

ZBTB20 promotes ferroptosis through inhibiting TMEM109 expression in glioblastoma cells

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Abstract. Ferroptosis is an iron-dependent type of regulated cell death which is dysregulated in several tumors, including glioblastoma (GBM). Zinc finger and BTB domain-containing protein 20 (*ZBTB20*), a transcription repressor, is expressed at low levels in GBM and suppresses GBM cell proliferation through the ERK signaling pathway. However, the effect of *ZBTB20* on ferroptosis has not been explored. The present study aimed to explore the role of *ZBTB20* in ferroptosis of glioma cells and its underlying mechanism. The present study demonstrated that both *ZBTB20* expression and ferroptosis levels in GBM cells were lower than that in normal glial cells. Gain- and loss-of-function experiments revealed that *ZBTB20* overexpression promoted ferroptosis and *ZBTB20* knockdown inhibited ferroptosis in GBM cells. Moreover, the results demonstrated that *ZBTB20* transcriptionally repressed the expression of transmembrane protein 109 (*TMEM109*) in GBM cells, assessed using dual-luciferase reporter and chromatin immunoprecipitation assays. *TMEM109* is mainly localized on the endoplasmic reticulum (ER) membrane of cells and regulates calcium leakage at the ER or sarcoplasmic reticulum. The present study revealed that *TMEM109* overexpression inhibited ferroptosis and *TMEM109* knockdown promoted ferroptosis in GBM cells. Using co-transfection experiments, it was further revealed that the promotive effect of *ZBTB20* can reverse the inhibitory effect of *TMEM109* on ferroptosis. In conclusion, the findings indicated that *ZBTB20* promotes ferroptosis in GBM cells through transcriptionally repressing the expression of *TMEM109*.

Introduction

Ferroptosis differs from apoptosis, autophagy and necrotic apoptosis (1). It is a type of programmed cell death consisting of lipid peroxidation induced by iron-dependent ions and reactive oxygen species (ROS) (2,3). Ferroptosis can be triggered by blocking the Xc-system [glutamate cystine reverse transporter, a dimer consisting of solute carrier (SLC)7A11 and SLC3A2, which mainly relies on SLC7A11 for its function] or by inhibiting glutathione (GSH)-dependent antioxidant reductase 4 (GPX4) (4,5). It has been reported that ferroptosis serves a key role in neurodegenerative diseases, ischemia-reperfusion injury, acute kidney injury and several types of cancer (6). Moreover, it has been reported that accumulation of Fe²⁺ notably contributes to the development of several cancers such as lung, liver and breast cancers (7,8). Cancer chemotherapeutic resistance is currently a major challenge in cancer treatment, and ferroptosis serves a pivotal role in cancer drug resistance, which in recent years has provided new opportunities for chemotherapy of insensitive cancers (9). Therefore, modulation of key ferroptosis genes to induce ferroptosis has emerged as a therapeutic strategy to combat cancer progression and chemotherapy resistance (10,11).

Glioma (neuroglioma) is currently the most common intracranial primary cancer in adults worldwide (12,13). Glioblastoma (GBM) is a highly aggressive cerebral malignant cancer characterized by massive neovasculogenesis, necrosis and a strong resistance to treatment (14). However, ferroptosis is understudied in gliomas. It has been reported that after analyzing five major types of programmed cell death in 1,750 patients with glioma from four independent databases, ferroptosis was identified as the most common type of programmed cell death (encompassing apoptosis, autophagy, ferroptosis, necrotic apoptosis and pyroptosis) in gliomas, and ferroptosis was associated with malignant cancer progression, a poor outcome and immunosuppression (15). Moreover, it has been reported that chemotherapeutics drugs (16) and cryptotanshinone (17) can have an effect on glioma cell ferroptosis (18). In addition, previous studies demonstrated that polypyrimidine tract binding protein 1 and NADPH regulate glioma

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ferroptosis by modulating oxidative stress pathways (19,20). Several recent studies have also reported that nanoparticle drugs (21) can target GBM across the blood-brain barrier and markedly optimize antitumor efficacy by activating ferroptosis (22,23). Furthermore, certain researchers have used nanosensitizers (24) to disrupt drug resistance mechanisms in GBM, providing new therapeutic avenues for glioma treatment (25). A study reported that circLRFN5 promotes ferroptosis by inhibiting the paired related homeobox 2/GTP cyclohydrolase 1 pathway, which in turn inhibits GBM progression (26). Nevertheless, although the role of ferroptosis in the pathogenesis of glioma and in targeted therapies has been studied in recent years, research has made limited progress. Moreover, exploring the role and mechanism of ferroptosis in the development of gliomas can help to improve glioma treatment.

The zinc finger family of proteins is one of the most important families regulating gene transcription in eukaryotes (27). Zinc finger and BTB domain-containing protein 20 (*ZBTB20*) is a member of this family whose main function is transcriptional repression (28). It may also serve an important role in hematopoiesis, oncogenesis and development, immune response and neurodevelopment (29). Previous studies have reported that mutations in the *ZBTB20* gene can lead to primrose syndrome (30,31). Furthermore, it has been reported that *ZBTB20* gene deletion can lead to anterior pituitary hypoplasia, developmental dwarfism and complete loss of mature prolactin (32). In addition, *ZBTB20* serves a crucial role in the development of several cancers (33). It has also been reported that *ZBTB20* can promote the growth of human hepatocellular carcinoma through the inhibition of forkhead box O1 (34,35), and *ZBTB20* promotes cell migration and invasion in gastric cancer through the inhibition of IKB α -induced NF- κ B activation (30). In neural precursor cells, the overexpression of *ZBTB20* has been reported to promote cell differentiation to astrocytes, and knockdown of *ZBTB20* inhibits cell differentiation to astrocytes (36). However, no association between *ZBTB20* and ferroptosis in glioma cells has been reported, to the best of our knowledge. However, it has been demonstrated that, in glioma cells, *ZBTB20* regulates the expression of oxidative stress-related proteins, which are important genes for ROS regulation (37). As ROS has been reported to be an important causative agent and signature feature in the process of ferroptosis (38,39), it was hypothesized that *ZBTB20* may be associated with the process of ferroptosis in glioma cells.

Transmembrane protein 109 (*TMEM109*), also known as Mitsugumin23, is a transmembrane protein found in the sarcoplasmic reticulum, endoplasmic reticulum (ER) and nuclear membrane of cells (40–43). It is involved in regulating the activity of voltage-gated ion channels and it has been reported that knockdown of *TMEM109* increases ultraviolet C-induced cellular DNA damage, leading to cell death (44). Previous research has also reported that *TMEM109* can promote Ca²⁺ release in skeletal muscle to cause muscle contraction (41). There are few studies on *TMEM109*, with one reporting that the ferroptosis pathway was altered in human cervical cancer cells with *TMEM109* knockdown (40). Therefore, it was hypothesized that *TMEM109* may be associated with ferroptosis.

