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ORIGINAL RESEARCH

RETRACTED ARTICLE: EZH2 contributes to 5-FU resistance in gastric cancer by epigenetically suppressing FBXO32 expression

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Background: Increasing evidence suggests the involvement of enhancer of zeste homologue 2 (EZH2) in chemoresistance of cancer treatment. Nevertheless, its function and molecular mechanisms in gastric cancer (GC) chemoresistance are still not well elucidated.

Materials and methods: In the present study, we investigated the functional role of EZH2 in 5-fluorouracil (5-FU) resistance of GC cells and discovered the underlying molecular mechanism.

Results: Results revealed that EZH2 was upregulated in 5-FU-resistant GC tissues and cell lines. High ZEH2 expression was correlated with poor prognosis of GC patients. EZH2 knockdown enhanced 5-FU sensitivity of AGS/5-FU and SGC-7901/5-FU cells. Moreover, EZH2 could epigenetically suppress FBXO32 expression. FBXO32 overexpression could mimic the functional role of downregulated EZH2 in 5-FU resistance. FBXO32 knockdown counteracted the inductive effect of EZH2 inhibition on 5-FU sensitivity of AGS/5-FU and SGC-7901/5-FU cells. Furthermore, EZH2 knockdown facilitated 5-FU sensitivity of 5-FU-resistant GC cells in vivo.

Conclusion: In summary, EZH2 depletion overcame 5-FU resistance in GC by epigenetically silencing FBXO32, providing a novel therapeutic target for GC chemoresistance.

Keywords: gastric cancer, 5-FU, enhancer of zeste homologue 2, FBXO32

Introduction

Gastric cancer (GC) is one of the most prevalent malignant neoplasms of digestive system, the third leading cause of cancer-related death worldwide.[1] Although remarkable progess has been achieved in diagnosis and therapy of GC in the past decade, prognosis for advanced GC patients remains poor.[2] Among many types of drugs applied to GC treatment, 5-fluorouracil (5-FU)-based chemotherapy is the mainstream therapeutic strategy.[3,4] Nevertheless, chemoresistance frequently occurs during chemotherapy, which is a key barrier to the efficacy of GC treatment.[5] Therefore, sequentially elucidating the underlying mechanism and discovering new therapeutic targets are imperative for developing effective therapies for GC patients.

Enhancer of zeste homologue 2 (EZH2), a vital catalytic subunit of PRC2, is a histone methyltransferase that epigenetically represses gene expression by promoting histone H3 lysine 27 trimethylation (H3K27me3).[6,7] EZH2 expression is aberrantly increased in cancers and closely related to the tumor progression, metastasis, and poor prognosis.[8–10] Although the oncogenic roles of EZH2 in cancers are extensively characterized, few studies have investigated the association of EZH2 with acquired drug resistance. Therefore, it is important to test whether inhibition of EZH2 will hold promise in the treatment of chemoresistant cancers.

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In this study, we aimed to investigate the expression pattern and functional role of EZH2 in GC 5-FU resistance as well as its underlying molecular mechanism. Our study found that the expression level of EZH2 was upregulated in GC tissues and cell lines, especially in 5-FU-resistant tissues and cells. Functionally, EZH2 knockdown sensitized 5-FU-resistant GC cells to 5-FU. Mechanically, silencing of EZH2 improved the sensitivity of GC cells to 5-FU through epigenetically suppressing FBXO32 expression. Our study revealed a novel EZH2/FBXO32 regulatory axis which could overcome 5-FU resistance in GC.

Materials and methods

Sample collection and cell culture

A total of 36 tumor tissues and matched adjacent normal tissues were collected from GC patients who underwent surgery at the Huaihe Hospital of Henan University from 2013 to 2017. All patients signed informed consents before this study. This study had acquired the approval from the ethics committee of the Huaihe Hospital of Henan University. The normalized RNA-seq data of stomach adenocarcinoma were downloaded from The Cancer Genome Atlas (TCGA) data portal website (https://cancergenome.nih.gov/).

Human fetal gastric epithelial cell line GES-1, human GC cell lines (AGS and SGC-7901), and their 5-FU-resistant cell lines (AGS/5-FU and SGC-7901/5-FU), and HEK293T cells were obtained from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 medium (Gibco, 2 mL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C with 5% CO_2.

Cell transfection

Empty pcDNA3.1 vector (Vector), pcDNA3.1-FBXO32 (FBXO32), siRNA against EZH2 (si-EZH2) or FBXO32 (si-FBXO32) and their negative control (si-con) were designed and synthesized by Genepharma (Shanghai, People's Republic of China). All cell transfections were performed using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) and then reverse transcribed into cDNA using PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan). EZH2 and FBXO32 expression levels were detected by qRT-PCR with the primes as follows: EZH2 forward, 5′-TTGTTGGCGGAAGCGTGTAAAATC-3′, EZH2 reverse, 5′-TCCCTAGTCCCGCGCAATGAGC-3′; FBXO32 forward, 5′-CCGGCATATGATGGATGCCCTGCAACTAGC-3′, FBXO32 reverse, 5′-GCTGGTCGACCTATGCCACTTAAGGAGAAC-3′.

5-FU sensitivity assay

The cell viability of AGS/5-FU and SGC-7901/5-FU cells and their parental cells was measured by MTT assay. 5-FU sensitivity was determined using the half-maximal inhibitory concentration (IC50) value. The IC50 value was calculated using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA).

Flow cytometric analysis

Cell apoptosis was evaluated using Annexin V–FITC/PI Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, People's Republic of China) as described previously.[11]

Chromatin immunoprecipitation (ChIP) assays

ChIP assay was performed to confirm the interaction between EZH2 and FBXO32 using the Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore, Bellerica, MA, USA). Briefly, AGS/5-FU and SGC-7901/5-FU cells were lysed in a complete ChIP lysis buffer. Then, cell lysates were incubated with protein A/G magnetic beads and antibodies against EZH2 (Active Motif, Carlsbad, CA, USA), H3K27me3 (Millipore) or IgG (Millipore). Finally, qRT-PCR analysis was performed to determine the enrichment of FBXO32 in the immunoprecipitated DNA. The primers used were as follows: forward, 5′-GCAGCTATACGAGGTAGGACTGGGG-3′; reverse, 5′-TTGCAGTGAGCTGAGATCGCGC-3′.

Western blot analysis

Western blotting was performed according to our previously reported protocol.[12] The primary antibodies anti-FBXO32 and anti-GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody anti-EZH2 was purchased from BD Biosciences (San Jose, CA, USA).

Lentivirus production and infection

shRNAs targeting the sequence of EZH2 or scrambled control were cloned into pLKO.1 vector (Addgene, Cambridge, MA, USA) to generate sh-EZH2 or sh-con lentivirus plasmids. sh-EZH2 or sh-con lentivirus plasmid was transfected into HEK293T cells together with ViraPower Lentiviral Packaging Mix (Thermo Fisher Scientific, Waltham, MA, USA). At 96 hours posttransfection, sh-EZH2 or sh-con virus supernatant was harvested to further infect AGS/5-FU

cells. Lastly, sh-EZH2 or sh-con stably-infected AGS/5-FU cells were screened with puromycin (Sigma-Aldrich, St Louis, MO, USA) for at least 1 week.

