

E2F1 promotes Warburg effect and cancer progression via upregulating ENO2 expression in Ewing sarcoma

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Abstract. Altered glucose metabolism is an important characteristic of cancer cells, which is referred to as Warburg effect or aerobic glycolysis. Ewing sarcoma (EWS) is a highly malignant tumor that occurs in children and adolescents. However, the functions of aerobic glycolysis in EWS remain to be elucidated. The present study identified a transcription factor, E2F transcription factor 1 (E2F1), as a new regulator of cancer the aerobic glycolysis and progression in EWS. The present study showed that *E2F1* modulated aerobic glycolysis in EWS cells by effecting glucose uptake, lactate production and ATP generation. Altered *E2F1* expression increased or decreased cell viability and invasion in EWS. Mechanistically, the results demonstrated that *E2F1* may promote the Warburg effect and cancer progression in EWS via upregulating enolase 2 expression. Generally, these findings indicated that *E2F1* involvement in the progression of EWS and could serve as a clinical therapeutic target in EWS.

Introduction

EWS is a highly aggressive bone- or soft tissue-associated tumor of childhood and young adults (1). Though advances

in the treatment of EWS have improved quality of life, the prognosis is still poor for early metastases and relapse (2). Therefore, it is urgently necessary to find the mechanism and relevant therapeutic targets of EWS.

Aerobic glycolysis is a well-recognized hallmark of malignant tumors (3), making it an attractive therapeutic target to inhibit tumor growth, including Ewing sarcoma (4). To meet high demands for growth, cancer cells exhibit a unique metabolic preference for catabolizing glucose to lactate even under aerobic conditions, a phenomenon described as Warburg effect or aerobic glycolysis (3). It has been proposed that transcription factors serve a crucial role in regulating aerobic glycolysis. For instance, acting as a principal regulator of glycolysis, hypoxia-inducible factor 1 α (HIF-1 α) contributes to aerobic glycolysis by the induction of the glucose transporters GLUT1 expression, thus enhancing cancer progression (5). Thus, identifying key regulators that regulate aerobic glycolysis could offer a novel direction for EWS treatment.

The present study, for the first time to the best of the authors' knowledge, identified transcription factor E2F1 as a pivotal regulator for aerobic glycolysis in EWS. Highly expressed *E2F1* in EWS predicted a poor prognosis. Moreover, the results revealed that *E2F1* facilitates Warburg effect and cancer progression by modulating the expression of enolase 2 (*ENO2*). Taken together, a new role of E2F1 in EWS was unearthed, which provide a promising therapeutic strategy for EWS.

Materials and methods

Access and analysis of public data. The Ewing sarcoma microarray datasets GSE17679 and corresponding clinical information were downloaded from Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). In addition, glycolysis genes were retrieved from the Kyoto Encyclopedia of Genes and Genomes ([https://www.kegg.jp](https://www.kegg.jp;); Table SI) and human transcription factor were downloaded from a public database (<http://humantfs.ccr.utoronto.ca>; Table SII). Data processing and quantile normalization were performed using R, a programming language and software environment for statistical computing (6). Differential expression analyses in different tumor tissues characterized by clinical characteristics

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were performed by the limma package (<http://www.bioconductor.org/packages/release/bioc/html/limma.html/>). Genes with $|\log_2\text{FoldChange}| > 1$ and $P < 0.05$ were identified as statistically significant. Then, overlapping analysis was performed to identify genes consistently associated with clinical characteristics of tumors, while their expression correlation and survival significance were also analyzed.

Cell culture. Human EWS cell lines RDES (HTB-166) and SK-ES-1 (HTB-86) were obtained from ATCC. Cells were cultured in RPMI medium (R8758; MilliporeSigma) containing 10% fetal bovine serum (F8318; MilliporeSigma), 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin (V900929; MilliporeSigma) at 37°C in a humidified atmosphere containing 5% CO_2 .

Plasmid transfections. EWS cells were cultured in six-well plates until cells reached $\geq 70\%$ confluence. Overexpression vector pCMV-HA-E2F1 or pDONR233-ENO2 and corresponding empty vector pCMV-HA or pDONR233, and shRNAs against E2F1 or ENO2 and corresponding empty vector lentiviral vector GV298 were transiently transfected using a Neofect DNA transfection reagent kit (cat. no. TF201201; Neofect Biotech Co., Ltd.). Briefly, 2 μg of plasmid DNA was diluted with 100 μl of serum-free medium mixed with 100 μl of serum-free medium containing 2 μl Neofect DNA transfection reagent (1 μg DNA:1 μl Neofect), which were incubated for 30 min at room temperature. Then the 200 μl plasmid/transfection reagent complex was added to the well, which were further incubated for 24–48 h at 37°C in a 5% CO_2 incubator, after which they were used for experiments. The knockout or overexpression efficiency was examined using a PCR-based method.

Glucose uptake, lactate and ATP assay. The present study employed a Glucose Uptake Assay kit and Lactate Assay kit (BioVision, Inc.) to determine glucose uptake level and lactic acid production, respectively. Briefly, the cells were seeded in a 96-well plate and incubated at indicated time and then the cell culture medium and cell supernatant were collected to determine glucose uptake and lactic acid production. Additionally, a ATP Colorimetric Assay kit was used to measure ATP production (BioVision, Inc.). All experiments are performed according to the manufacturer's protocol.

MTT assays. Cell proliferation was determined using MTT assays. Human EWS cell lines RDES or SK-ES-1 cells were seeded in a 96-well plate with 1×10^5 /well in 100 μl RPMI, allowed to grow for the indicated times at 37°C in a 5% CO_2 incubator. Thereafter, MTT solution (final concentration, 5 mg/ml) was added to the wells and the cells were incubated for 4 h at 37°C in a 5% CO_2 incubator. Then, removed the medium and 100 μl dimethylsulfoxide (DMSO) was added to dissolve formazan crystals with agitated cell culture plate for 5 min at room temperature. The absorbance was recorded on a microplate reader Elx800 (Bio-Tek, Winooski, Vermont, USA) at 570 nm.