To date, the association between *ZBTB20* and *TMEM109* is unclear. Therefore, the present study aimed to assess the role of *ZBTB20* and *TMEM109* in GBM cells, and the association between *ZBTB20*, ferroptosis and *TMEM109* in GBM.

Materials and methods

Cell cultures and reagents. The U251 glioma cells (cat no. TCHu58) and U87MG cells [cat. no. TCHu138], the cell line as a GBM of unknown origin) were purchased from the National Collection of Authenticated Cell Cultures. The cell line was authenticated by short tandem repeat analysis and subjected to mycoplasma detection at the National Collection of Authenticated Cell Cultures. The normal human glial cells HA1800 (cat no. 1800; ScienCell; <https://sciencellonline.com/en/human-astrocytes/>) and glioma cells were cultured in high-sugar DMEM medium (Biological Industries; Sartorius AG) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin (Corning, Inc.) and 1% streptomycin (Corning, Inc.) at 37°C in a 5% CO₂ incubator (Thermo Fisher Scientific, Inc.).

***ZBTB20* overexpression and knockdown transfection.** The lentiviral supernatants used for *ZBTB20* overexpression and knockdown were purchased from Shanghai Jikai Genetics Corporation. The biotech company has not disclosed the sequence of the negative control (NC) short hairpin RNA (shRNA). The target sequences of *ZBTB20* knockdown were as follows: shZBTB20-1, 5'-GCATGTGTCTGACGGATA AGT-3'; shZBTB20-2, 5'-GCACTGGACTTCAGGATA AGT-3'; and shZBTB20-3, 5'-GCCAAACACTTCTAGAGA AAT-3'. The control group (Vector group) of the overexpression group (*ZBTB20* group) was transfected with cells containing empty pReceiver-Lv105 lentivirus, and the control group (shZBTB20 group) of the knockdown group (shZBTB20 group) was transfected with cells containing psi-LVRU6GP with a scrambled sequence (5'-GCTTCGCGCCGTAGTCTTA-3'; GeneCopoeia, Inc.). When the cell confluence reached 50%, the viral transfection operation (MOI=10) was carried out. A total of 48 h after transfection, the fluorescence intensity was observed under microscope to evaluate the transfection effect. After successful transfection, the cells can be passaged and cultured for one week before proceeding with subsequent experimental operations.

***TMEM109* vectors and lentiviral transfection.** The lentiviral supernatants used for *TMEM109* overexpression and knockdown were purchased from Shanghai Jikai Genetics Corporation. The biotech company has not disclosed the sequence of the NC shRNA. The target sequences of *TMEM109* knockdown were as follows: shTMEM109-1, 5'-GCATGTGTTCAAAGCCATTCT-3'; shTMEM109-2, 5'-GCCATCTCATCAGCCATTCT-3'; and shTMEM109-3, 5'-ACCCAGATAGGTCGATCTGTG-3'. Uniformly planted glioma cells with favorable growth status were cultured in 6-well plates (Wuxi NEST Biotechnology Co., Ltd.), with ~2x10⁵ cells per well. Once the cells adhered to the plate and reached ~60% confluence, the old medium was discarded. Newly prepared lentiviral mixture was then added to the cells, which consisted of DMEM, lentiviral solution and transfection

reagent. The mixture was placed in an incubator and incubation was continued. After 48 h, the culture plate was removed and the fluorescence intensity was observed under the microscope to determine the infection efficiency of the cells. The old medium containing the virus solution was aspirated and replaced with serum-free DMEM to continue culturing and passaging. Half of the infected cells were used to determine infection efficiency, whilst the remaining cells were screened for stable infection using neomycin or puromycin (2 µg/ml) screening, depending on the virus design. Blank control wells were set up, and screening was stopped when the cells in the blank group were completely eliminated.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells that had been administered TRIzol® reagent (Corning, Inc.) after being washed three times with PBS. The RNA was then used to create cDNA using reverse transcription, following the instructions of the Reverse Transcription Kit (Takara Bio, Inc.). The conditions were set as follows: 37°C for 15 min; 85°C for 5 sec; and cooling down to 4°C. The cDNA was amplified using an Amplification Kit for PCR experiments (Takara Bio, Inc.). The procedure was set to tick SYBR Green method, CT method. The conditions were set as follows: 95°C for 5 sec; 95°C for 5 sec; 55°C for 30 sec; and 72°C for 30 sec. The number of cycles was set to 40. The primer sequences used were as follows: *ZBTB20* forward, 5'-GACAGGATCTACTCGGCACTC-3' and reverse, 5'-ACTGCGCCGCTGTAAAAAGA-3'; *TMEM109* forward, 5'-TGGGGAAAGCATGTGTTCAAA-3' and reverse, 5'-TGGTGCAAAGTCTCGACGG-3'; and *GAPDH* forward, 5'-GGAAGCTTGTTCATCAATGGAAATC-3' and reverse, 5'-TGATGACCCTTTTGGCTCCC-3'.

JASPAR. The promoter sequence of the target gene is obtained from NCBI database, and the upstream 2 kb region is adjusted according to the direction of the gene chain. The target transcription factor was searched on the JASPAR website (<https://jaspar.elixir.no/>), and the appropriate motif version was selected. Then the promoter sequence of the target gene in FASTA format was analyzed by the JASPAR-Scan tool to find the possible binding sites of transcription factors.

Western blotting. The cultured cells were collected using a cell scraper and lysed using RIPA lysate (cat. no. P0013B; Beyotime Institute of Biotechnology). The protein concentration was determined using bicinchoninic acid (BCA) method. Proteins (20 µg/20 µl) were then extracted and solubilized using SDS-PAGE (10% acrylamide), and then transferred onto a PVDF membrane (MiliporeSigma; Merck KGaA). The membrane was blocked with 5% bovine serum albumin at room temperature for 2 h in TBST (0.05% Tween; Beijing Solarbio Science & Technology Co., Ltd.) and subsequently incubated at 4°C for 16 h in primary antibody diluent (1:1,000-10,000) after the blocking process was completed. Western blot analysis employed several primary antibodies, including β-actin (cat. no. AC026; ABclonal Biotech Co., Ltd.), *ZBTB20* (cat. no. A7970; ABclonal Biotech Co., Ltd.), *GPX4* (cat. no. A1933; ABclonal Biotech Co., Ltd.), *SLC7A11* (cat. no. 26864-1-AP, Proteintech Group, Inc.) and *TMEM109* (cat. no. bs-19950R; BIOSS). Peroxidase-coupled secondary

antibodies (1:1,000-2,000, cat. no. ab6721; Abcam) were then used for 1 h incubation at room temperature, and the signals were detected using ECL Ultra Sensitive Luminescent Liquid (Strong; Applygen Technologies, Inc.). Exposure imaging was performed using the fully automatic chemiluminescence imaging system (Tanon 4600, Tanon Science and Technology Co., Ltd.) to analyze relative intensities with the assistance of ImageJ Lab software (ImageJ 2.0; National Institutes of Health).