Xenograft tumor model

The animal experiments were performed according to the national standard of the care and use of laboratory animals and got the approval of the Ethics Committee of Huaihe Hospital of Henan University. Approximately 1.0×10^7 AGS/5-FU cells stably infected with sh-EZH2 or sh-con were subcutaneously inoculated into the posterior flank of BALB/c-nude mice (4 weeks old) from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, People's Republic of China). One week later, mice were intraperitoneally injected with 6 mg/kg 5-FU or same volume of PBS every week according to indicated groups (n=5 each group): sh-con + PBS, sh-EZH2+ PBS, sh-con +5-FU, sh-EZH2+5-FU. The tumor sizes were measured with a digital caliper and volume was calculated using the following equation: volume = (length × width^2/2). After 42 days, the mice were sacrificed, and the tumors were dissected out for weight assessment, qRT-PCR, and Western blot assay.

Statistical analysis

All data were evaluated as means ± SD. Student's *t*-test and one-way analysis of variance were used to calculate the statistical difference using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant when *P*-value <0.05.

Results

EZH2 was upregulated in 5-FU-resistant GC tissues and cell lines

To investigate the role of EZH2 i[illegible] we first examined EZH2 expression in GC tissue[illegible] from TCG[illegible] databases. The results indicated that EZH2 [illegible]pression wa[illegible] significantly increased in GC tumor ti[illegible]sues co[illegible]red wit[illegible]normal tissues (Figure 1A). To co[illegible]firm the[illegible]result[illegible] further explored EZH2 expression i[illegible]6 pa[illegible]d GC tumor or adjacent normal tissues by qR[illegible]-PCR an[illegible]ysis. Co[illegible]sistently, EZH2 was significantly [illegible]pr[illegible]ulated in G[illegible]ssues compared with adjacent normal tissues ([illegible]gure 1B). Additionally, compared with 5-[illegible]nsitive GC [illegible]sues, EZH2 expression was remarkably elevated in 5-FU-resistant GC tissues (Figure 1C). To further e[illegible]lore the expression of EZH2 in GC cells,

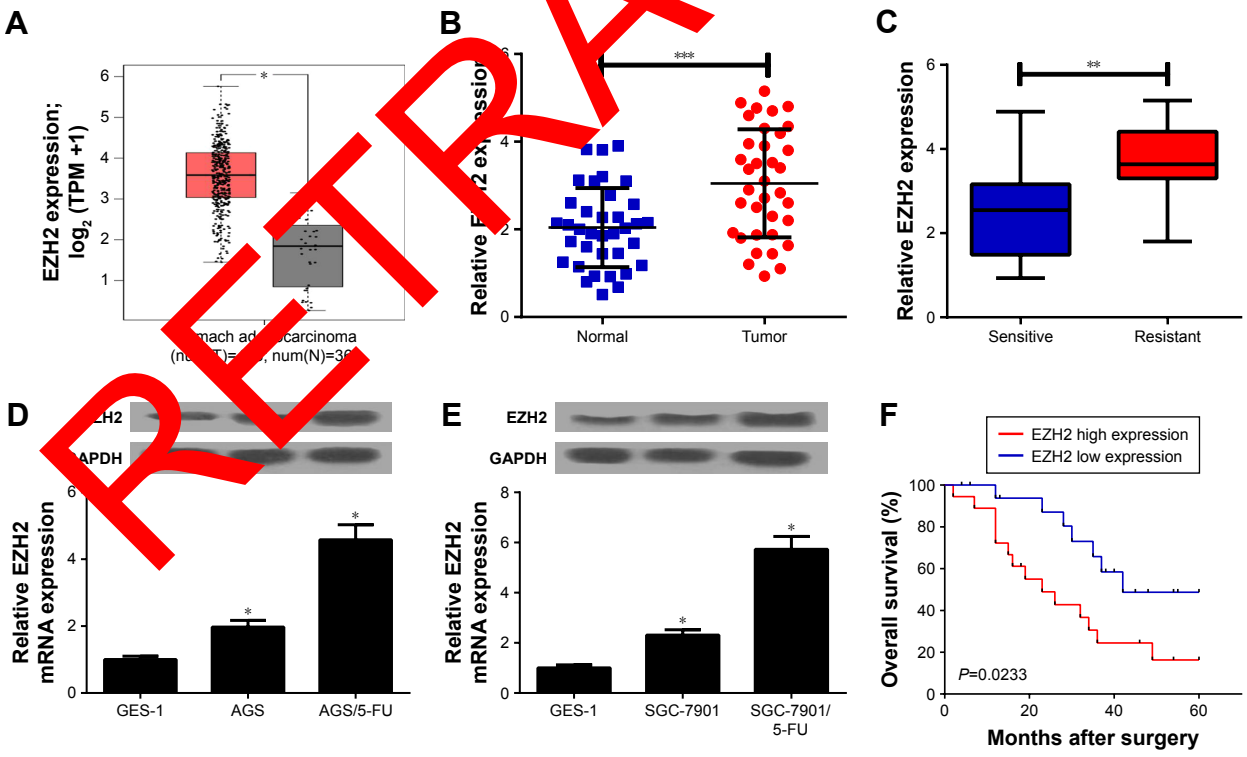

Figure 1 EZH2 was upregulated in 5-FU-resistant GC tissues and cell lines.

Notes: In (**A**) EZH2 expression in GC tumor (red) or normal (gray) tissues from TCGA dataset was analyzed. qRT-PCR analysis indicated the EZH2 expression levels in 36 paired GC tumor or adjacent normal tissues (**B**), 5-FU-sensitive or 5-FU-resistant GC tissues (**C**). qRT-PCR and Western blot analyses detected EZH2 mRNA and protein expression levels in 5-FU-resistant GC cell lines (AGS/5-FU and SGC-7901/5-FU) and their parental cells (AGS and SGC-7901) or human fetal gastric epithelial cell line GES-1 (**D**, **E**). (**F**) The overall survival was evaluated by Kaplan–Meier curve between low and high EZH2 expression groups. **P*<0.05, ***P*<0.01, and ****P*<0.001.

Abbreviations: 5-FU, 5-fluorouracil; EZH2, enhancer of zeste homologue 2; GC, gastric cancer; qRT-PCR, quantitative real-time PCR; TCGA, The Cancer Genome Atlas.

qRT-PCR and Western blot analyses were performed in GC parental cell lines (AGS and SGC-7901), 5-FU-resistant cell lines (AGS/5-FU and SGC-7901/5-FU), and normal fetal gastric epithelial cell line GES-1, respectively. The results displayed that EZH2 mRNA and protein expression levels were dramatically improved in AGS and SGC-7901 cells compared with GES-1 cells (Figure 1D and E). Notably, AGS/5-FU and SGC-7901/5-FU cells exhibited higher EZH2 mRNA and protein levels than their parental cells (Figure 1D and E). Kaplan–Meier survival analysis revealed that patients with high EZH2 level had a low overall survival (P=0.0233) compared with that with low EZH2 level (Figure 1F). Taken together, these data demonstrated that upregulation of EZH2 may be implicated with 5-FU resistance in GC.

