of primer set used for PCR are shown. D, The activity of *GLUT3* promoter reporter vectors with (GLUT3-Luc and GLUT3(s)-Luc) or without predicted YY1 binding site (GLUT3(del)-Luc) in *YY1*-silenced HCT116^{WT} (left) and HCT116^{p53null} (right) cells. E, The activities of GLUT3-Luc, GLUT3(s)-Luc and GLUT3(del)-Luc in *YY1*-overexpressed HCT116^{WT} (left) and HCT116^{p53null} (right) cells. F, The activity of *GLUT3* promoter reporter vector with mutated predicted YY1 binding site (GLUT3(mut)-Luc) in *YY1*-silenced HCT116^{WT} (left) and HCT116^{p53null} (right) cells. G, The activity of GLUT3(mut)-Luc in *YY1*-overexpressed HCT116^{WT} (left) and HCT116^{p53null} (right) cells. H, I, The activity of *GLUT3* promoter reporter vector (H) and *GLUT3* mRNA expression level (I) in HCT116^{p53null} cells overexpressing *YY1*^{T372R} mutant. Cells transfected with shCon or pcCon were used as control. β-actin was used for qPCR normalization. Quantitative data were expressed as mean ± SEM from 3 independent experiments. $^{**}P < .01$. NS, not significant; pcCon, pcDNA3.1(+)

To this end, we established HCT116^{p53null}/Con, HCT116^{p53null}/shYY1 and HCT116^{p53null}/shYY1/GLUT3 stable cell lines (Supplementary Figure S6) and analyzed their tumorigenic potential upon subcutaneous transplantation into BALB/c-nu/nu mice. We found that *GLUT3* overexpression significantly restored the tumorigenic potential of HCT116^{p53null} cells suppressed by *YY1* silencing (Figure 6A,B). Immunohistochemistry of tissue sections from the xenografted tumors confirmed the protein expression levels of YY1 and GLUT3 in the xenografted tumors: GLUT3 was downregulated in the tumor generated from HCT116^{p53null}/shYY1 cells (Figure 6C). Furthermore, Ki67 staining results also showed that the number of proliferative cells was conspicuously lower in tumors generated from *YY1*-