ROS fluorescence imaging. Cells were pre-cultured on 96-well plates at a density of 3,000 cells per well for 24 h. After washing the plates with PBS, they were incubated with DMEM containing 10 µM dihydroethidium (cat. no. 50102ES02; Shanghai Yeasen Biotechnology Co., Ltd.) for 2 h at room temperature or 37°C. Subsequently, images of the plates were captured using a confocal microscope (GE Healthcare).

Fe²⁺ detection experiment. Intracellular Fe²⁺ content was determined using an Fe²⁺ content detection kit (cat. no. BC5415; Beijing Solarbio Science & Technology Co., Ltd.). Cells were treated with Fe²⁺ detection reagent 1, broken up using ultrasonic crushing and centrifuged at 4°C (1,000 x g, 20 min). Reagent 2 was then added and mixed and left to stand for 10 min. The treated reagent was spread onto 96-well plates, incubated at 37°C for 30 min and then the absorbance at 593 nm was detected using an Automatic Elisa Analyzer (PERLONG, <http://www.prolong.com.cn/productinfo.php?anclss=5&nclss=5&id=5>). Finally, the Fe²⁺ content of the cells was calculated according to the formula of the kit.

Malondialdehyde (MDA) detection experiment. MDA content was determined using an MDA content detection kit (cat. no. S0131S; Beyotime Institute of Biotechnology). The thiobarbituric acid storage solution and MDA detection working solution were prepared in advance and then the standard sample was used to make a standard curve. The sample was subsequently processed according to the instructions in the reagent kit and an Automatic Elisa Analyzer was used to detect the absorbance of the sample at 532 nm. Finally, the MDA content of the cells was calculated according to the formula of the kit.

Dual luciferase reporter gene assay. Plasmids purchased from Shanghai GeneChem Co., Ltd. were constructed using the *TMEM109* gene sequence (GenBank accession no. NC_000011.10) and the *ZBTB20* gene sequence (GenBank accession no. NC_000003.12) obtained from GenBank. These plasmids were used to create cell lines with overexpression and knockdown of *TMEM109* for experimental purposes. 293T cells (Stem Cell Center of Zhengzhou University) in logarithmic growth phase were suspended and plated in 12-well plates at a density of ~2.5x10⁵ cells per well, depending on cell morphology. The cells were cultured overnight in a 37°C, 5% CO₂ incubator. The cells were then treated with a dual luciferase reporter gene kit (cat. no. RG027; Beyotime Institute of Biotechnology) and divided into four groups: Promoter-NC + NC, promoter-NC + *ZBTB20*, promoter-NC and promoter-*ZBTB20*. The prepared DNA was added into 100 µl PBS and mixed evenly. Subsequently, 4.8 µl HiGene

transfection reagent (cat. no. C1506; Applygen Technologies, Inc.) was applied and the mixture was added to the well plates of the divided groups after 15 min of mixing and resting. After 48 h, the medium was replaced with DMEM medium and incubated further. Finally, the plate was removed and washed with PBS three times. The appropriate amount of lysis solution was then added to fully lyse the cells. The solution was collected in a centrifuge tube and centrifuged at low temperature at $12,000 \times g$ for 5 min. The supernatant was then collected for subsequent experiments. Subsequently, the firefly and sea kidney luciferase reagents were configured. Then, 50 μ l supernatant were taken and added to the special 96-well plate. A total of 5 duplicate wells were set up for each sample. Finally, detection was performed using a multifunctional Automatic Elisa Analyzer with a 2-sec interval and 10-sec assay time. Then calculated following the provided instructions.

Chromatin immunoprecipitation (ChIP) assay. U251 cells with a healthy growth status were selected and fixed at room temperature for 10 min in a 1% formaldehyde solution once they reached ~90% confluence. After cross-linking, the nucleus and chromatin were digested, followed by ChIP, elution and de-cross-linking. The DNA was then purified and analyzed using RT-qPCR (cat. no. RR037A; Takara Bio, Inc.). The transcription conditions were as follows: The reverse transcription reaction was carried out at 37°C for 15 min for 3 cycles, followed by the period of reverse transcriptase inactivation at 85°C for 5 sec. Additionally, 10 μ l PCR products were extracted from each tube for PCR experiments; the cycling conditions included an initial holding period at 95°C for 5 min, followed by a two-step PCR program consisting of 40 cycles of 95°C for 5 sec and 60°C for 10 sec.

Statistical analysis. Statistical analyses were performed using Prism 8.0.2 software (Dotmatics). Statistical significance was evaluated using one-way analysis of variance, followed by Tukey's post hoc test for multiple comparisons. The results are presented as the mean $\pm$ standard deviation and were repeated in ≥ 3 independent experiments. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ZBTB20 expression and ferroptosis level is decreased in GBM cells. To assess the role of ZBTB20 in ferroptosis, the expression of ZBTB20 and ferroptosis level between GBM cells (U251 and U87MG) and normal glial cells (HA1800) was first compared. The results revealed that the expression of ZBTB20 in U251 and U87MG cells was lower than that in HA1800 cells (Fig. 1A, B, F and G), whilst the expression of TMEM109, SLC7A11 and GPX4 was higher (Fig. 1A, C-F and H-J). GPX4 is an intracellular antioxidant enzyme and its reduced level leads to the accumulation of lipid peroxides, which promotes ferroptosis (45). SLC7A11, an ferroptosis regulator, is a regulator upstream of GPX4 (46).

The results also demonstrated that both ROS (Fig. 1K and L) and Fe^{2+} (Fig. 1M) levels were lower in U251 and U87MG cells compared with HA1800 cells. Moreover, patients with high ZBTB20 expression had longer overall survival (Fig. 1N), but those with high TMEM109 expression had shorter overall

survival (Fig. 1O). These results indicated that a significant reduction in the level of ferroptosis in GBM cells was accompanied by an increase in ZBTB20 expression, suggesting that ZBTB20 may be involved in the regulation of ferroptosis.

