**Supplementary information**

# Aerobic glycolysis in colon cancer is repressed by naringin via the *HIF1A* pathway

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## Materials and methods

### 1. JASPAR analysis

The positional weight matrix (PWM) of the transcription factor *HIF1A* was predicted on the JASPAR website (https://jaspar.genereg.net/). Subsequently, we used NCBI to search for the promoter region of the *ENO2* gene, that is, the DNA sequence of the transcription start site (TSS site, −1000 to 100, https://www.ncbi.nlm.nih.gov/gene). The promoter sequence was input into the JASPAR website to evaluate the binding site of *HIF1A*.

### 2. Western blot

Colon cancer cells were pretreated for 36–48 hours according to the experimental groups. The culture medium was discarded, and the cells were washed

3 times with phosphate buffer. After the colon cancer cells were scraped and centrifuged at 1000 g, RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) was used to lyse the precipitated cells. The bicinchoninic acid (BCA) kit (Beyotime Biotechnology, Shanghai, China) was used to determine the protein concentration to ensure 30 μg protein per sample well. A 12% polyacrylamide gel (including SDS) was used to separate the bands of interest by electrophoresis at a constant voltage of 80 volts for 150 min. Subsequently, a constant current of 300 mA was used to transfer the target protein to the activated nitrocellulose membrane. A 5% bovine serum albumin (BSA) solution was used to block the membrane for 1 hour at room temperature and incubated overnight with the primary antibody at 4 °C. After the membrane was incubated with the secondary antibody at room temperature for one hour, the ECL exposure solution was used to emit light in a dark room.

**3. Extracellular acidification rate (ECAR)**

An XF24 extracellular flux analyser (Seahorse Bioscience, North Billerica, MA) was used to detect the rate of extracellular acidification. Briefly, pretreatment of colon cancer cells seeded on XF24-well plates was performed 24 hours before the test. In addition, the probe was hydrated overnight in a 37 °C carbon dioxide-free constant temperature incubator. In this assay, standard concentrations of glucose, 2-deoxyglucose (2DG), and oligomycin were added to the dosing hole in sequence. The Seahorse instrument was used to analyze the data. The measurement protocol followed the manufacturer's instructions.

**4. Matrigel invasion**

Matrigel was prepared to melt overnight at 4 °C, while the experimental test tube and pipette tip were precooled. Before the cells were digested, Matrigel gel was diluted with cold serum-free medium. A sterile transwell chamber with a diameter of 8 μm was prepared, and 100 μL of diluted gel was added to each chamber. The Matrigel was coagulated at a constant temperature of 37 °C for 1 hour. The cells in the culture flask were digested, resuspended and counted. A total of $5\times10^5$ cells/mL fetal bovine serum (FBS)-free medium was added, and 100 μL of cell suspension was added to the upper chamber. Then, 500 μL of cell culture medium containing 10% FBS was added to the lower chamber. At 37 °C, the cells grew for 24–36 hours. The cell culture medium was discarded, and the cells were fixed with 4% paraformaldehyde. A cotton swab was used to wipe off the noninvasive cells in the upper chamber. Then, 500 μL of 0.1% crystal violet was added to each well, the chamber was placed in it, and the membrane was immersed in the culture medium, removed after 30 min at 37 °C, and washed with phosphate-buffered saline (PBS). The cells were observed and counted under a microscope. GraphPad 5.0 (GraphPad Software, San Diego, USA) was used for statistical analysis.

**5. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay**

Colon cancer cells were uniformly seeded in a 96-well plate, with 10,000 cells per well. Transfection or dosing was performed 48 hours before the test. Four hours before the test, fresh medium containing 0.5% MTT (Beyotime Biotechnology,

Shanghai, China) was replaced in a dark environment, and the culture was continued in a 37 °C constant temperature incubator. Subsequently, the medium was discarded, and the excess medium was washed with PBS. Then, 100 μL of DMSO (Beyotime Biotechnology, Shanghai, China) was added to each well and shaken for 15 min without light. This MTT-DMSO mixture was detected at an absorbance of 570 nm, and GraphPad 5.0 (GraphPad Software, San Diego, USA) was used to analyze the data.