Matrigel invasion assay. Cell invasion assay was performed using membranes with precoated Matrigel at 37°C for 30 min

(200 $\mu\text{g/ml}$; BD Biosciences). Briefly, 1×10^5 cells/well were added to the upper chambers and 500 μl medium containing 10% FBS was added to the bottom chamber. Following incubation at 37°C and 5% CO_2 for 24 h, invaded cells were stained with 1% crystal violet for 15 min at room temperature. Subsequently, the invasive cells were examined and counted using a light microscope (magnification, $\times 200$) in five random microscopic fields.

Gene overexpression and knockdown. Human genes *E2F1* (Gene ID: 1869) overexpression vector with pCMV-HA was purchased from Addgene (cat. no. 24225) and *ENO2* (Gene ID: 2026) overexpression vector with pDONR233 was purchased from AtaGenix (cat. no. AtBC002745). Oligonucleotides specific for short hairpin (sh)RNAs against *E2F1* and *ENO2* were designed and purchased from Genechem Co., Ltd. with lentiviral vector GV298. The nucleotide sequences were as follows: *E2F1* (sh-*E2F1* #1: TCTGCCACCATAGTCTCG AGA, sh-*E2F1* #2: CTCGAGCAAAGTCACAGTCGA), *ENO2* (sh-*ENO2* #1, CAAGGGAGTCATCAAGGACAA, sh-*ENO2* #2 CGCCTGGCTAATAAGGCTTTA). The negative control was sh-Sch: AACGGACTCGAGTCCGTTTAC.

Reverse transcription-quantitative (RT-q) PCR. Total RNA (1×10^6 cells/well in a 6-well plate) was isolated with RNeasy Mini kit (Takara Bio, Inc.) according to the manufacturer's protocols and RNA absorbance measured by an ultra-micro spectrophotometer at 260 nm (Thermo Fisher Scientific, Inc.). The obtained RNA ($\sim 2.0 \mu\text{g}$) was used to conduct reverse transcription reactions with Transcriptor First Strand cDNA Synthesis kit (Takara Bio, Inc.) at 37°C for 1 h based on the manufacturer's protocols. RT-qPCR with luminariscolor hiGreen qPCR master mix (Fermentas; Thermo Fisher Scientific, Inc.) was conducted using ABI Prism 7700 Sequence Detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the qPCR: 50°C for 2 min and 95°C for 10 min; 36 cycles of 95°C for 10 sec and 60°C for 60 sec. The primer sequences were synthesized by TSINGKE Biotech Co., Ltd. Each 20- μl PCR reaction components contained 10 μl hiGreen qPCR Mix, 3 μl cDNA, 0.6 μl Forward Primer (10 μM) and 0.6 μl Reverse Primer (10 μM) and finally by adding nuclease-free H_2O to 20 μl . This experiment was repeated three times. The transcript levels were normalized to β -actin and analyzed by $2^{-\Delta\Delta C_q}$ method (7). The following primer sets were used: for human *E2F1*: 5'-CGCTATGAGACCTCACTGAAT-3' (forward) and 5'-CACTGGATGTGGTTCTTGGAC-3' (reverse); *ENO2*: 5'-AGGTGCAGAGGTCTACCATAC-3' (forward) and 5'-AGC TCCAAGGCTTCACTGTTC-3' (reverse); *PFKM*: 5'-AGC TGCCTACAACCTGGTGA-3' (forward) and 5'-TCCACT CAGAACGGAAGGTGT-3' (reverse); *TPII*: 5'-AGTGACTAA TGGGGCTTTTACTG-3' (forward) and 5'-GCCCAATCA GTCATCTGACTC-3' (reverse); β -actin: 5'-CATGTACGT TGCTATCCAGGC-3' (forward) and 5'-CTCCTTAATGTC ACGCACGAT-3' (reverse).

Western blot analysis. Cellular protein was extracted using 1X cell lysis buffer (Promega Corporation) and the cellular protein concentration was measured by bicinchoninic acid protein assay kit (Promega Corporation). The obtained