ZBTB20 promotes ferroptosis in U251 GBM cells. Previous research reported that ZBTB20 inhibits cell proliferation and promotes apoptosis in GBM cells (47). The present study evaluated the role of ZBTB20 in ferroptosis through gain- and loss-of-function experiments (Fig. 2A, B, E and F). U251 GBM cells stably expressing ZBTB20 were obtained by lentiviral infection and puromycin screening. The results of western blotting revealed that the expression of SLC7A11 and GPX4 was lower in GBM cells with ZBTB20 overexpression, compared with that in the vector control (Fig. 2A, C and D), and higher in GBM cells with ZBTB20 knockdown, compared with that in the shRNA control (Fig. 2E, G and H). Furthermore, both ROS (Fig. 2I-K) and Fe^{2+} (Fig. 2L and M) levels were higher in GBM cells with ZBTB20 overexpression and lower in GBM cells with ZBTB20 knockdown compared with wild-type cells. In U87MG glioma cells, the aforementioned results were consistent (Fig. S1).

ZBTB20 transcriptionally represses TMEM109 expression in U251 GBM cells. Although the present study demonstrated that ZBTB20 promotes ferroptosis, the existing literature cannot adequately explain the mechanisms involved. Genes transcriptionally repressed by ZBTB20 were validated via RT-qPCR. By integrating Jasper online predictions (<https://jaspar.genereg.net/>) of ZBTB20's promoter binding regions, TMEM109 was ultimately identified as the target gene for further investigation, and gain- and loss-of-function experiments demonstrated that ZBTB20 repressed TMEM109 expression through transcriptional repression (Fig. 3A-H). ZBTB20 acts as a transcription factor, and in order to assess whether ZBTB20 directly transcriptionally regulates TMEM109, ChIP (Fig. 3I) and dual-luciferase reporter gene experiments (Fig. 3J) were performed. The results revealed that ZBTB20 can directly bind to the TMEM109 promoter region and inhibit its expression. The results of PCR (Fig. 1A-D) and western blotting (Fig. 1E-H) showed that in U251 and U87MG cells (Fig. S2), the transcriptional inhibitory effect of ZBTB20 on TMEM109 remained consistent.

ZBTB20 promotes ferroptosis through repressing TMEM109 expression in U251 GBM cells. TMEM109 has been reported to be a Ca^{2+} channel located in the membrane of the ER (48). It was hypothesized that TMEM109 alters intracellular calcium homeostasis, and as Ca^{2+} alteration is one of the inducing factors of ferroptosis (49), TMEM109 may be involved in the regulation of ferroptosis. To assess this, U251 cells were infected with lentivirus carrying TMEM109 cDNA, and GBM cells with TMEM109 overexpression and knockdown were obtained. The results of western blot analysis revealed that the expression of SLC7A11 and GPX4 was higher in GBM cells with TMEM109 overexpression compared with that in the vector control cells (Fig. 4A-D), and lower in GBM cells with TMEM109 knockdown compared with that in the shRNA control cells (Fig. 4E-H). The results also demonstrated that TMEM109 overexpression decreased ROS levels (Fig. 4I-K) and Fe^{2+} content (Fig. 4L and M), whereas TMEM109

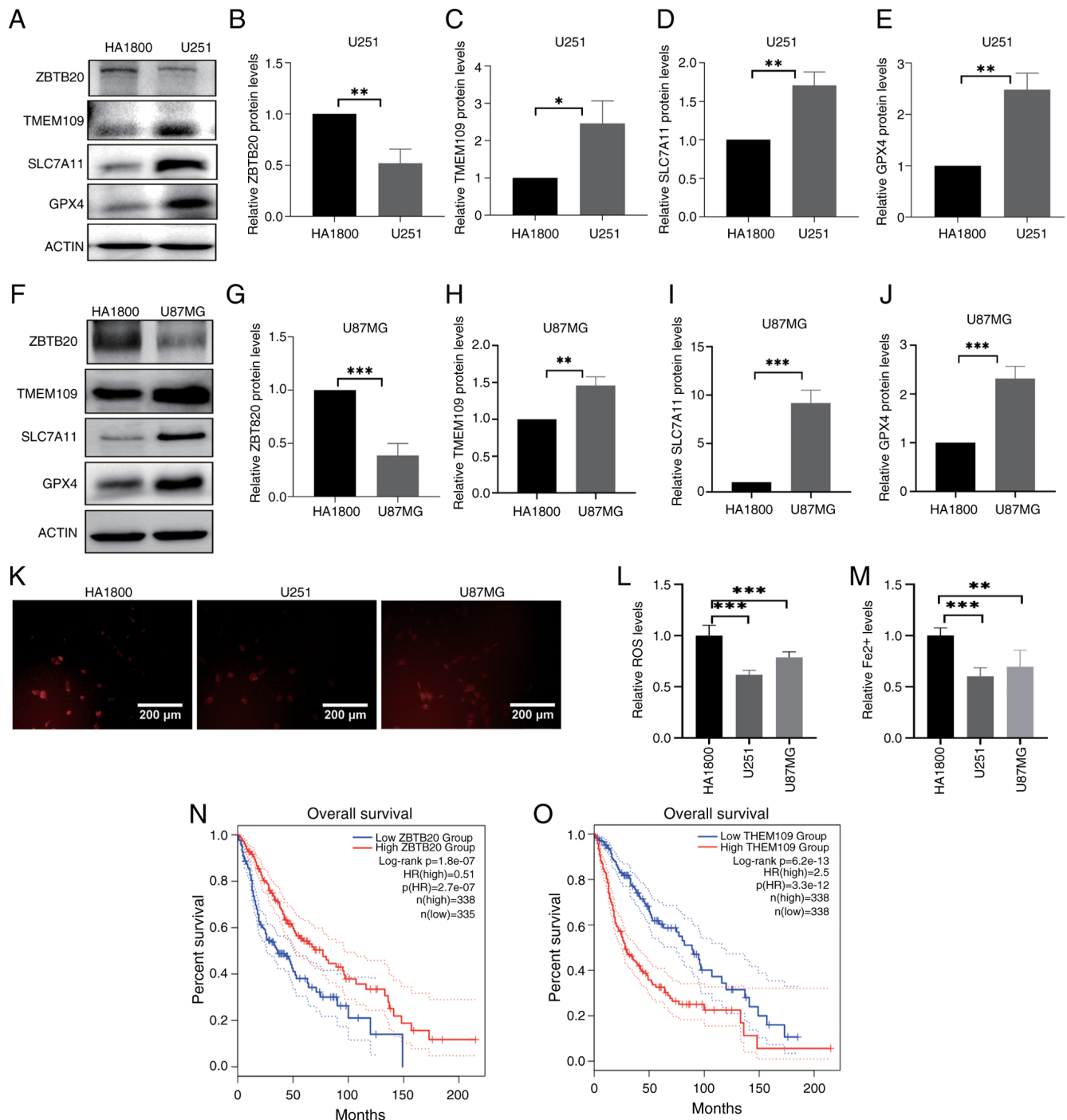


Figure 1. ZBTB20 expression and ferroptosis level in GBM cells. (A-E) Expression of ZBTB20, TMEM109, SLC7A11 and GPX4 in U251 and HA1800 cells detected by (A) western blotting and (B-E) the corresponding histograms. (F-J) Expression of ZBTB20, TMEM109, SLC7A11 and GPX4 in U87MG and HA1800 cells detected by (F) western blotting and (G-J) the corresponding histograms. (K and L) ROS level detected by fluorescence microscopy and the corresponding histogram. (M) Histogram of Fe^{2+} content. (N) Overall survival of ZBTB20. (O) Overall survival of TMEM109. Data are shown as the mean $\pm$ standard deviation, n=3. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. ZBTB20, zinc finger and BTB domain containing 20, GBM, glioblastoma; TMEM109, transmembrane protein 109; SLC7A11, solute carrier family 7 member 11, GPX4, glutathione peroxidase 4; ROS, reactive oxygen species.

knockdown induced the opposite results (Fig. 4I-M). Furthermore, the findings revealed that patients with high TMEM109 expression had shorter overall survival (Fig. 1O). The same result of TMEM109 inhibiting ferroptosis was also demonstrated in U87MG cells (Fig. S3).