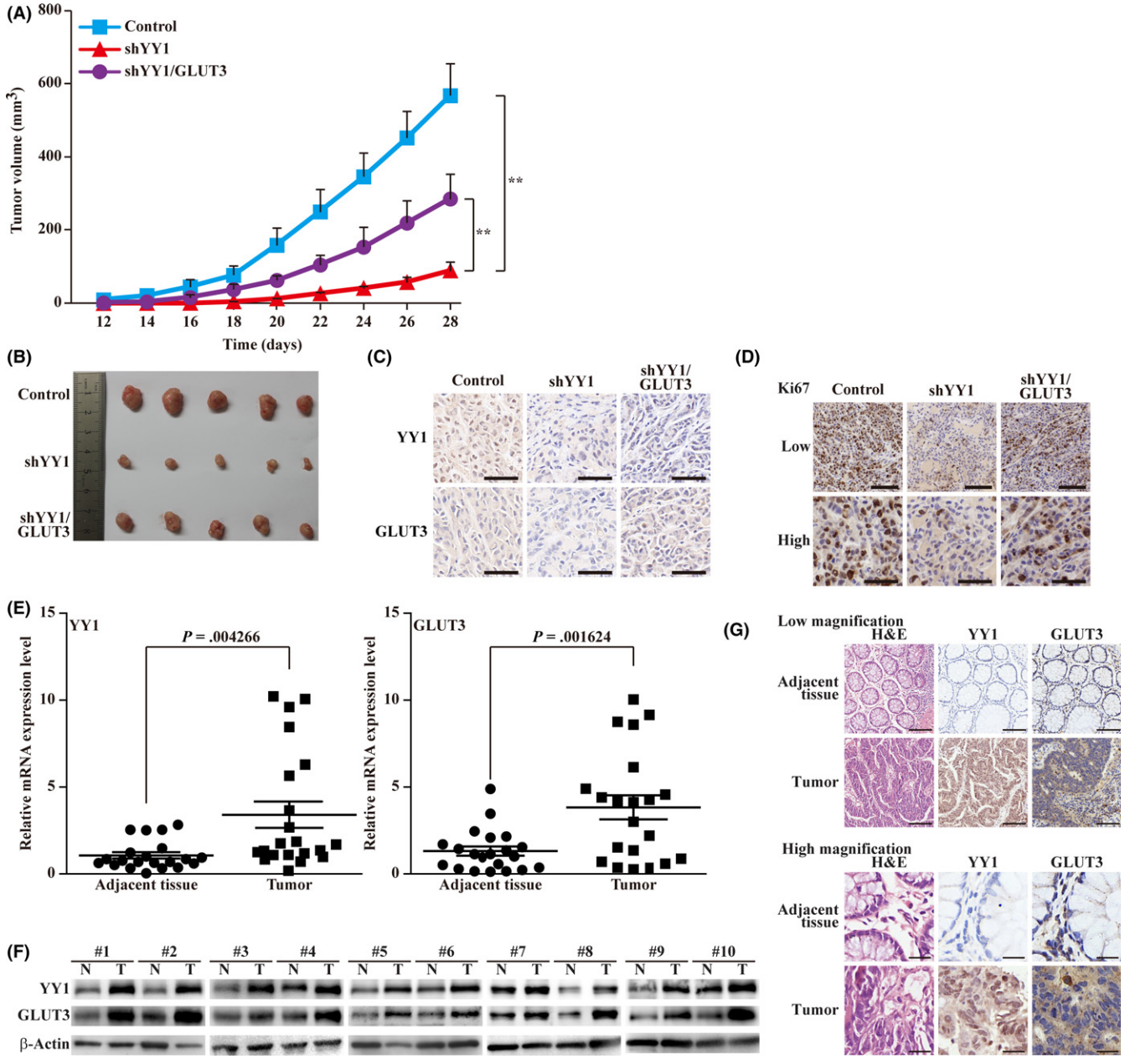

FIGURE 6 Yin Yang 1 (YY1)/glucose transporter 3 (GLUT3) axis regulates tumorigenesis in a p53-independent manner. A, B, Tumorigenesis potential of HCT116^{p53null}/Con, HCT116^{p53null}/shYY1 and HCT116^{p53null}/shYY1/GLUT3 stable cell lines were examined in vivo by subcutaneous injection into Balb/c-nu/nu mice (n = 5). Volumes of the tumors generated were measured at indicated time points (A), and the representative images are shown (B). C, Immunohistochemistry staining images against YY1 and GLUT3 in the tissue sections of xenografted tumors in Balb/c-nu/nu mice injected with indicated cell lines (scale bars: 50 μm). D, Ki67 staining of the xenografted tumor in Balb/c-nu/nu mice injected with indicated cell lines. Low (scale bars: 100 μm) and high (scale bars: 50 μm) magnification images are shown. E, The mRNA expression levels of YY1 and GLUT3 in human clinical colon carcinoma and the corresponding adjacent tissue were determined using qPCR. F, G, The protein expression levels of YY1 and GLUT3 in human clinical colon carcinoma and the corresponding adjacent tissue were determined using western blotting (F) and immunohistochemistry staining against YY1 and GLUT3 (G, scale bars: 100 μm for low magnification, and 20 μm for high magnification). β-actin was used for qPCR normalization and western blotting loading control. $^{**}P < .01$

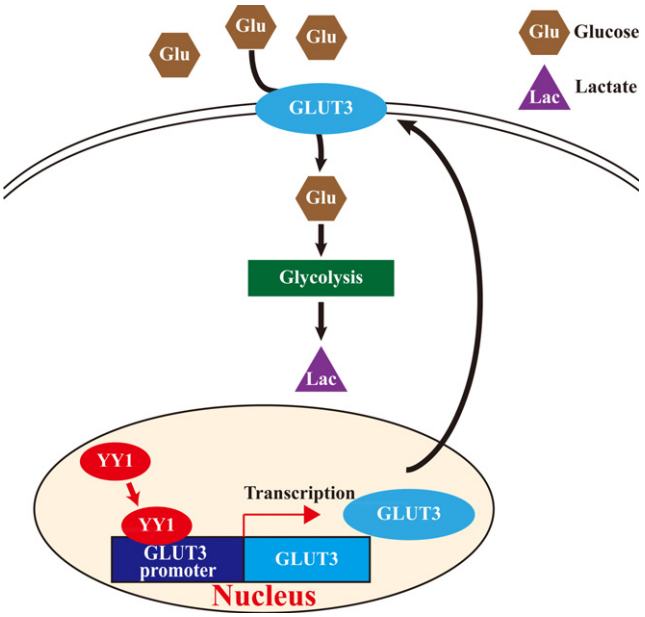

FIGURE 7 Schematic diagram showing the mechanism of Yin Yang 1 regulation on the tumorigenesis via direct transcriptional regulation on glucose transporter 3

silenced cells, while *GLUT3* overexpression significantly restored it (Figure 6D).

Finally, compared with adjacent normal tissues, *YY1* and *GLUT3* mRNA levels were aberrantly upregulated in clinical human colon carcinoma tissues (Figure 6E). Concomitantly, YY1 and GLUT3 protein expression levels were also upregulated in tumor lesions, as shown by the western blotting and immunohistochemistry results (Figure 6F,G).

In summary, our study elucidated a novel mechanism fundamental to the role of YY1 in tumorigenesis: YY1 induces tumor cell metabolic shift, proliferation and, subsequently, tumorigenesis in a p53-independent manner through the direct activation of *GLUT3*, which, in turn, enhances aerobic glycolysis and provides tumor cells with the energy and biosynthesis they need for high proliferation (Figure 7).