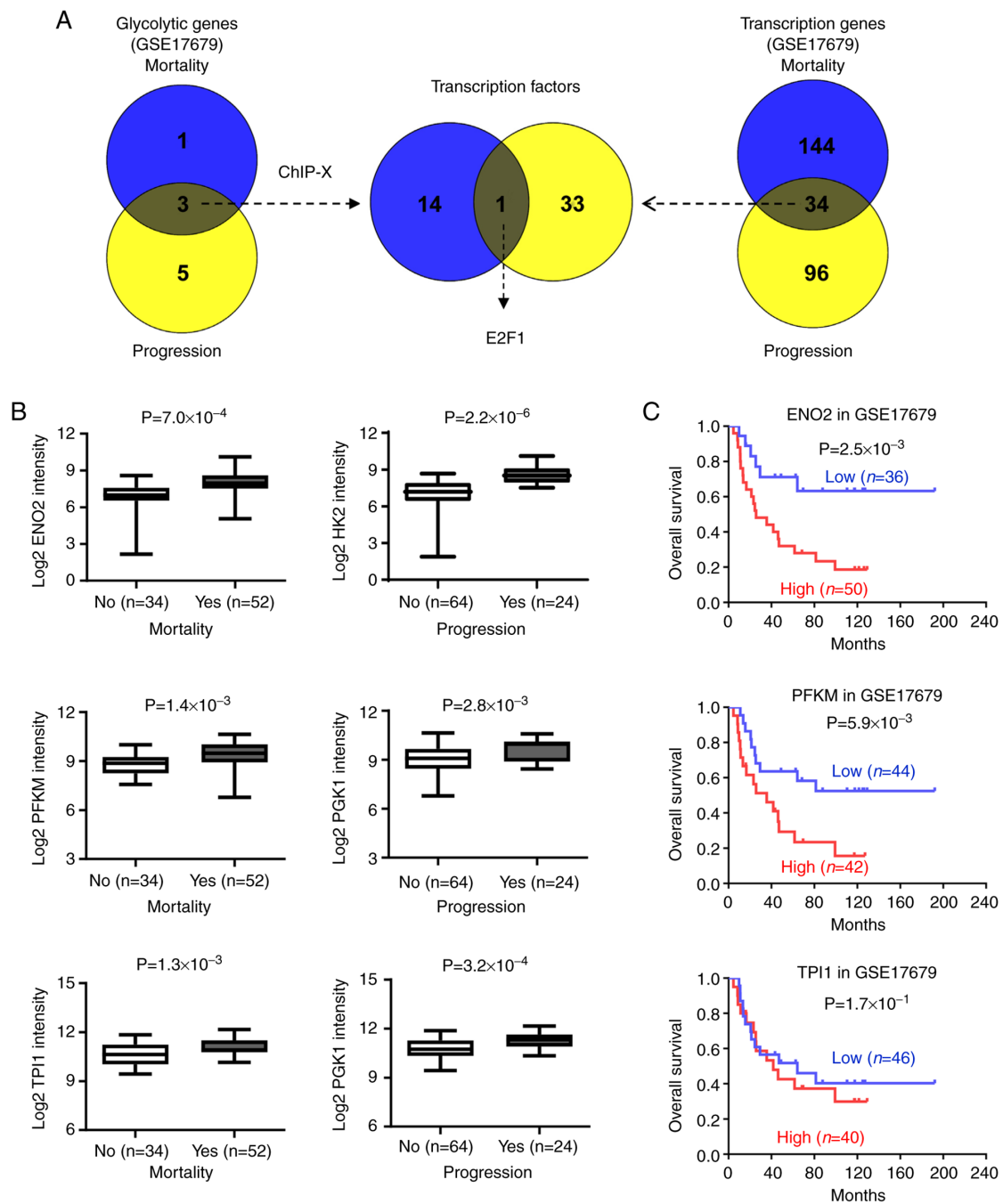


Figure 1. E2F1 involvement in aerobic glycolysis in EWS. (A) (left and right panel) Venn diagrams showing different expressed glycolytic genes and transcription factors ($P < 0.05$) in EWS (GSE17679) associated with the status of mortality and progression, respectively; (middle panel) overlapping analysis with 15 transcription factors regulating three glycolytic genes analyzed by ChIP-X. (B) The relative *ENO2* (upper panel), *PFKM* (middle panel) and *TPI1* (lower panel) levels in EWS (GSE17679) with the mortality and progression. (C) Kaplan-Meier curves showing the overall survival in EWS (GSE17679) with high or low levels of *ENO2*, *PFKM* and *TPI1* (cutoff values=7.474, 9.177 and 10.900). Student's t-test compared the difference in panel B. Log-rank test for survival comparison in panel C. E2F1, E2F transcription factor 1; EWS, Ewing sarcoma.

proteins (~30 μ g) were separated by electrophoresis with 4 to 20% precast polyacrylamide gel (Invitrogen; Thermo Fisher Scientific, Inc.) and transferred onto a polyvinylidene fluoride membrane. Then the membrane was blocked with PBS containing 5% non-fat milk at room temperature for 1 h. Subsequently, the membranes were incubated with primary antibodies against rabbit anti-human antibodies against E2F1 (1:1,000; cat. no. A19579, ABclonal), rabbit anti-human antibodies against *ENO2* (1:1,000; A3118, ABclonal), rabbit anti-human antibodies against *PFKM* (1:1,000; cat. no. A5477,

ABclonal), rabbit anti-human antibodies against *TPI1* (1:1,000; cat. no. A2579, ABclonal), or rabbit anti-human antibodies against β -actin (1:50,000; cat. no. AC026, ABclonal) at 4°C overnight. After washing with TBS containing 0.1% Tween-20 (TBST), the membranes were incubated with horseradish peroxidase-conjugated goat Anti-Rabbit IgG secondary antibodies at room temperature for 1 h (1:5,000, cat. no. AS014, ABclonal). Enhanced chemiluminescence substrate kit (Thermo Fisher Scientific, Inc.) was used for the chemiluminescent detection of signals with autoradiography