Subsequently, co-transfected cells highly expressing both TMEM109 and ZBTB20 were constructed. RT-qPCR (Fig. 5A-D) and western blotting (Fig. 5E-I) results revealed

that the expression of both SLC7A11 and GPX4 was significantly higher in co-transfected cells, whilst ROS levels (Fig. 5J and K) and Fe^{2+} content (Fig. 5L) were significantly lower compared with cells overexpressing ZBTB20 alone. Furthermore, the MDA detection experiment revealed that the content of MDA was significantly higher in co-transfected cells compared with ZBTB20 overexpression cells (Fig. 5M). These results suggested that the promoting effect of ZBTB20 on

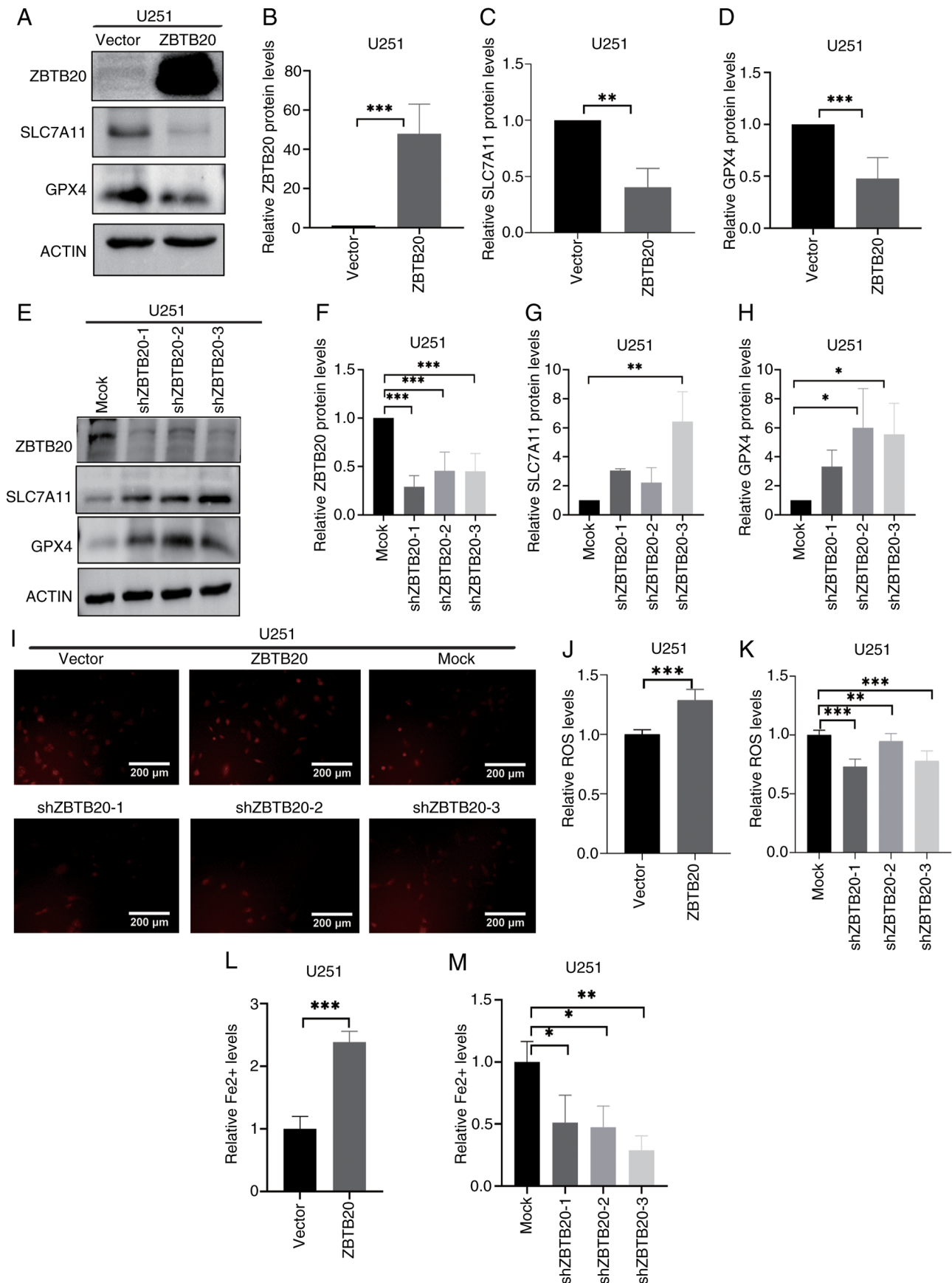


Figure 2. ZBTB20 promotes ferroptosis in U251 GBM cells. (A-D) Expression of ZBTB20, SLC7A11 and GPX4 detected by (A) western blotting and (B-D) the corresponding histograms in GBM cells with ZBTB20 overexpression. (E-H) Expression of ZBTB20, SLC7A11 and GPX4 detected by (E) western blotting and (F-H) the corresponding histograms in GBM cells with ZBTB20 knockdown. (I-K) ROS levels detected by (I) fluorescence microscopy and the corresponding histogram in GBM cells with ZBTB20 (J) overexpression and (K) knockdown. (L and M) Histograms of Fe²⁺ content in GBM cells with ZBTB20 (L) overexpression and (M) knockdown. *P<0.05, **P<0.01 and ***P<0.001. ZBTB20, zinc finger and BTB domain containing 20, GBM, glioblastoma; TMEM109, transmembrane protein 109; SLC7A11, solute carrier family 7 member 11, GPX4, glutathione peroxidase 4; ROS, reactive oxygen species.