EZH2 knockdown overcame 5-FU resistance of GC cells

To estimate the resistance of AGS/5-FU and SGC-7901/5-FU cells to 5-FU, IC50 of 5-FU was determined by MTT assay in AGS/5-FU and SGC-7901/5-FU cells and their parental cells. Compared with parental cells, AGS/5-FU and SGC-7901/5-FU cells showed poor response to 5-FU, as evidenced by the increased IC50 (Figure 2A and B). To further investigate the role of EZH2 in 5-FU-resistant GC cells, EZH2 siRNAs (si-EZH2 #1, si-EZH2 #2, or si-EZH2 #3) or si-con were transfected into AGS/5-FU and SGC-7901/5-FU cells. qRT-PCR and Western blot analyses revealed that EZH2 siRNAs demonstrably declined EZH2 mRNA and protein expression levels in AGS/5-FU and SGC-7901/5-FU cells (Figure 2C and D), especially in si-EZH2 #1 treated group. Remarkably, EZH2 knockdown enhanced the sensitivity of AGS/5-FU and SGC-7901/5-FU cells to 5-FU, proved by EZH2 siRNAs-mediated decrease of IC50 (Figure 2E and F; Figure S1A and B). To further determine the effect of EZH2 on 5-FU-induced apoptosis, flow cytometry analysis was performed in AGS/5-FU and SGC-7901/5-FU cells exposed to 30 μg/mL 5-FU. As expected, EZH2 depletion remarkably enhanced 5-FU-induced apoptosis in AGS/5-FU and SGC-7901/5-FU cells (Figure 2G and H; Figure S1C and D). Together, silencing of EZH2 improved 5-FU sensitivity in GC cells.

EZH2 epigenetically suppressed FBXO32 expression in GC cells

Previous studies demonstrated that EZH2 could target FBXO32.[13,14] Hence, we further investigated whether EZH2 epigenetically suppressed FBXO32 in 5-FU-resistant GC cells. First, Chipbase database (http://rna.sysu.edu.cn/chipbase/) was used to analyze the correlation between EZH2 and FBXO32 in 450 GC tissue samples from TCGA databases. The results exhibited that EZH2 expression was negatively associated with FBXO32 level in GC tissue samples (Figure 3A). Western blot analysis indicated that EZH2 knockdown significantly elevated FBXO32 expression in AGS/5-FU and SGC-7901/5-FU cells (Figure 3B; Figure S2A and B). To confirm that FBXO32 is transcriptionally repressed by EZH2 in our GC cells, we performed ChIP assays to evaluate the enrichment of EZH2 and the H3K27me3 on the FBXO32 promoter. The results revealed that EZH2 knockdown dramatically decreased EZH2 and H3K27me3 recruitment in the FBXO32 promoter in AGS/5-FU and SGC-7901/5-FU cells (Figure 3C and D). All these data suggested that EZH2 epigenetically suppressed FBXO32 expression in GC cells.

FBXO32 overexpression enhanced 5-FU sensitivity of GC cells

To further investigate the role of FBXO32 in 5-FU-resistant GC cells, we first detected FBXO32 expression in 5-FU-resistant AGS/5-FU and SGC-7901/5-FU cells and their parental cells. As expected, FBXO32 mRNA and protein expression levels were acutely downregulated in AGS/5-FU and SGC-7901/5-FU cells (Figure 4A and B). Then, FBXO32 overexpressing vector (FBXO32) or empty vector (Vector) was transfected into AGS/5-FU and SGC-7901/5-FU cells. qRT-PCR and Western blot analyses revealed that FBXO32 mRNA and protein levels were remarkably increased in FBXO32 transfecting AGS/5-FU and SGC-7901/5-FU cells (Figure 4C and D). Moreover, FBXO32 overexpression improved the sensitivity of AGS/5-FU and SGC-7901/5-FU cells to 5-FU, proved by FBXO32-mediated decrease of IC50 (Figure 4E and F). To further confirm that FBXO32 could overcome 5-FU resistance in GC cells, the parent cells AGS and SGC-7901 were transfected with si-con or si-FBXO32. qRT-PCR and Western blot analyses demonstrated that FBXO32 was successfully knocked down by si-FBXO32 (Figure S3A and B). Moreover, FBXO32 knockdown rendered AGS and SGC-7901 cells resistant to 5-FU (Figure S3C and D). To further determine the effect of FBXO32 on 5-FU-induced apoptosis, flow cytometry analysis was performed in AGS/5-FU and SGC-7901/5-FU cells exposed to 30 μg/mL 5-FU. As expected, FBXO32 overexpression remarkably enhanced