**6. Dual luciferase reporter assay**

Dual luciferase reporter vectors containing *HIF1A* and *ENO2* were constructed. Specifically, the PWM site of *HIF1A* was searched on the JASPAR (http://jaspar.genereg.net/) website, cloned three times and then inserted into the pGL3.0-Basic vector. Additionally, the *ENO2* promoter region containing the *HIF1A* binding site was searched on the JASPAR website and cloned into the pGL3.0-Basic vector. Then, 50,000 colon cancer cells were uniformly seeded in a 24-well plate, and after the cells were completely attached, they were transfected with the dual luciferase reporter gene vector and pRL-TK vector. After 24 hours of transfection, the cells were washed with PBS and dried, and 100 μL of cell lysate was added (Beyotime Biotechnology, Shanghai, China), pipetted and mixed thoroughly. Then, 10 μL of lysate was pipetted into a 1 mL EP tube. Then, 50 μL of luc buffer and stop buffer were added to the EP tube, and a GloMax 20/20 Luminometer (Promega, USA) was used to detect the fluorescence intensity of firefly and sea cucumber

luciferase. The relative fluorescence intensity was calculated and statistically analyzed by GraphPad5.0 (GraphPad Software, San Diego, USA).

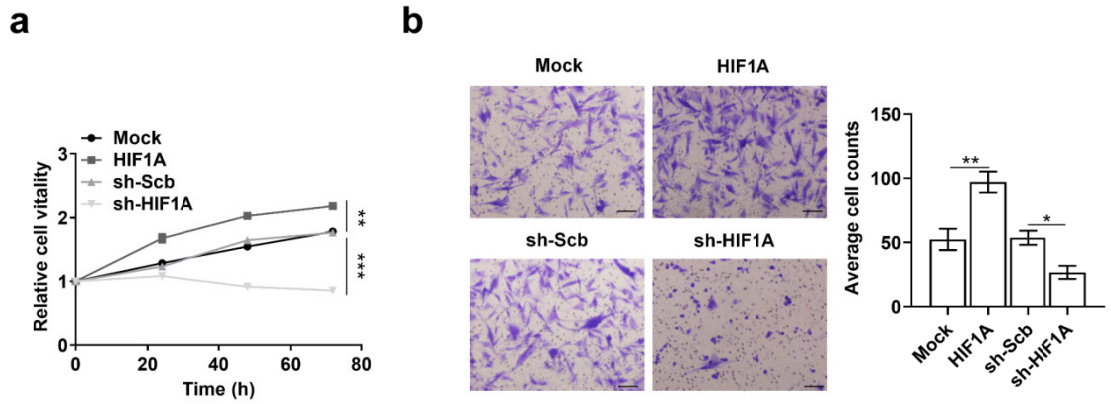


**Fig. S1 Promotes aerobic glycolysis in colon cancer. (a) MTT and (b) Matrigel invasion assays were used to test the effects of *HIF1A* on the proliferation and invasion of colon cancer cells. Scale bar: 100 μm. Data are presented as mean±SD, *n*=3. $^{*}$ *P*<0.05, $^{**}$ *P*<0.01, $^{***}$ *P*<0.001. *HIF1A*: hypoxia inducible factor-1α; SD: standard deviation.**