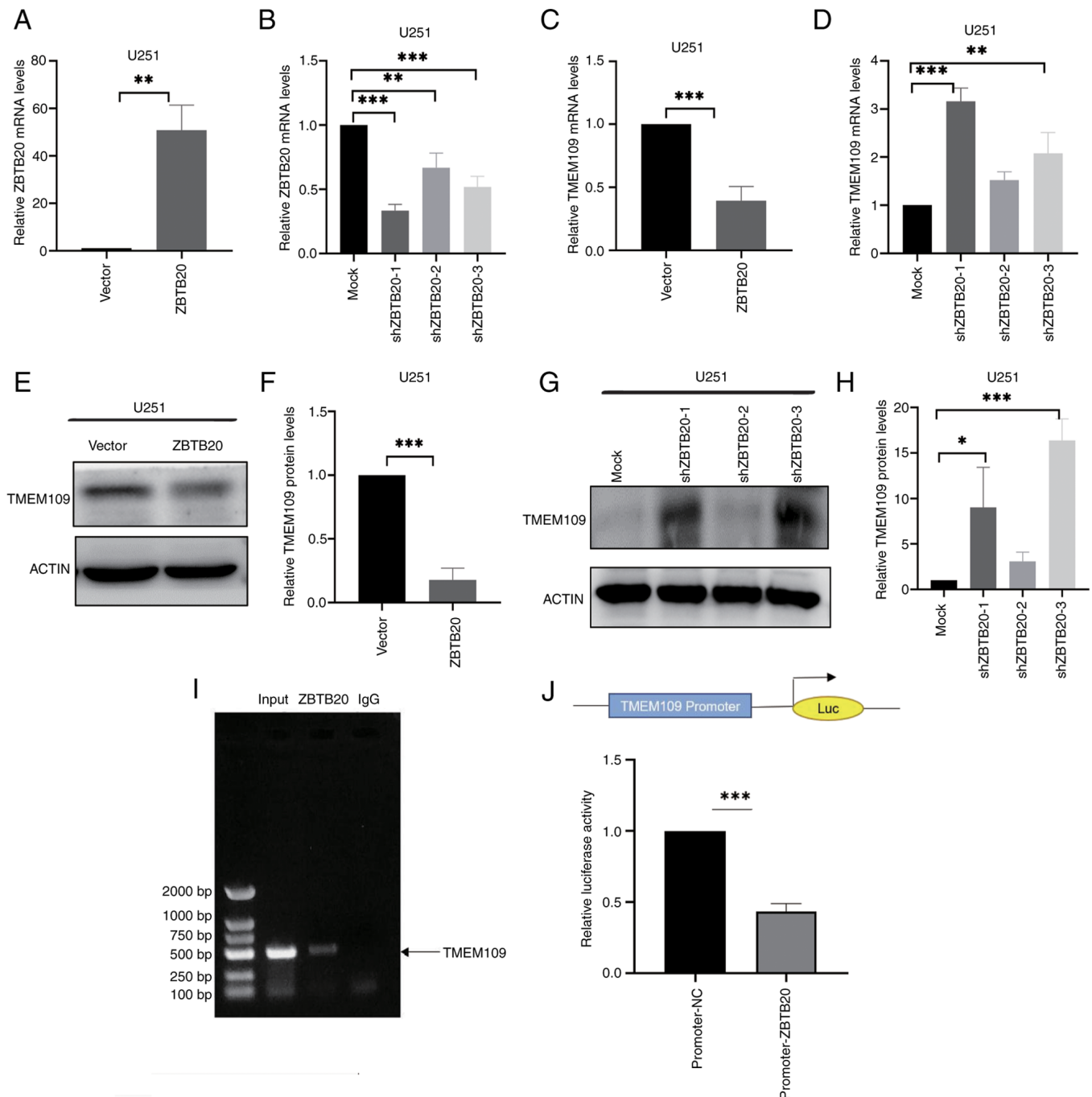


Figure 3. ZBTB20 transcriptionally represses TMEM109 expression in U251 GBM cells. (A-D) Expression of (A and B) ZBTB20 and (C and D) TMEM109 in GBM cells with ZBTB20 overexpression detected by reverse transcription-quantitative PCR with the corresponding histogram. (E-H) Expression of ZBTB20 and TMEM109 in GBM cells with ZBTB20 knockdown detected by (E and G) western blotting with (F and H) the corresponding histogram. (I) Results of chromatin immunoprecipitation assay. (J) Results of the dual-luciferase reporter gene experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. ZBTB20, zinc finger and BTB domain containing 20, GBM, glioblastoma; TMEM109, transmembrane protein 109.

ferroptosis was realized by inhibiting TMEM109 expression. In U87MG cells, TMEM109 reversed the iron-depletion effect promoted by ZBTB20, in consistency with the aforementioned results (Fig. S4).

Discussion

The present study demonstrated that ZBTB20 promotes ferroptosis in GBM cells through transcriptionally inhibiting TMEM109 expression. Furthermore, the results illustrate the

regulatory mechanism of ferroptosis in GBM and highlight the role of ZBTB20 and TMEM109 in it, thus providing new ideas for clinical treatment of GBM. Moreover, whilst the present study demonstrated a positive effect of ZBTB20 on ferroptosis in glioma cells, other studies have reported that ZBTB20 can promote the proliferation of hepatocellular carcinoma (34) and gastric cancer (30), suggesting that ZBTB20 serves different roles on progression in different cancer tissues.

Numerous studies have also reported that ferroptosis is involved in the oncogenesis and development of several