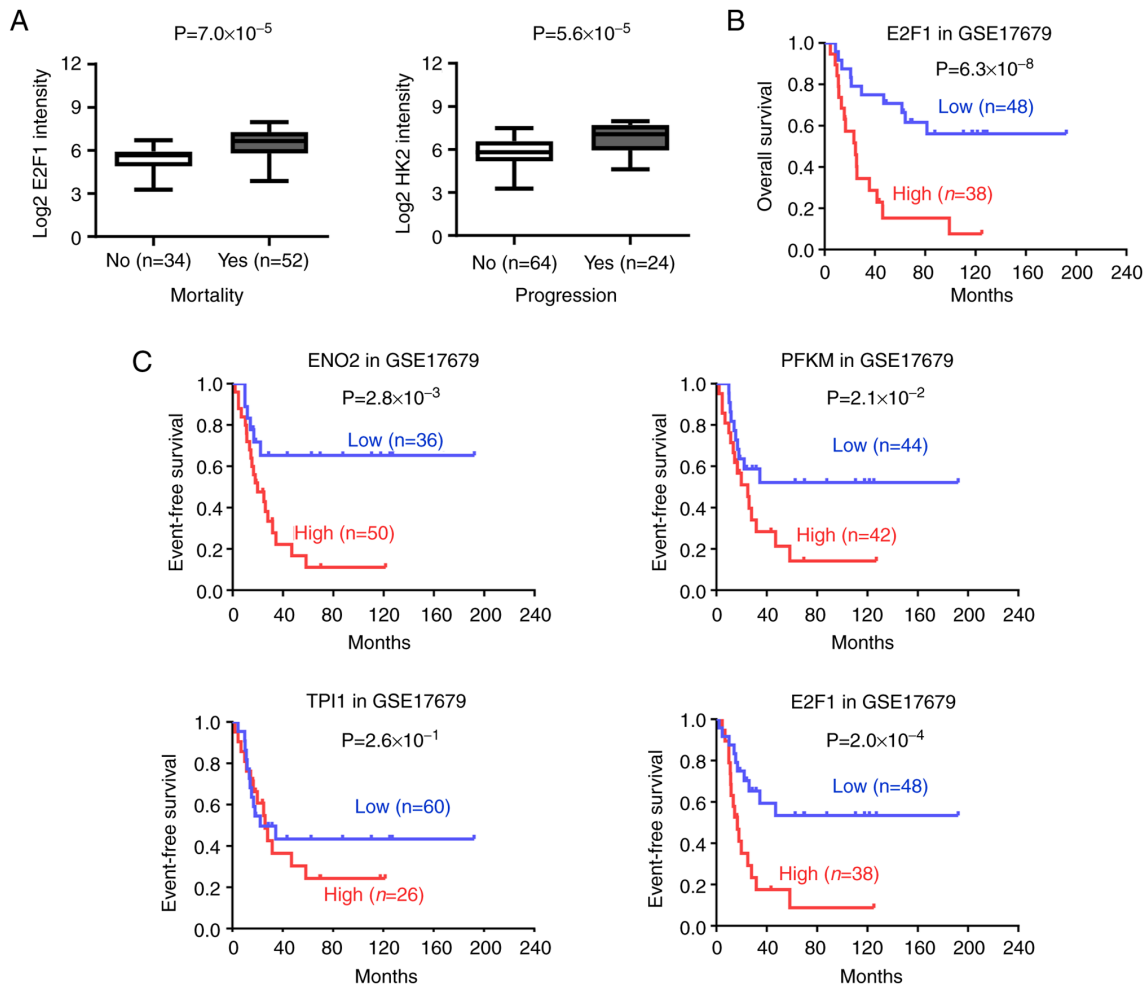


Figure 2. Expression of *E2F1* and glycolytic genes in tumor tissues. (A) The relative *E2F1* levels in EWS (GSE17679) with the mortality and progression. (B) Kaplan-Meier curves showing the overall survival in EWS (GSE17679) with high or low expression of *E2F1* (cutoff values=5.998). (C) Kaplan-Meier curves showing the overall survival in EWS (GSE17679) with high or low expression of *ENO2*, *PFKM*, *TPI1*, and *E2F1* (cutoff values=7.347, 9.177, 10.900 and 5.998). Student's t-test compared the difference in panel A. Log-rank test for survival comparison in panels B and C. *E2F1*, *E2F* transcription factor 1; EWS, Ewing sarcoma.

film (Cytiva). Protein expression was quantified by densitometry using the Image-Pro Plus 6.0 imaging software (Media Cybernetics, Inc.) with β -actin as the loading control.

Statistical analysis. Statistical analyses used are detailed in the figure legends. A two-tailed unpaired t-test was used to compare data between two independent groups. One-way ANOVA with Bonferroni's multiple comparison post hoc test was used to compare mean differences between data with multiple groups. For survival analyses, cutoff of gene expression was defined by average values, survival curves were analyzed by log-rank (Mantel-Cox) analysis. Pearson correlation analysis and all the other statistics were performed in GraphPad Prism 9 (GraphPad Software). Data are presented as mean \pm standard error of the mean (SEM). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

***E2F1* involvement in aerobic glycolysis in EWS.** To identify key regulators of aerobic glycolysis in EWS, the present study

performed a bioinformatics analysis base on a public EWS dataset (GSE17679) and identified one and eight glycolytic genes ($P < 0.05$) were correlated with varied status of mortality and progression (Fig. 1A). Based on the above data, three glycolytic genes (*ENO2*, *PFKM* and *TPI1*) were found to be related with the status of mortality and progression (Fig. 1A and Table SIII). Further, 15 potentially transcription factors were found regulating these three glycolytic genes analyzed by ChIP-X (8). In addition, 34 transcription factors were consistently associated with the status of mortality and progression in EWS dataset (Fig. 1A), which were further overlapping analysis with previous result (Fig. 1A and Table SIII). Clearly, *E2F1* as the only transcription factor potential associated with aerobic glycolysis in EWS (Fig. 1A and Table SIII). Further analysis revealed that higher expression of *ENO2*, *PFKM*, *TPI1* and *E2F1* were observed in patients with EWS with mortality ($P=7.0 \times 10^{-4}$, $P=1.4 \times 10^{-3}$, $P=1.3 \times 10^{-3}$ and $P=7.0 \times 10^{-5}$) and progression ($P=2.2 \times 10^{-6}$, $P=2.8 \times 10^{-3}$, $P=3.2 \times 10^{-4}$ and $P=5.6 \times 10^{-5}$) (Figs. 1B and 2A). More importantly, log-rank test of EWS cases indicated that patients with high *ENO2*, *PFKM* and *E2F1* expression had poorer overall survival, but not *TPI1* (Figs. 1C and 2B). Consistently, patients with high