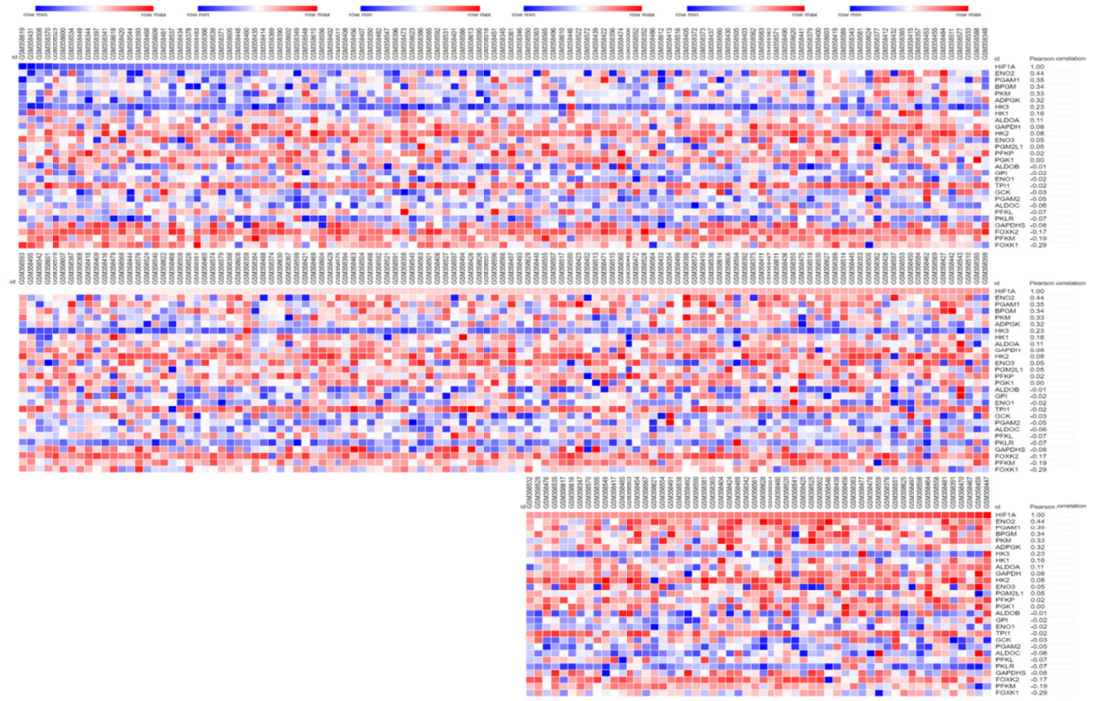

**Fig. S2 The heatmap shows the glycolytic core genes associated with *HIF1A* in colon cancer RNA-seq (GSE14333, *n*=226).**