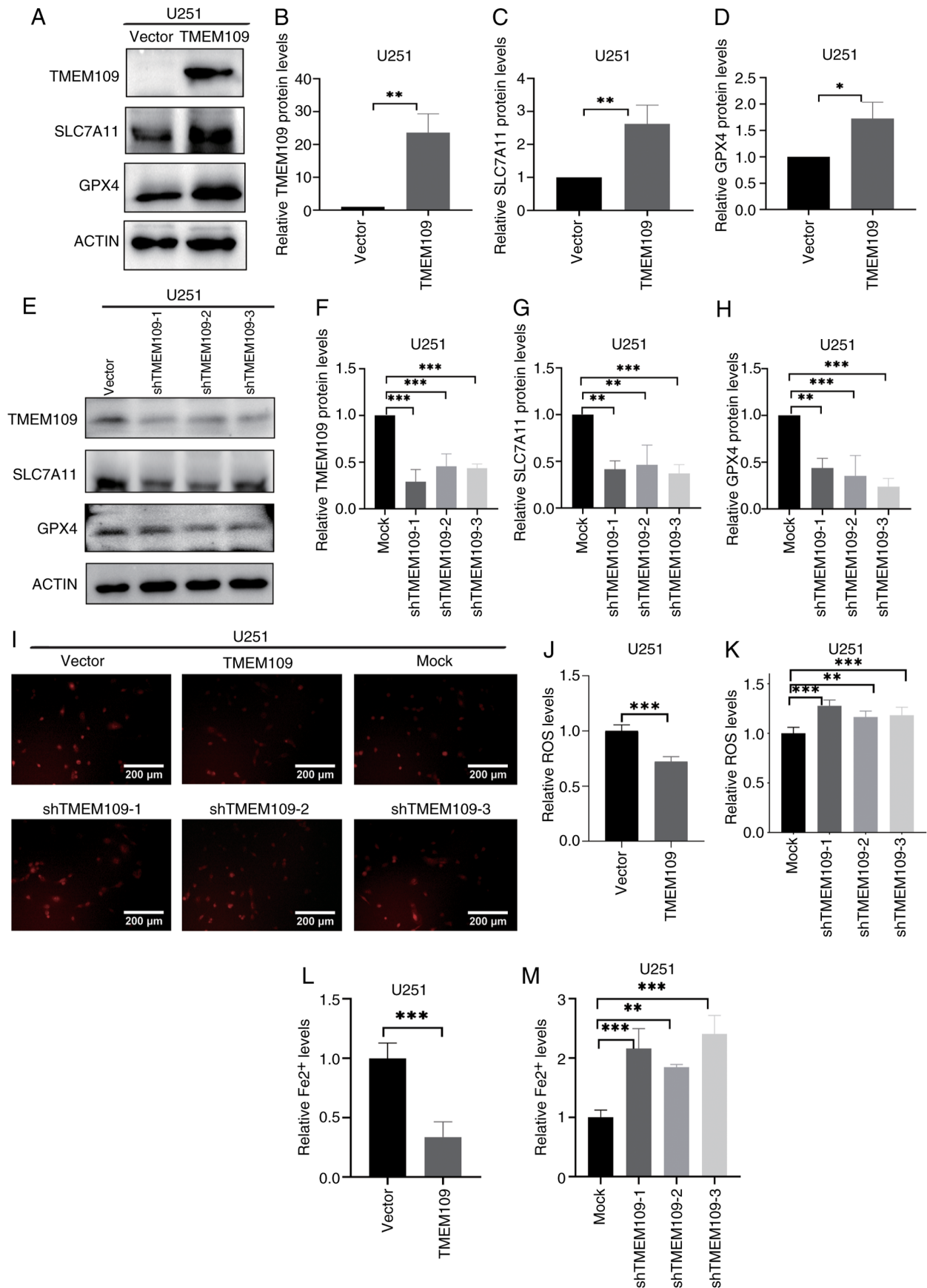


Figure 4. TMEM109 represses ferroptosis in U251 GBM cells. (A-D) Expression of ZBTB20, SLC7A11 and GPX4 detected by (A) western blotting and (B-D) the corresponding histograms in GBM cells with TMEM109 overexpression. (E-H) Expression of ZBTB20, SLC7A11 and GPX4 detected by (E) western blotting and (F-H) the corresponding histograms in GBM cells with TMEM109 knockdown. (I-K) ROS levels detected by (I) fluorescence microscopy and the corresponding histogram in GBM cells with TMEM109 (J) overexpression and (K) knockdown. (L and M) Histograms of Fe²⁺ content in GBM cells with TMEM109 (L) overexpression and (M) knockdown. *P<0.05, **P<0.01 and ***P<0.001. ZBTB20, zinc finger and BTB domain containing 20, GBM, glioblastoma; TMEM109, transmembrane protein 109; SLC7A11, solute carrier family 7 member 11, GPX4, glutathione peroxidase 4; ROS, reactive oxygen species.