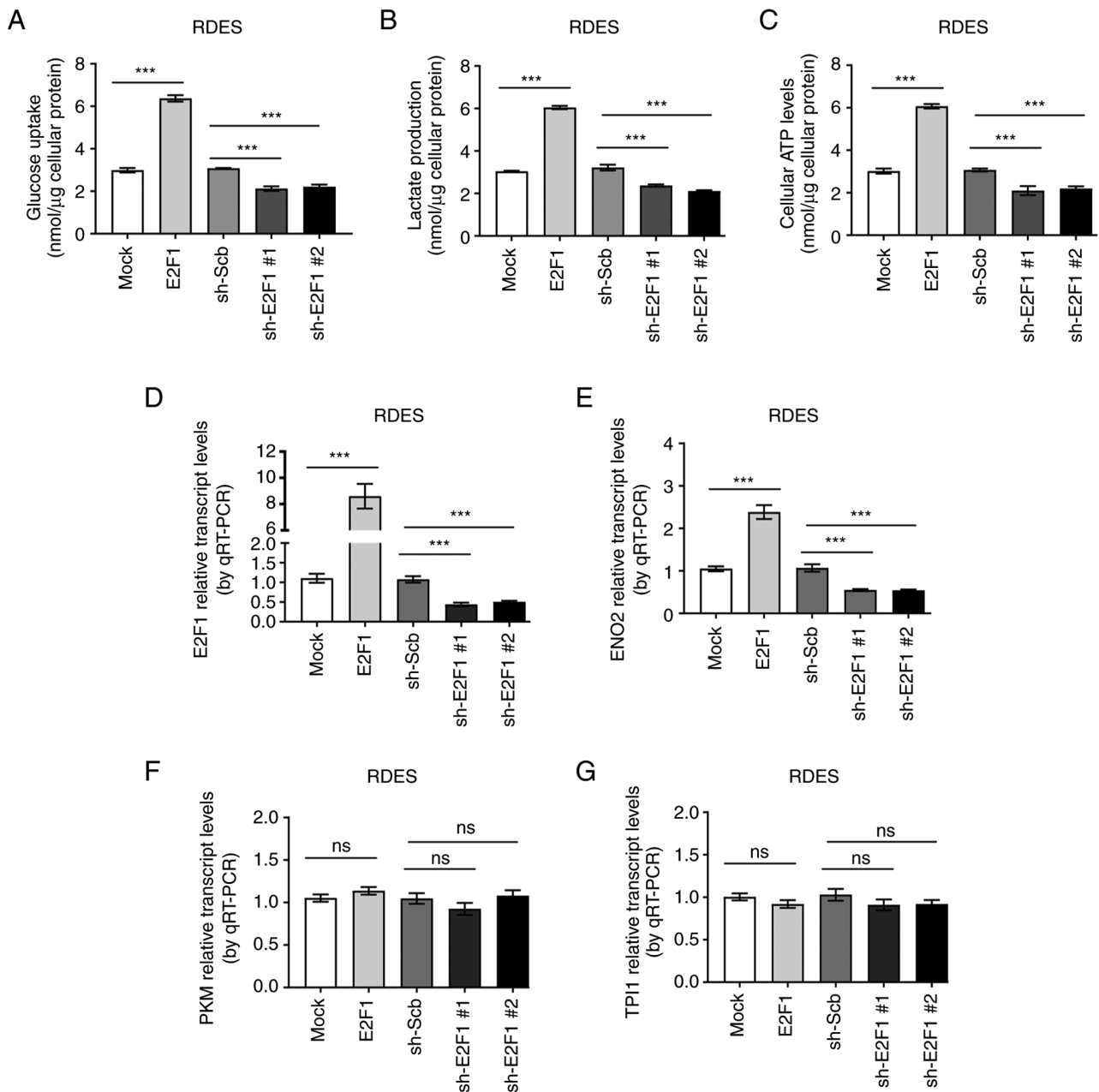


Figure 3. E2F1 regulates aerobic glycolysis. The levels of (A) glucose uptake, (B) lactate production and (C) cellular ATP levels were detected in RDES cells upon the transfection of the indicated plasmids. Reverse transcription-quantitative PCR (normalized to β -actin, n=3) revealing the transcript levels of (D) *E2F1*, (E) *ENO2*, (F) *PFKM* and (G) *TP1I* in RDES cells treated with stably transfected as indicated. Student's t-test compared the difference in Mock vs. E2F1. One-way ANOVA followed by post-hoc Bonferroni's test was used to compare the difference in sh-Scb vs. sh-E2F1 #1 or sh-E2F1 #2. ***P<0.001 vs. Mock or sh-Scb; ns, no significance; E2F1, E2F transcription factor 1; sh, short hairpin.

ENO2, *PFKM* and *E2F1* had poorer event-free survival in EWS, but not *TP1I* (Fig. 2C). These findings indicated that E2F1 involvement in aerobic glycolysis in EWS.

E2F1 regulates aerobic glycolysis gene expression of ENO2. To characterize the roles of E2F1 on aerobic glycolysis in EWS, glucose uptake, lactate production and ATP levels were evaluated under enhanced or decreased *E2F1* expression. As expected, forced expression of *E2F1* increased the glucose uptake, lactate production and ATP levels in EWS cells, while silencing of *E2F1* led to decrease the glucose uptake, lactate production and ATP levels (Fig. 3A-C). To further functionally characterize E2F1 regulating aerobic glycolysis,

the expression of putative target genes were measured. The result validated that stable overexpression of *E2F1* in EWS cell line RDES resulted in increased expression of *ENO2*, while silencing of *E2F1* led to decreased expression of *ENO2* (Figs. 3D-E and 4A-C). Meanwhile, the level of *PFKM* and *TP1I* was not affected by the altered expression of *E2F1* (Figs. 3F-G and 4D-E). In line with the above findings, mining of public datasets revealed that there was a positive expression correlation between *E2F1* and *ENO2*, even *PFKM* and *TP1I* ($R=0.263$, $P=1.3 \times 10^{-2}$, $R=0.424$, $P=3.8 \times 10^{-5}$ and $R=0.369$, $P=4.0 \times 10^{-4}$) (Fig. 4F-H). Collectively, these data demonstrated that E2F1 may affect aerobic glycolysis in EWS cells via regulating *ENO2* expression.