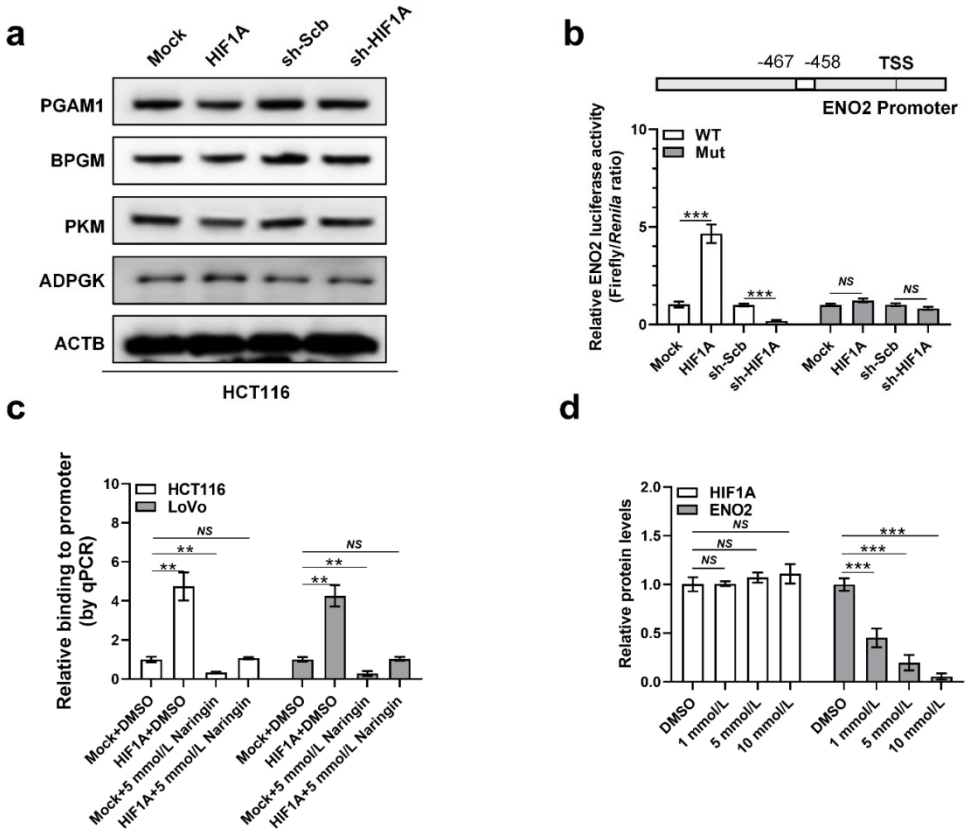


**Fig. S3** ***ENO2*** **is a key glycolytic target gene of** ***HIF1A.*** **(a) Western blot results showed that** ***HIF1A*** **had no effect on the expression of other glycolytic genes (*PGAM1*, *BPGM*, *PKM*, *ADPGK*) in HCT116 cells. (b) This panel shows the regulatory effect of** ***HIF1A*** **on the activity of the** ***ENO2*** **mutant and wild-type promoter regions. The deletion site is located in the −467 to −458 region of the** ***ENO2*** **TSS. (c) ChIP assays verify the enrichment levels of** ***HIF1A*** **in the** ***ENO2*** **promoter region. (d) This panel shows the relative protein expression levels of** ***HIF1A*** **and** ***ENO2*** **in Figure 4H (standard by background grayscale and β-actin expression value). Data are presented as mean±SD, $n$=3. $^{**}$ $P$<0.01, $^{***}$ $P$<0.001. NS: no significance;** ***HIF1A*****: hypoxia inducible factor-1α;** ***PGAM1*****: recombinant phosphoglycerate mutase 1;** ***BPGM*****: bisphosphoglycerate mutase;** ***PKM*****: pyruvate kinase;** ***ADPGK*****: adenosine diphosphate (ADP)-dependent glucokinase; SD: standard deviation; DMSO: dimethyl sulfoxide.**

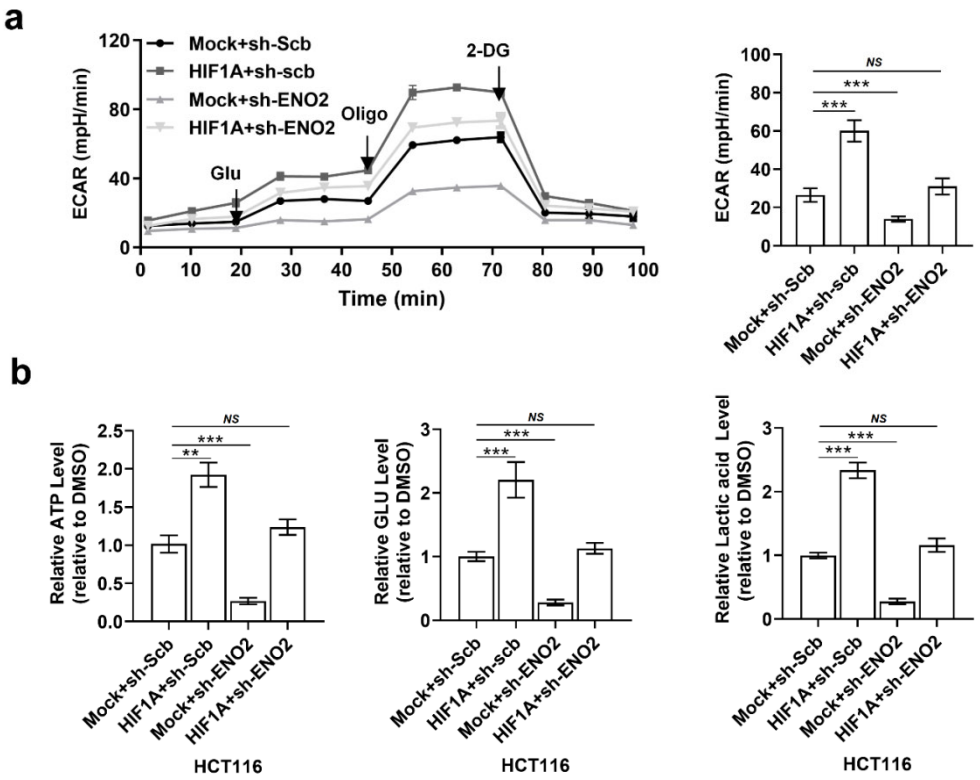


**Fig. S4 Knockdown of *ENO2* inhibits glycolysis of colon cancer cells. (a, b) Over expression *HIF1A* and knocked down *ENO2* in HCT116 cells to detect the ECAR and glycolytic metabolites. Data are presented as mean±SD, *n*=3. $^{**}$ *P*<0.01, $^{***}$ *P*<0.001. NS: no significance; *HIF1A*: hypoxia inducible factor-1α; *ENO2*: enolase 2; ECAR: extracellular acidification rate; ATP: adenosine triphosphate; SD: standard deviation; mpH: 1/1000 pH; Glu: glucose; Oligo: oligomycin; 2-DG: 2-deoxyglucose.**