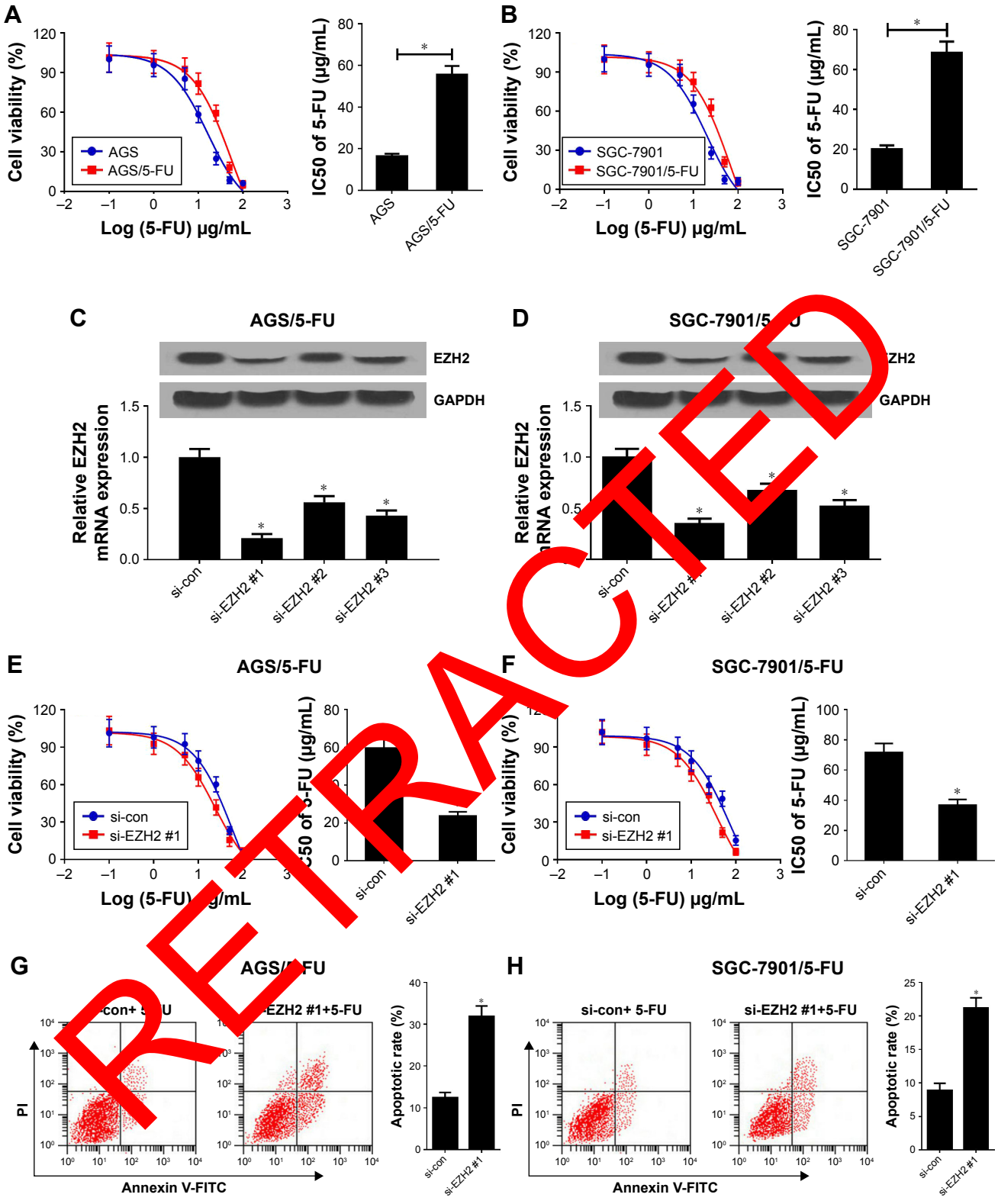

Figure 2 Knockdown of EZH2 overcame 5-FU resistance of GC cells.

Notes: (**A**, **B**) The cell viability was determined by MTT assay in AGS/5-FU and SGC-7901/5-FU cells and their parental cells exposed to different concentrations of 5-FU (0.1, 1, 5, 10, 25, 50, 100 μg/mL) for 48 hours. (**C**, **D**) qRT-PCR and Western blot analyses were performed in AGS/5-FU and SGC-7901/5-FU cells transfected with EZH2 siRNAs (si-EZH2 #1, si-EZH2 #2 or si-EZH2 #3) or si-con. (**E**, **F**) AGS/5-FU and SGC-7901/5-FU cells transfected with si-EZH2 #1 or si-con were treated with various concentrations of 5-FU (0.1, 1, 5, 10, 25, 50, 100 μg/mL) for 48 hours and cell viability was evaluated by MTT assay. (**G**, **H**) Cell apoptosis was determined by flow cytometry analysis in si-EZH2 #1 or si-con transfected AGS/5-FU and SGC-7901/5-FU cells after treatment with 30 μg/mL of 5-FU. *$P<0.05$.

Abbreviations: 5-FU, 5-fluorouracil; EZH2, enhancer of zeste homologue 2; GC, gastric cancer; qRT-PCR, quantitative real-time PCR; si-con, negative control.

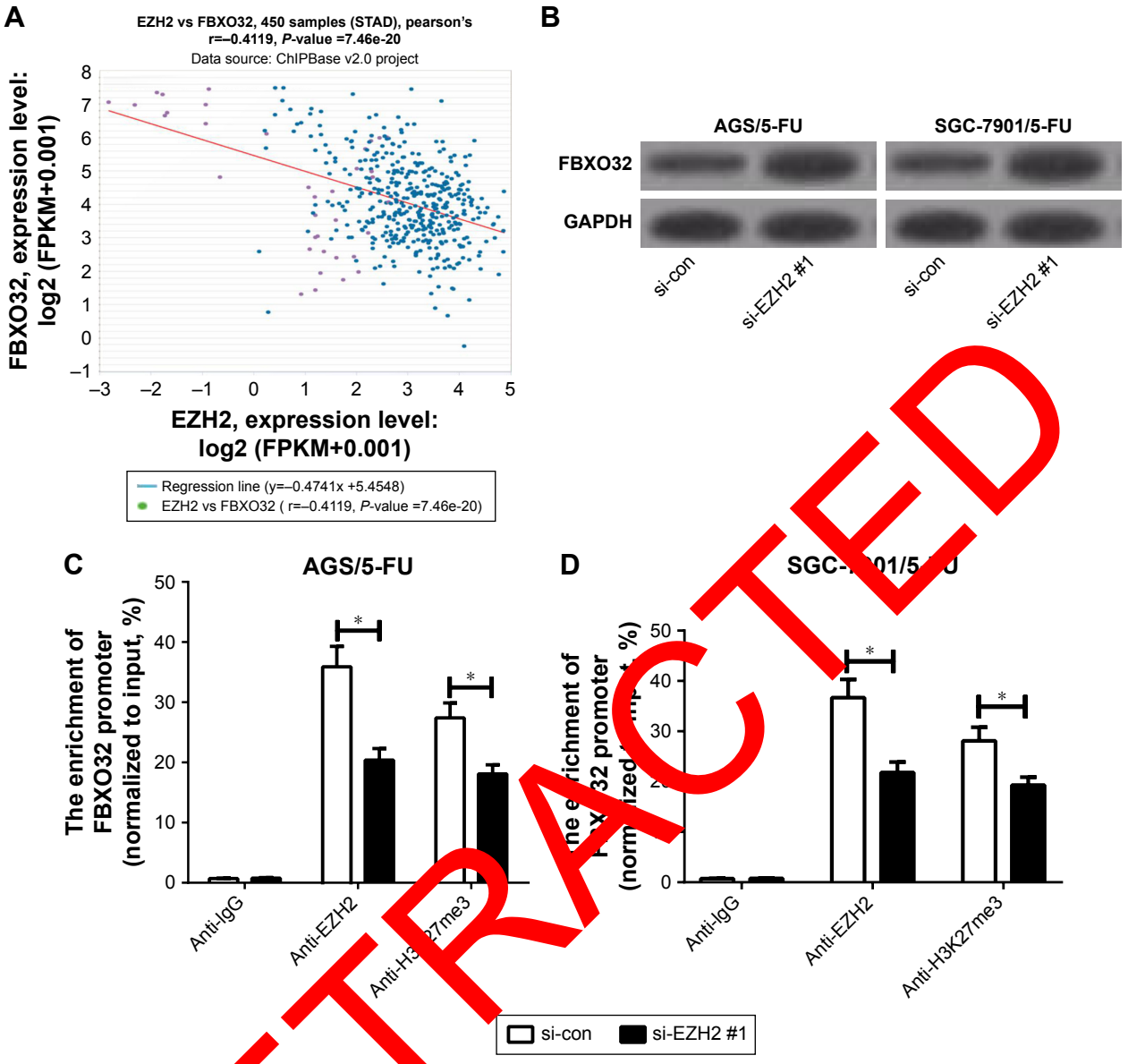

Figure 3 EZH2 directly inhibited FBXO32 expression in GC cells.

Notes: (**A**) Pearson correlation coefficient analysis between EZH2 and FBXO32 in 450 tumor tissue samples of GC. (**B**) FBXO32 protein levels in AGS/5-FU and SGC-7901/5-FU cells transfected with si-con, si-EZH2 #1. (**C**, **D**) ChIP followed by qRT-PCR analysis was performed to evaluate the enrichment of the FBXO32 promoter on EZH2 and H3K27me3 in AGS/5-FU and SGC-7901/5-FU cells. *$P<0.05$.