4 | DISCUSSION

Highly proliferative tumor cells tend to shift their metabolism toward aerobic glycolysis,[2] thus producing ATP as well as glycolytic intermediates needed for the synthesis of macromolecules. Through aerobic glycolysis, tumor cells convert the majority of glucose to lactate, which conditions their microenvironment to favor metastasis.[1,34] Our previous results showed that YY1 could positively regulate *GLUT1* expression under hypoxia by stabilizing HIF-1α protein.[24] However, tumor cells prefer glycolysis even when oxygen is sufficient. Our findings clearly showed that YY1 enhances *GLUT3* transcription and glucose uptake into tumor cells under normoxia and, thus, is a novel positive regulator of tumor cells aerobic glycolysis. These findings are the first to reveal the relation between YY1 and GLUT3, the glucose transporter with high affinity for glucose.

Glucose transporter proteins form a superfamily of membrane transporters that mediate the transport of monosaccharides, polyols and other small carbon compounds across the membranes of eukaryotic cells.[35] According to the prediction by UCSC Genome Browser, among *GLUT* family members, *GLUT1*, *GLUT3* and *GLUT8* are predicted to have YY1 transcriptional regulation; however, the prediction score for *GLUT8* is significantly lower than others. These predictions conform with our results showing that YY1 could not significantly affect *GLUT8* transcription. It is also noteworthy that while YY1 slightly affects *GLUT6* expression, we could not find putative YY1 binding sequence on its promoter, indicating that YY1 regulation on its expression might occur indirectly. Among the YY1-regulated GLUT family members, GLUT3 has the strongest glucose affinity,[8,11] and, indeed, knocking down *YY1* in *GLUT1*-silenced colon carcinoma cells still suppressed glucose consumption, lactate production and intracellular ATP level (Supplementary Figure S7). Our results clearly showed that YY1 is a critical positive regulator of *GLUT3*. GLUT3 was initially identified in fetal skeletal muscle[36] and was later found to be predominantly expressed in neurons and brains.[8,35] While its expression, normally, is strongly tissue-specific, high GLUT3 expression has been observed in various tumors, including colorectal carcinomas and brain tumors.[9,10] Furthermore, GLUT3 is involved in anti-angiogenic therapy resistance as well as in driving cancer stem cell phenotype.[36,37] The critical roles of GLUT3 are most plausibly due to its high activity as a glucose transporter, with higher affinity for glucose than other Class I GLUT family members, including the widely expressed GLUT1.[7,8] Indeed, GLUT3 has been reported as a potential target for anticancer therapy.[38] Our novel findings show that GLUT3 is positively regulated by YY1, which is highly expressed in various tumors, and, thus, elucidate a novel pathway for GLUT3 upregulation in tumor cells. Furthermore, these results indicate that a GLUT3-targeting therapeutic strategy could be achieved by targeting YY1.

Kawauchi et al[28] report that GLUT3 expression is regulated by tumor suppressor p53 through the IKK-NF-κB pathway, while Sui et al and Gronroos et al[20,39] report that YY1 can suppress the transcription and stabilization of p53. Intriguingly, our results clearly showed that YY1 regulation of the transcriptional activity of GLUT3 and tumor cell aerobic glycolysis occurs even when p53 is lacking, and that this p53-independent regulation is critical for YY1-induced tumorigenesis in p53-null tumor cells. Activation of endogenous p53 has become a major strategy in treating cancers;[40] however, p53 mutation and aberrant expression are commonly found in cancers.[41] Thus, these results strongly suggest that *YY1* silencing is a potential therapeutic strategy for treating tumors, irrespective of their p53 status.

Herein, we also showed that oxaliplatin, a drug commonly used for colon carcinoma, suppresses p53-null tumor cell proliferation by regulating the YY1/GLUT3 axis, suggesting that YY1 might be crucial for the antiproliferative effect of oxaliplatin. Although the detailed mechanism regarding how oxaliplatin suppresses YY1

expression needs to be further elucidated, these novel findings provide new insight into the pharmacology of oxaliplatin, as well as evidence for the potential of targeting YY1/GLUT3 axis as a therapeutic strategy.

In conclusion, we unraveled a novel role of YY1 in tumorigenesis by linking its oncogenic characteristic with glucose entry into the cells, the pacesetter of tumor cell aerobic glycolysis. These findings further suggest that YY1 is a crucial factor in tumorigenesis, and that it is a good potential target for treating cancers, irrespective of the p53 status.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

V. K. and S. W. conceived the project, designed the experiments, performed data analysis, and interpretation of the experimental results. Y. W. performed most of the experiments and performed data analysis. C. H. carried out part of the vector constructions, the colony formation assay and the animal experiments. Y. L. carried out EdU-incorporation and colony formation assays. H. Z. collected human clinical samples and performed clinical samples analysis. All authors were involved in writing the manuscript and had final approval of the submitted and published versions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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