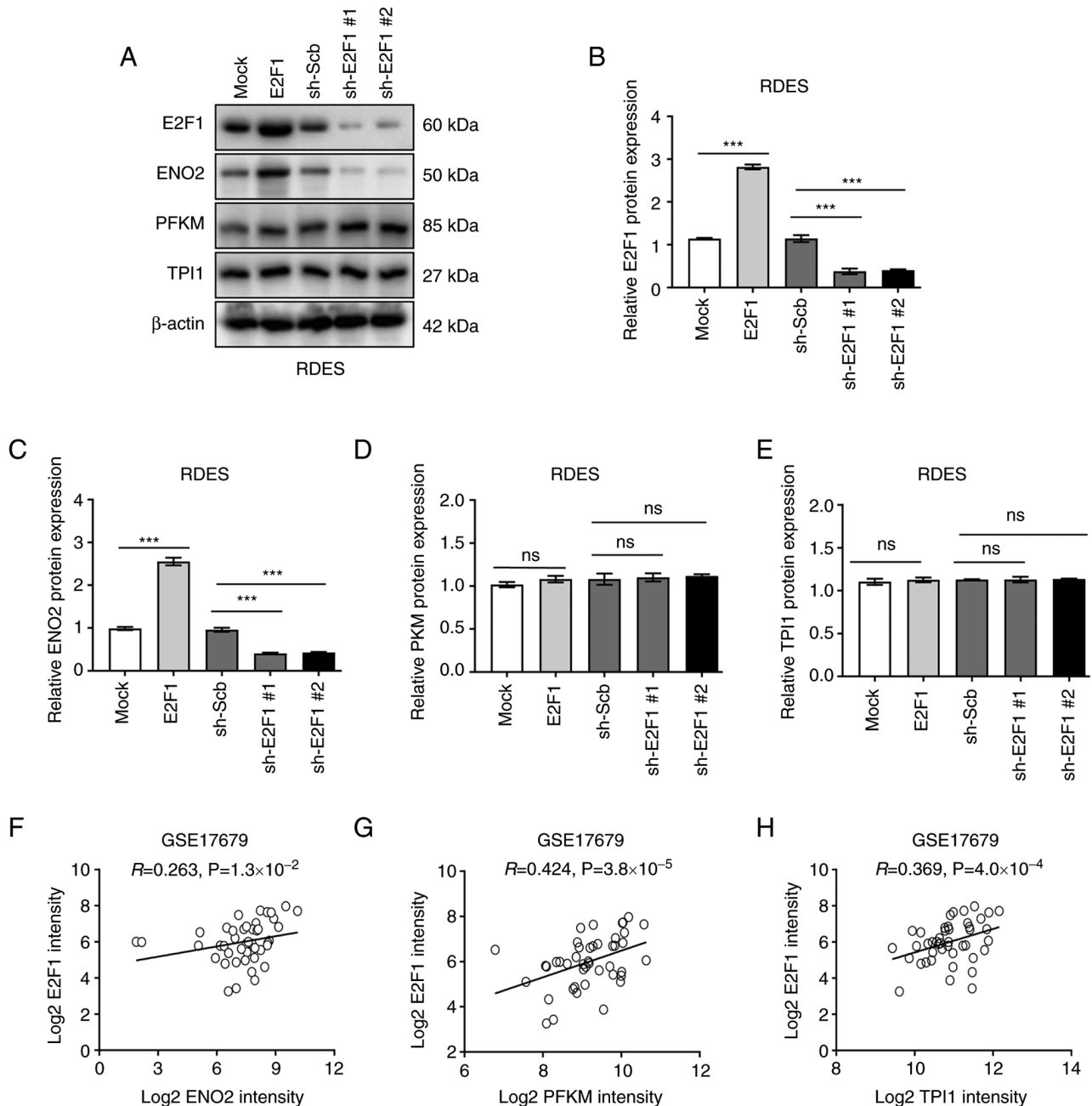


Figure 4. E2F1 regulates aerobic glycolysis gene expression of *ENO2*. (A) Western blot assays revealed the protein levels of E2F1, ENO2, PFKM and TPI1 in RDES cells treated with stably transfected as indicated. (B-E) Protein expression was quantified by densitometry using Image-Pro Plus with β -actin as the loading control and statistics were performed in GraphPad Prism 9. (F-H) The positive gene expression correlation between *E2F1* and (F) *ENO2*, (G) *PFKM* and (H) *TPI1*. Student's t-test compared the difference in Mock vs. E2F1. One-way ANOVA followed by post-hoc Bonferroni's test was used to compare the difference in sh-Scb vs. sh-E2F1 #1 or sh-E2F1 #2. *** $P < 0.001$ vs. Mock or sh-Scb; ns, no significance; E2F1, E2F transcription factor 1; sh, short hairpin.

E2F1 regulates EWS progression via ENO2. To further assess the functional roles of *E2F1* in EWS, EWS cells with stable overexpression and knockdown of *E2F1* were established. The accumulation of endogenous *E2F1* significantly accelerated cell viability and invasion of RDES or SK-ES-1 cells, whereas knockdown of endogenous *E2F1* led to a significant reduce in cell viability and invasion (Fig. 5A-D). Additionally, silenced or enhanced expression of *ENO2* partially rescued the changes in cell viability and invasion of EWS induced by the forced expression or silencing of *E2F1* (Fig. 5A-D). In conclusion, these data demonstrated that *E2F1* regulates EWS progression via *ENO2*.

Discussion

Altered aerobic glycolysis is a well-recognized characteristic of cancer cells, as elevated glycolytic flux provides essential anabolic to sustain cancer cell proliferation and metastasis (9). Thus, targeting aerobic glycolysis in cancer cells remains an attractive therapeutic way and several small organic molecules, such as 3-bromopyruvate are able to block aerobic glycolysis and repress tumor progression (10). As glycolysis progress is mediated by numerous enzymes, investigating the regulation mechanism of glycolysis genes provide an improved understanding about cancer therapy. Emerging