Abbreviations: 5-FU, 5-fluorouracil; ChIP, chromatin immunoprecipitation; EZH2, enhancer of zeste homologue 2; GC, gastric cancer; qRT-PCR, quantitative real-time PCR; si-con, negative control.

5-FU-induced apoptosis in AGS/5-FU and SGC-7901/5-FU cells (Figure 4G and H). Taken together, elevated EZH2 improved 5-FU sensitivity in GC cells.

EZH2 knockdown facilitated 5-FU sensitivity of GC cells through inhibiting FBXO32 expression

To further investigate whether EZH2 exerted its functional role in 5-FU resistance of GC cells through regulating FBXO32 expression, AGS/5-FU and SGC-7901/5-FU cells were transfected with si-con, si-EZH2 #1, or si-EZH2 #1+ si-FBXO32. qRT-PCR and Western blot analyses exhibited that transfection of si-EZH2 #1 increased FBXO32 expression in AGS/5-FU and SGC-7901/5-FU cells, which was particularly reversed by FBXO32 knockdown (Figure 5A and B). MTT assay revealed that downregulation of EZH2 enhanced 5-FU sensitivity of AGS/5-FU and SGC-7901/5-FU cells; nevertheless, the inductive effect of EZH2 inhibition on 5-FU sensitivity of AGS/5-FU and SGC-7901 /5-FU cells was strikingly eliminated by FBXO32 silencing

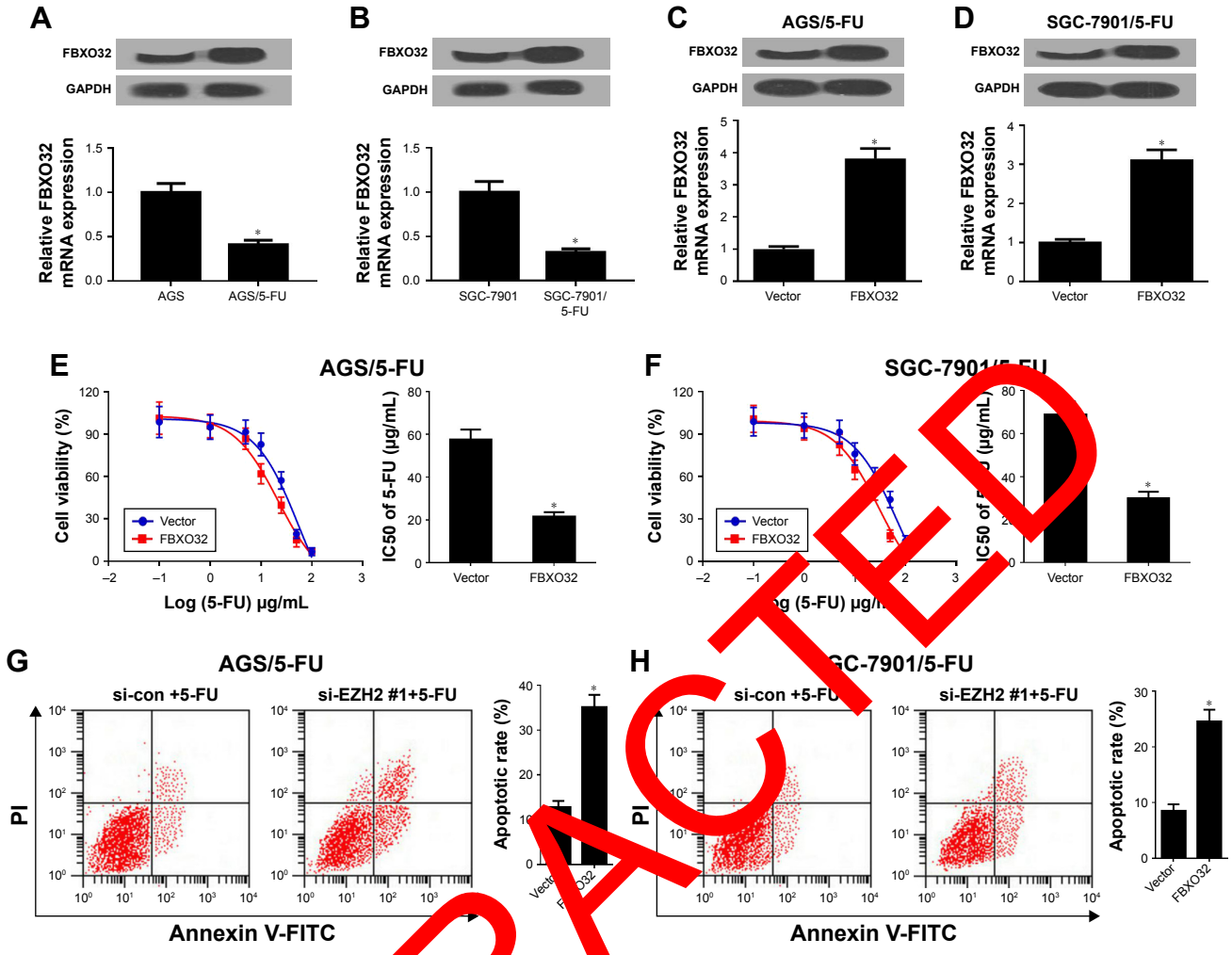

Figure 4 Overexpression of FBXO32 improved 5-FU resistance.
Notes: (**A**, **B**) FBXO32 mRNA and protein levels were detected in 5-FU resistant AGS/5-FU and SGC-7901/5-FU cells and their parent cells. AGS/5-FU and SGC-7901/5-FU cells were transfected with Vector or FBXO32, followed by determination of FBXO32 expression by qRT-PCR and Western blot analyses (**C**, **D**), IC50 of 5-FU by MTT assay (**E**, **F**), and cell apoptotic rate by flow cytometry analysis (**G**, **H**). $^{*}P<0.05$.
Abbreviations: 5-FU, 5-fluorouracil; GC, gastric cancer; IC50, half maximal inhibitory concentration; qRT-PCR, quantitative real-time PCR; si-con, negative control.

(Figure 5C and D). Furthermore, introduction of si-FBXO32 significantly destroyed the inductive effect of downregulated EZH2 on apoptosis in AGS/5-FU and SGC-7901/5-FU cells (Figure 5E and F). Generally, these results demonstrated that EZH2 knockdown facilitated 5-FU resistance of GC cells through suppressing FBXO32 expression.