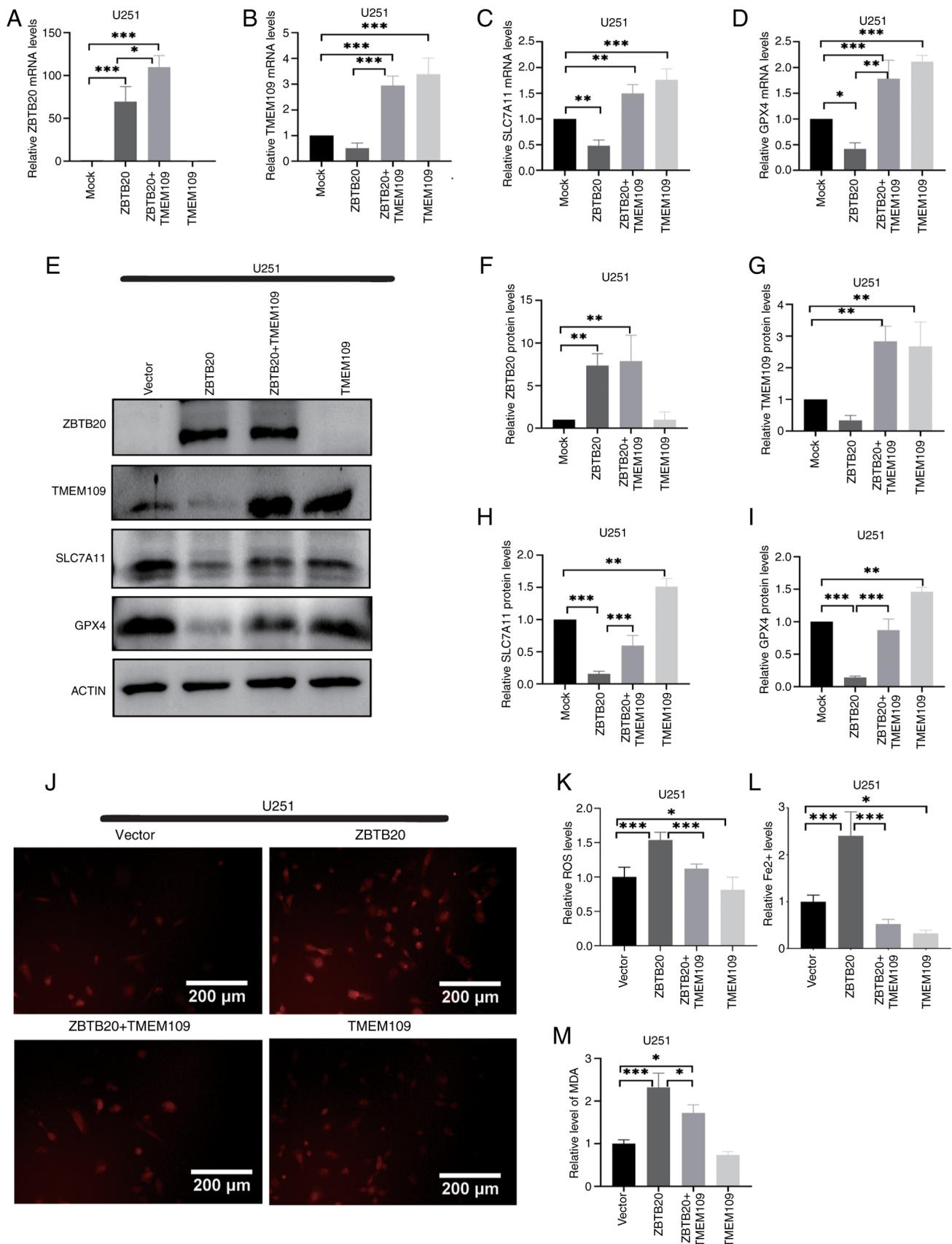


Figure 5. ZBTB20 promotes ferroptosis through repressing TMEM109 expression in U251 GBM cells. (A-D) Expression of ZBTB20, TMEM109, SLC7A11 and GPX4 detected by reverse transcription-quantitative PCR and the corresponding histograms in GBM cells with ZBTB20 and/or TMEM109 overexpression. (E-I) Expression of ZBTB20, TMEM109, SLC7A11 and GPX4 detected by (E) western blotting and (F-I) the corresponding histograms in GBM cells with ZBTB20 and/or TMEM109 overexpression. (J and K) ROS levels detected by (J) fluorescence microscopy and (K) the corresponding histogram. (L) Histograms of Fe²⁺ content in GBM cells with ZBTB20 and/or TMEM109 overexpression. (M) Histograms of MDA content in GBM cells with ZBTB20 and/or TMEM109 overexpression. *P<0.05, **P<0.01 and ***P<0.001. ZBTB20, zinc finger and BTB domain containing 20, GBM, glioblastoma; TMEM109, transmembrane protein 109; SLC7A11, solute carrier family 7 member 11, GPX4, glutathione peroxidase 4; ROS, reactive oxygen species; MDA, malondialdehyde.

cancers. For example, cystatin SN regulates the stability of GPX4 protein through OTU deubiquitinase, ubiquitin aldehyde binding 1, which inhibits ferroptosis and promotes metastasis of gastric cancer (50). Additionally, it has been reported that, compared with paraneoplastic tissue, the expression level of STAT3 (a ferroptosis inhibitor), is markedly increased in gliomas (51); this indicates that the ferroptosis level in gliomas is relatively low. Heat shock protein 90 induces acyl-CoA synthetase long chain family member 4-dependent ferroptosis in gliomas by dephosphorylating Ser637 at the Drp1 locus (52). Another study using the Chinese Glioma Gene Atlas database reported that ferroptosis-related genes were differentially expressed in GBM and paraneoplastic tissues (53). The present study demonstrated that the level of ferroptosis was significantly reduced in GBM cells compared with in human normal glial cells.

It has been reported that ZBTB20 can increase ROS levels in GBM cells by regulating the stress-inducible protein Sestrin3 (54,55), and that ROS are an important regulator in ferroptosis (56). The present study demonstrated that ZBTB20 increased ROS levels and promoted ferroptosis using gain- and loss-of-function experiments. The expression of two key genes in ferroptosis (57), SLC7A11 and GPX4, was assessed in the present study to measure ferroptosis. SLC7A11 is a key gene in the antioxidant damage pathway in ferroptosis and is located on the cell membrane. The light chain subunit SLC7A11, a functional subunit, and the heavy chain subunit SLC3A2, which maintains the stability of SLC7A11 (58), form the cystine-glutamate reverse transporter (Xc-system). SLC7A11 has a high degree of specificity for cystine and glutamate, and its main function is to participate in the uptake of cystine and the release of glutamate (59), which promotes the synthesis of GSH (60), avoids cellular exposure to oxidative stress damage, and maintains the redox balance inside the cell, thus preventing cell death caused by membrane lipid peroxidation (15). Several studies have reported that SLC7A11 is highly expressed in several solid malignant cancers, such as breast cancer, pancreatic cancer, ovarian cancer and glioma, and has an association with drug resistance (4,15). Currently, SLC7A11 has become a research focus in anticancer therapy. The main role of SLC7A11 is to increase the synthesis of glutathione, promote the activity of GPX4, and enhance the ability of cells to resist oxidative damage (61). GPX4 can attenuate lipid peroxidation toxicity and maintain homeostasis of the membrane lipid bilayer (5). GPX4 depends on GSH to catalyze the conversion of peroxides to alcohols, and GSH deficiency directly causes inactivation of GPX4, which further triggers ferroptosis (62). Meanwhile, SLC7A11 can regulate intracellular GSH expression; therefore, SLC7A11 exists as an upstream regulator of GPX4 (63). The present study demonstrated that ZBTB20 decreased the expression of SLC7A11 and GPX4, indicating that ZBTB20 may be involved in the SLC7A11/GPX4 antioxidant damage pathway to regulate ferroptosis.

Furthermore, the present study revealed that the regulatory effect of ZBTB20 on ferroptosis was partly through transcriptional repression of TMEM109. Previous research has reported that TMEM109 overexpression altered the cytoplasmic Ca^{2+} concentration and caused calcium

imbalance (64), and calcium imbalance contributes to ferroptosis (64-66). Downregulation of membrane-spanning 4-domains subfamily A member 15 expression, which is localized in the ER, promotes luminal Ca^{2+} accumulation, which inhibits lipid elongation and desaturation, driving lipid droplet dispersion and the formation of shorter, more saturated ether lipids, thereby protecting phospholipids from peroxidation and preventing ferroptosis in cells (65). Inositol trisphosphate receptor channel-mediated calcium release promotes ferroptosis in SH-SY5Y neuroblastoma cells (64). RyR receptor-mediated calcium release contributes to the inhibition of GPX4 and thus induces ferroptosis in primary hippocampal neurons (66). Overall, it was hypothesized that the regulatory effect of TMEM109 on ferroptosis may be associated with its regulation of calcium leakage.

However, there are certain limitations of the present study that need to be improved. First, although the results demonstrated a regulatory effect of TMEM109 on ferroptosis and suggested an association with Ca^{2+} imbalance, altered Ca^{2+} levels were not measured to validate this argument. Second, the way in which the inhibitory effect of ZBTB20 on SLC7A11 and GPX4 expression was realized was not further explored.

In conclusion, in the present study, it was found that ZBTB20 promotes ferroptosis by repressing the expression of TMEM109 through transcription. And ferroptosis serves a pivotal role in cancers, which in recent years has provided new opportunities for treatment of cancers (9). Therefore, the present study provides a new therapeutic approach for combating the progression of glioma and chemotherapy resistance.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XC and ML designed experimental programs and contributed to molecular biology testing and cell experiments. PD and HC contributed to analysis and interpretation of data. WW and XN contributed to the conception of the study and assisted with performing the analysis with constructive discussions. RW and LZ contributed to cellular experiments and cell immunofluorescence. XC and PD confirm the authenticity of all the raw data. All authors read and approved the final version of the 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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