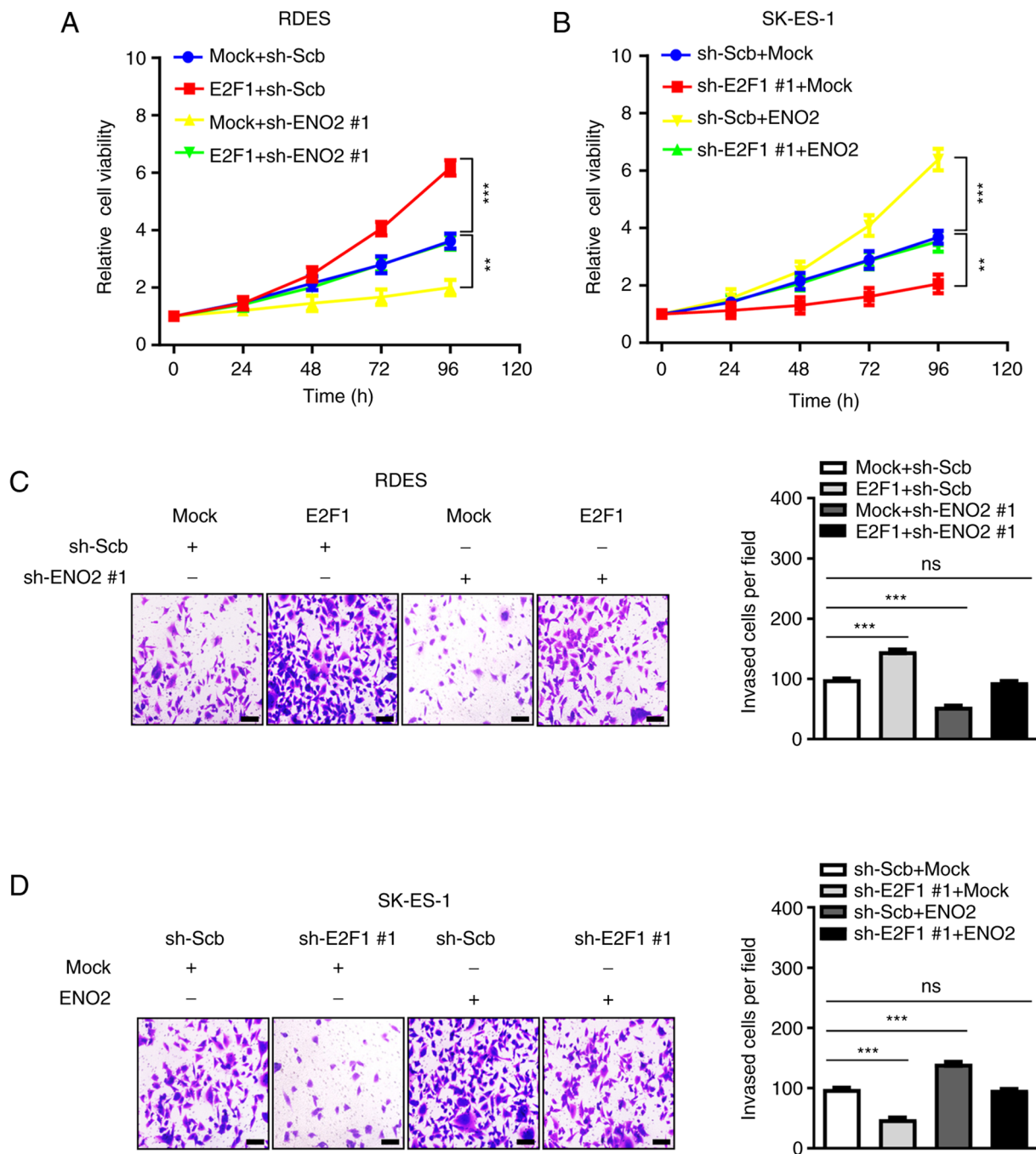


Figure 5. E2F1 regulates EWS progression via *ENO2*. (A) MTT colorimetric assay indicating the viability of RDES cells treated stably transfected as indicated (n=3). (B) MTT colorimetric assay indicating the viability of SK-ES-1 cells treated with stably transfected as indicated (n=3). (C) Representative images (left panel) and quantification (right panel) of Matrigel invasion assays showing the invasion of RDES cells treated with stably transfected as indicated (n=3). (D) Representative images (left panel) and quantification (right panel) of Matrigel invasion assays showing the invasion of SK-ES-1 cells treated with stably transfected as indicated (n=3). Scale bars in images: x200 magnification scale bar, 100 μ m. Student's t-test compared the difference in panel A and B. One-way ANOVA with Bonferroni's multiple comparison post hoc test compared the difference in panel C and D. **P<0.01, ***P<0.001 vs. Mock + sh-Scb; ns, no significance; E2F1, E2F transcription factor 1; sh, short hairpin.

evidence demonstrates that transcription factors regulate glycolysis in various cancers. For example, the oncogenic transcription factor c-MYC regulates expression of glycolytic genes, including enolase 1 (*ENO1*) and lactate dehydrogenase A (*LDHA*), eventually enhancing aerobic glycolysis (11). Additionally, HIF1 α through activating pyruvate dehydrogenase kinase 1 (*PDK1*) modulates aerobic glycolysis (12). Similarly, depletion of *EWS-FLI1*, the oncogenic driver of EWS, results in a decrease in *LDHA* levels in preclinical

models of EWS, thus eventually impairing glycolysis and affecting cell survival. The current study uncovered *E2F1* as a critical regulator of aerobic glycolysis and EWS progression. It demonstrated that *E2F1* is a driver of EWS and promoted the aerobic glycolysis and progression of EWS cells by regulating expression of *ENO2*.

E2F1 belongs to the E2F transcription factor family that is involved in numerous cellular processes (13,14). In human tumors, aberrant E2F1 is been found in various types of cancer,