EZH2 knockdown enhanced 5-FU sensitivity in tumors in vivo

To further confirm the functional role of EZH2 in 5-FU resistance in vivo, AGS/5-FU cells infected with sh-con or sh-EZH2 were subcutaneously injected into the nude mice to generate a xenograft model, followed by treatment with 5-FU or PBS. The data revealed that EZH2 knockdown or 5-FU treatment significantly suppressed tumor growth, evidenced by the reduction in tumor volume (Figure 6A) and tumor weight (Figure 6B). Moreover, simultaneous EZH2 knockdown and 5-FU treatment resulted in a more distinct inhibition on tumor growth, suggesting downregulation of EZH2 facilitated the sensitivity of GC cells to 5-FU in vivo (Figure 6A and B). Additionally, qRT-PCR assay revealed that EZH2 mRNA levels were lowered, while FBXO32 expression was elevated in tumors after sh-EZH2 introduction or 5-FU treatment (Figure 6C), especially after combination of sh-EZH2 introduction and 5-FU treatment. Western blot assay revealed that EZH2 knockdown or 5-FU exposure greatly lowered EZH2 but improved FBXO32 protein levels in tumor tissues (Figure 6D). The combination of EZH2 knockdown and 5-FU exposure led to much reduced EZH2 and elevated FBXO32 protein expression (Figure 6D). All these data demonstrated that EZH2 depletion enhanced 5-FU sensitivity of GC cells in vivo.

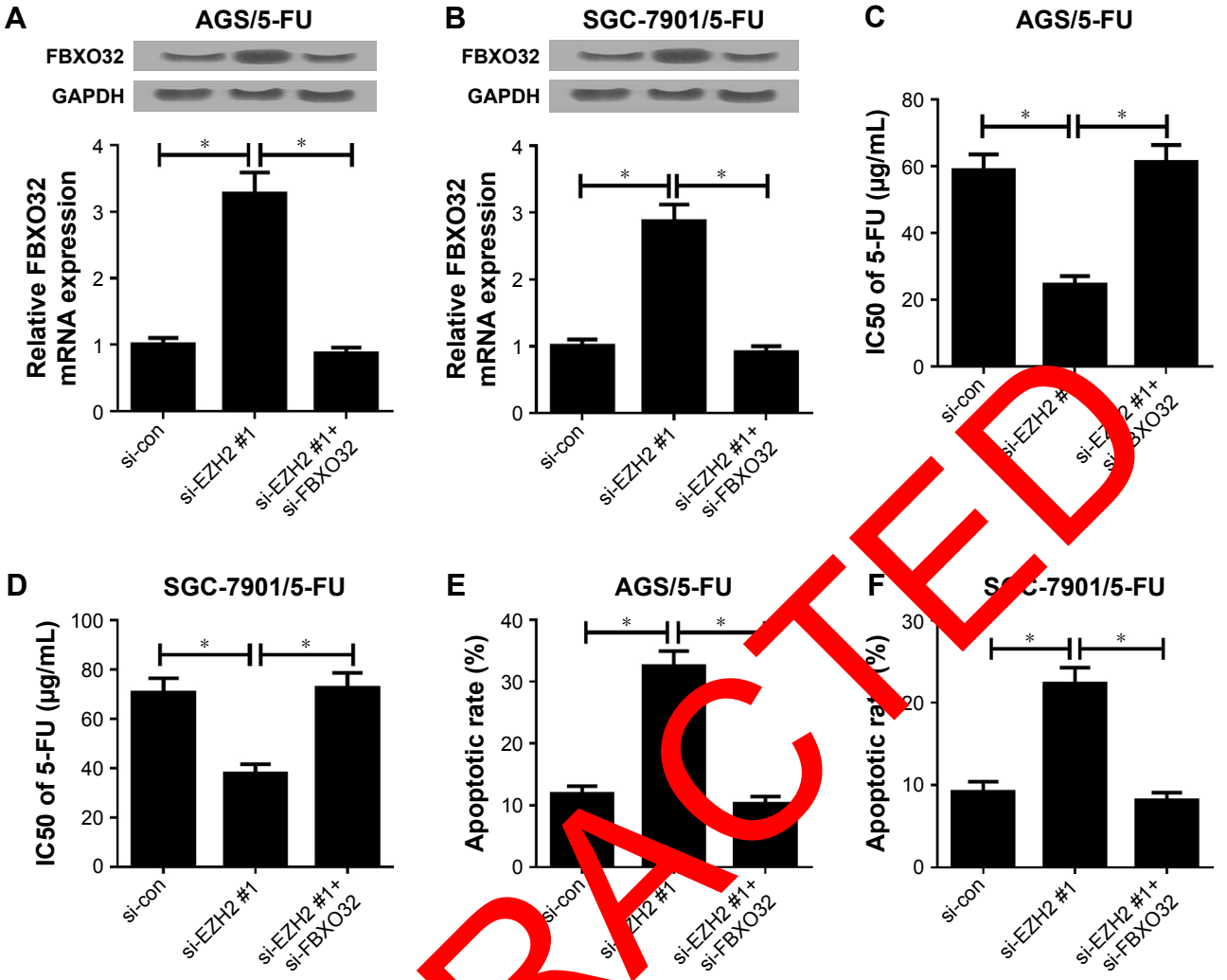

Figure 5 FBXO32 knockdown reversed the enhancing effect of upregulated EZH2 on 5-FU sensitivity of GC cells.
Notes: AGS/5-FU and SGC-7901/5-FU cells were transfected with si-con, si-EZH2 #1, or si-EZH2 #1+ FBXO32, followed by determination of FBXO32 expression by qRT-PCR and Western blot analyses (**A**, **B**), IC50 of 5-FU by MTT assay (**C**, **D**), and cell apoptotic rate by flow cytometry analysis (**E**, **F**). *$P<0.05$.
Abbreviations: 5-FU, 5-fluorouracil; EZH2, enhancer of zeste homolog 2; GC, gastric cancer; IC50, half-maximal inhibitory concentration; qRT-PCR, quantitative real-time PCR; si-con, negative control.

Discussion

Acquiring 5-FU resistance leads to limited therapeutic effectiveness for GC patients in the clinic at present. Hence, it is essential to investigate the molecular mechanism underlying 5-FU resistance and identify novel targets for 5-FU resistance prevention and therapy. In this study, we found that EZH2 expression was significantly increased in 5-FU-resistant GC tissues and cells. Moreover, EZH2 knockdown sensitized AGS/5-FU and SGC-7901/5-FU cells to 5-FU by promoting cell apoptosis. More importantly, downregulation of EZH2 facilitated the sensitivity of GC cells to 5-FU via epigenetically suppressing FBXO32 expression. Therefore, EZH2 was confirmed to play a critical role in 5-FU resistance, providing a promising therapeutic target for 5-FU resistance in GC.

Elucidating the molecular mechanism underlying 5-FU resistance could contribute to developing reasonable and effective therapies to overcome 5-FU resistance. Our results demonstrated that EZH2 was upregulated in 5-FU-resistant GC tissues and cells, and EZH2 inhibition sensitized AGS/5-FU and SGC-7901/5-FU cells to 5-FU. Apart from our findings, several other studies have reported that abnormal EZH2 expression was implicated with 5-FU resistance in cancers. For example, EZH2 expression was elevated in hepatocellular carcinoma, and EZH2 depletion sensitized multidrug-resistant Bel/5-FU cells to 5-FU by suppressing MDR1 expression.[15,16] Moreover, EZH2-mediated DPYD inhibition through regulating H3K27me3 at the DPYD promoter contributed to 5-FU resistance.[17] Prominently, miR-124-mediated inhibition

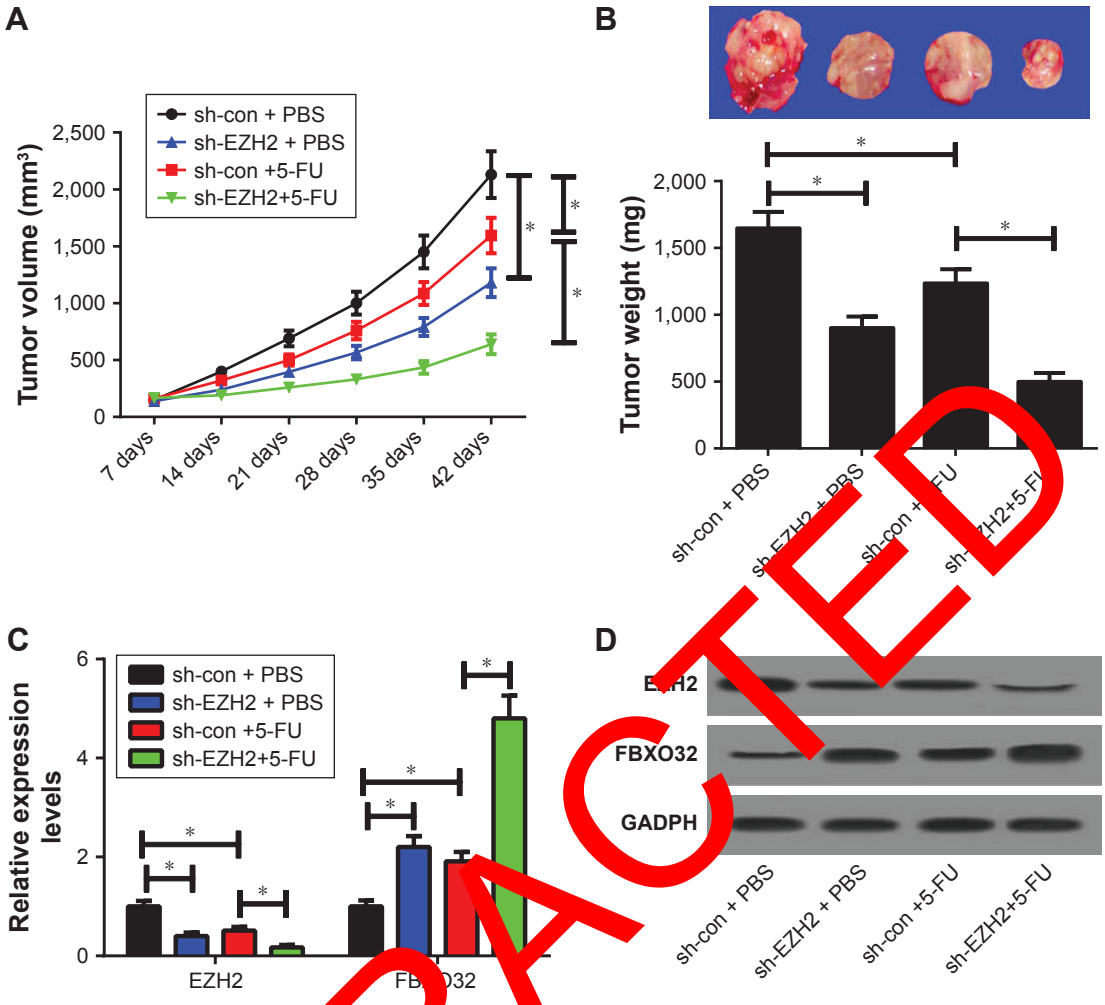

Figure 6 EZH2 knockdown sensitized GC cells to 5-FU in vivo.
Notes: AGS/5-FU cells stably transfected with sh-con or sh-EZH2 were subcutaneously inoculated into the nude mice, followed by treatment with PBS or 5-FU. Mice were euthanized to remove tumor masses at 42 days after inoculation. (**A**) The tumor volumes were measured with a caliper at indicated time points. (**B**) The representative photographs and average weights of resected tumors. (**C**) qRT-PCR analysis of EZH2 and FBXO32 mRNA levels in excised tumor tissues. (**D**) Western blot assay of EZH2 and FBXO32 protein levels in excised tumor tissues. *$P<0.05$.
Abbreviations: 5-FU, 5-fluorouracil; EZH2, enhancer of zeste homologue 2; GC, gastric cancer; qRT-PCR, quantitative real-time PCR.

of EZH2 expression suppressed proliferation, induced apoptosis, and resensitized the treatment outcome of 5-FU in GC.[18] All these findings suggested that EZH2 inhibition could be used as a promising therapeutic strategy for 5-FU resistance cancers.

The precise mechanism by which upregulated EZH2 expression confers 5-FU resistance is uncertain. Hence, the functional mechanism of EZH2 was further investigated in the present study. Previous studies demonstrated that EZH2 could target FBXO32.[13,14] However, the interaction between EZH2 and FBXO32 in GC was still unclear. In the present study, ChIP assay verified that EZH2 directly inhibited FBXO32 expression in AGS/5-FU and SGC-7901/5-FU cells. FBXO32 (also known as atrogin-1) is a member of the F-box protein family and constitutes one of the four subunits of the ubiquitin protein ligase complex.[19,20] Mounting evidence has revealed the emerging role of FBXO32 in tumorigenesis.[13,21,22] Moreover, FBXO32 was reported to be downregulated in ovarian cancer and GC, suggesting that FBXO32 may function as a tumor suppressor.[23,24] Prominently, overexpression of FBXO32 induced apoptosis and enhanced chemosensitivity to cisplatin in ovarian cancer.[23] Similarly, our data revealed that FBXO32 overexpression could overcome 5-FU resistance in AGS/5-FU and SGC-7901/5-FU cells. Furthermore, FBXO32 inhibition reversed the inductive effect of EZH2 knockdown on the sensitivity of AGS/5-FU and SGC-7901/5-FU to 5-FU. All these data demonstrated that EZH2 inhibition sensitized 5-FU-resistant GC cells to 5-FU through epigenetically silencing FBXO32 in GC.