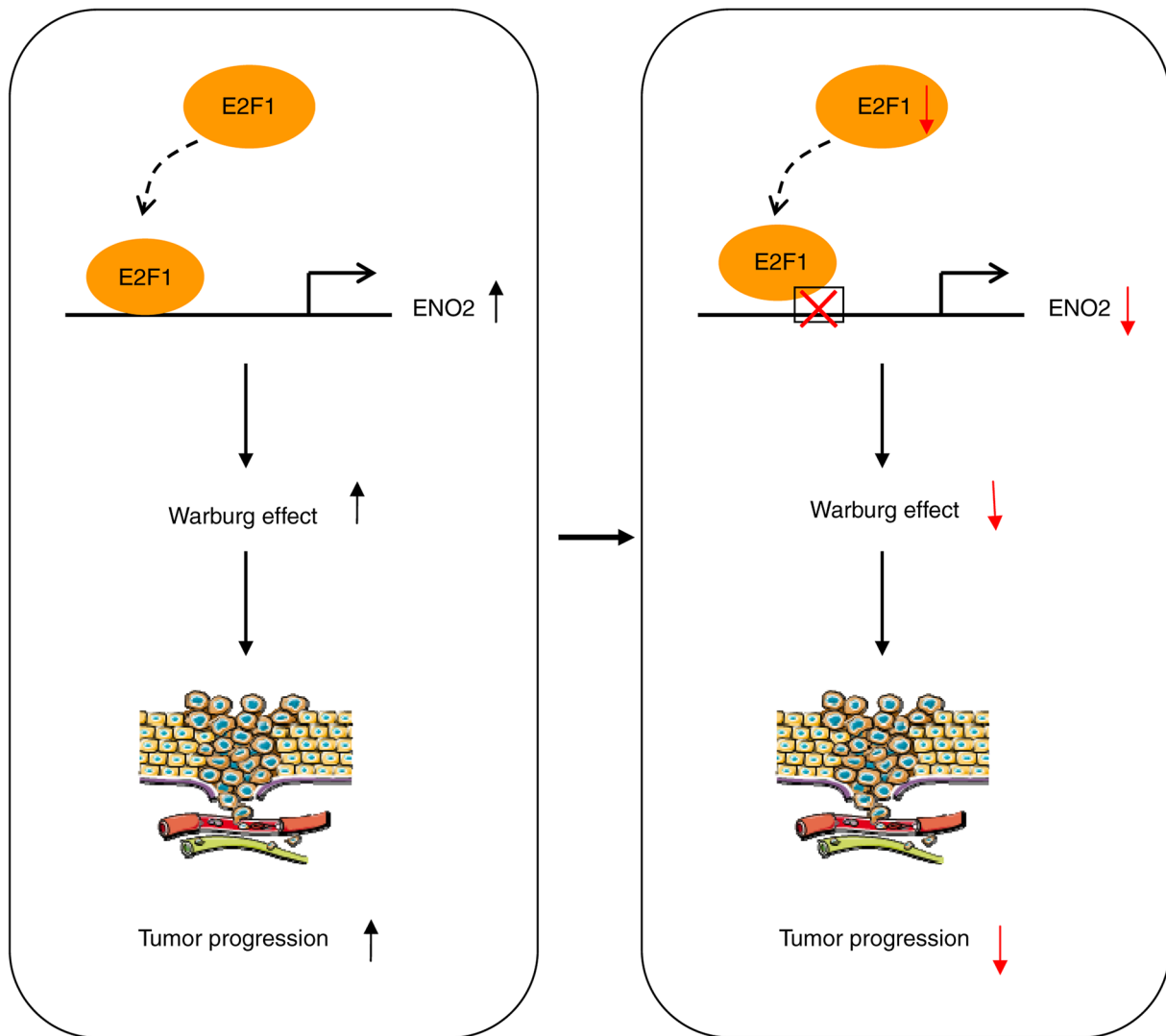


Figure 6. Schematic diagram of the mechanism. Schematic depicting the mechanisms underlying E2F1-promoted Warburg effect and cancer progression: as a transcription factor, E2F1 promotes expression of ENO2, resulting in enhanced Warburg effect and cancer progression in EWS. E2F1, E2F transcription factor 1.

leading to the unfavorable prognosis of patients with cancer. In neuroblastomas, *E2F1* regulates *MYCN* gene expression, which is the most important molecular marker of neuroblastomas (15). Additionally, studies indicate an emerging role of *E2F1* in regulating aerobic glycolysis. For instance, *E2F1* is involved in the development of liver pathology by regulating glycolysis process (16). In breast cancer, *E2F1* transcriptionally regulates *SEC61G* expression result in modulate glycolysis, leading to cancer development and metastasis (17). The findings of the present study indicated that *E2F1*, facilitated cancer progression and aerobic glycolysis in EWS via regulating the expression of *ENO2*.

ENO2, also known as neuro-specific enolase (NSE), is primarily expressed by mature neurons and cells of neuronal origin (18,19). Serving as a key glycolytic enzyme in glycolysis, *ENO2* is responsible for the conversion of β -glycerophosphate into dihydroxyacetone phosphate (20). *ENO2* is a well-established biomarker for neuroblastoma, small-cell lung cancer and other tumors (21,22). In gastric cancer, studies have found that elevated *METTL3* expression

through activating *GLUT4* and *ENO2* expression promotes tumor angiogenesis and glycolysis (23). However, the roles of *ENO2* in EWS remain to be elucidated. In present study, high *ENO2* expression was closely associated with progression and poor prognosis in EWS. More importantly, it was demonstrated that *ENO2* was the target of E2F1, which mediated aerobic glycolysis and cancer progression in EWS.

The present study has several limitations. First, it only explored the role of *E2F1* *in vitro*, thus *in vivo* studies are needed to further investigate the effects of *E2F1* on glycolysis and cancer progression in EWS. Second, *E2F1* is associated with cell cycle progression and *E2F1* can modulate genes expression in a cell cycle-dependent or independent manner (24). Here, the results of the present study demonstrated that *E2F1* can regulate the expression of *ENO2*, although without full understanding of the clear mechanism. To clarify the mechanism that *E2F1* regulated *ENO2* expression in EWS, special inhibitors of cell cycle or E2F1 are needed to be administered in follow-up studies.

In summary, the present study demonstrated that *E2F1* was a prognostic biomarker and associated with glycolysis in EWS.

Mechanistically, *E2F1* regulated *ENO2* expression to induce glycolysis and promote cancer progression in EWS (Fig. 6). Therefore, *E2F1* might be a potential predictor and therapeutic target for EWS. These results extend the understanding of EWS.

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Availability of data and materials

The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

Authors' contributions

XJ, ZC, and JZ designed and performed the experiments. JH, GY and YL were involved in designing the figures and analyzing the data. HY and TL designed the research, revised the manuscript for important intellectual content and drafted the manuscript. HY and TL confirm the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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