In conclusion, our study demonstrated that knockdown of EZH2 enhanced 5-FU sensitivity of GC cells. Importantly, the enhacing effect of downregulated EZH2 on 5-FU sensitivity might be mediated by FBXO32 in GC cells, providing a promising therapeutic strategy to overcome 5-FU resistance in GC.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

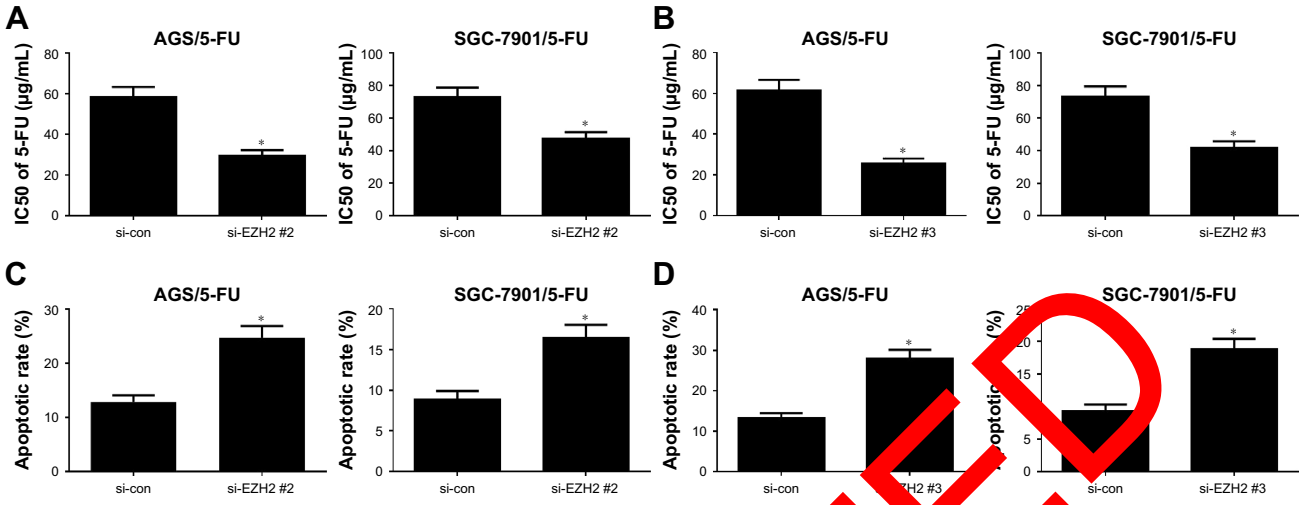

Figure S1 (**A**, **B**) AGS/5-FU and SGC-7901/5-FU cells transfected with si-EZH2 #2, si-EZH2 #3 or si-con were treated with various concentrations of 5-FU (0.1, 1, 5, 10, 25, 50, 100 μg/mL) for 48 h and cell viability was evaluated by MTT assay. (**C**, **D**) Cell apoptosis was determined by flow cytometer analysis in si-EZH2 #2, si-EZH2 #3 or si-con transfected AGS/5-FU and SGC-7901/5-FU cells after treatment with 30 μg/mL of 5-FU.

Note: *$P<0.05$.

Abbreviations: 5-FU, 5-fluorouracil; EZH2, enhancer of zeste homologue 2; si-con, negative control.

Figure S2 (**A**, **B**) FBXO32 protein levels were detected by Western blot analysis in AGS/5-FU and SGC-7901/5-FU cells transfected with si-con, si-EZH2 #2, or si-EZH2 #3.

Abbreviations: 5-FU, 5-fluorouracil; EZH2, enhancer of zeste homologue 2; si-con, negative control.

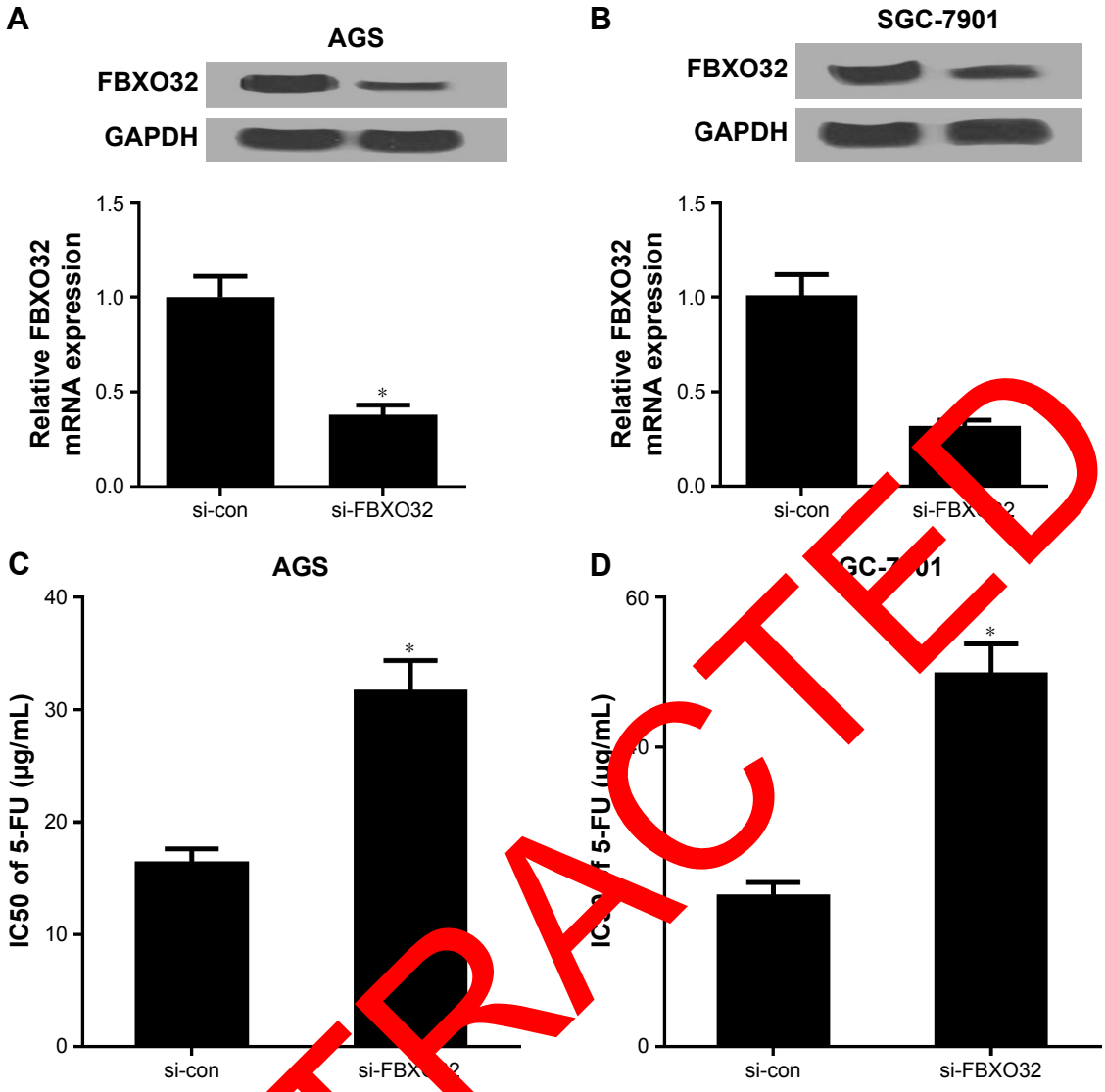

Figure S3 AGS and SGC-7901 cells were tr[illegible]fected [illegible] si-con or si-FB[illegible]O32, followed by determination of FBXO32 expression by qRT-PCR and Western blot analyses (**A**, **B**), IC50 of 5-FU by MTT assay (**C**, **D**).
Note: *$P<0.05$.
Abbreviations: 5-FU, 5-fluorour[illegible], IC50, half maximal inhibitory concentration; EZH2, enhancer of zeste homologue 2; si-con, negative control.