TMBIM1 promotes EMT by stimulating autophagic degradation of E-cadherin via AMPK/mTOR/ULK1 axis in human gliomas

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Research Article

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Abstract

Background

Gliomas are the most common malignant tumors of the central nervous system in adults, glioblastoma is notorious for its highly metastatic and recurrent, accounting for approximately 50% of all gliomas. Exploring its molecular mechanism is urgently needed for the treatment and prognosis evaluation of gliomas. Transmembrane BAX inhibitor motif-containing 1 (TMBIM1) has been reported to be associated with non-alcoholic steatohepatitis, tumor and other diseases. However, the role of TMBIM1 in GBM and the underlying mechanisms remains unclear.

Methods

The expression level and prognostic value of TMBIM1 in gliomas were investigated by public datasets, and further confirmed by western blot and immunohistochemistry (IHC) in our tissues. Intracranial xenograft model, IHC and western blot were used to evaluate the functional role of TMBIM1.

Results

TMBIM1 was over-expressed in GBM, and its high expression reduced the survival time of glioma patients. TMBIM1 induced EMT and autophagy, and inhibition of autophagy reverses TMBIM1-regulated EMT in vitro and in vivo. Intracranial xenograft model showed the survival time of mice in TMBIM1 knockdown group treated with chloroquine (CQ)was significantly prolonged. The loss of E-cadherin expression is considered the foundation of EMT, and we subsequently demonstrated that TMBIM1 stimulating autophagic degradation of E-cadherin via AMPK/mTOR/ULK1 axis.

Conclusion

Our study provides a novel mechanism for the regulation of EMT in the process of gliomas metastasis, indicating that inhibition of TMBIM1 activity to attenuate autophagy may be a potential strategy for the treatment of gliomas.

Introduction

Glioma is one of the most common primary intracranial tumors in adults, accounting for more than 70% of malignant brain tumors, among which GBM is the most malignant type(1, 2). However, despite the combination of tumor resection, radiotherapy and chemotherapy, the median survival of GBM is only 12–18 months and the 5-year survival rate is less than 5%(3). GBM is notorious for its high metastasis and recurrence ability, which may be the key reason why the treatment of GBM cannot meet the medical needs. Therefore, exploring the molecular mechanisms underlying the highly invasive and metastatic capacity of GBM may help to improve patient outcomes.

Epithelial-mesenchymal transition (EMT) allows epithelial cells to acquire mesenchymal characteristics and enhance the ability of metastasis and invasion(4). Studies have found that EMT is considered to be the main driving force of tumor deterioration, and plays a crucial role in tumor recurrence, metastasis and chemotherapy resistance(5). The loss of E-cadherin during cancer progression is fundamental to the development of EMT. SPHK1 is a regulator of sphingolipid metabolites and could induced EMT in hepatoma cells by accelerating the lysosomal degration of CDH1. Moverover, overexpression of SPHK1 led to the interaction between TRAF1 and BECN1, which in turn activated lysine ubiquitination of BECN by promoting autophagy(6) Currently, the role of autophagy-lysosomal degradation pathway in regulating EMT process had been extensively studied in cancers, inclduing glioma(7, 8)

TMBIM1 is a membrane protein localized in endosomal/lysosomes and plays crucial role in vascular remodeling and mediating cystic medical degeneration. TMBIM1 was involved in regulating cell apoptosis and maintaining intracellular calcium balance(9). TMBIM1 expression was found to be elevated in colerectal cancer tissues and its genetic alteration obviously associated with high risk of lymph node metastasis and distant metastasis(10). Hongliang Li et.al. reported that TMBIM1 inhibits adipocyte proliferation and improves obesity-related metabolic diseases by promoting lysosomal degradation of TLR4(11, 12). Lysosomal degradation is a fundamental process during autophagy which is a self-digesting process. Impairment of the autophagy-lysosomal degradation pathway has been associated with cancer.

Considering the importance of TMBIM1 in regulating autophagy -lysosomal pathway, we tried to find potential mechanism under which TMBIM1 regulated the EMT process of glioma. In our study, we demonstrated that TMBIM1 was over-expressed in GBM, and high TMBIM1 expression predicted poor prognosis in GBM and lower-grade gliomas (LGG). Moreover, our research found TMBIM1 induced autophagy and EMT-mediated cell metastasis. Further research confirmed that TMBIM1 induces autophagic degradation of E-cadherin by mediating AMPK/mTOR/ULK1 axis. Our study revealed the mechanism of TMBIM1 regulating EMT in GBM, and provided a new strategy for the treatment of GBM patients.

Materials And Methods

Bioinformatics

A total of five glioma datasets were obtained from GlioVis portal (http://gliovis.bioinfo.cnio.es)(13), including TCGA-GBM, TCGA-GBMLGG, CGGA, Rembrandt and Gravendeel datasets. The disease-free survival of TMBIM1 in glioma patients was acquired from GEPIA online website(14).

Human tissue samples

Paraffin-embedded glioma tissue microarray contained 111 glioma tissues and 8 normal brain tissues. All tissue samples were obtained in the Department of Neurosurgery, Renmin Hospital of Wuhan University from March 2016 to June 2019. Details of clinical information for all patients was presented in

Table S1. The other 5 non-tumor brain tissues (NBT) and 18 GBM tissues were collected from March 2019 to March 2021 for Western blot (WB) analysis. All NBT were obtained from patients with severe brain injury who need surgery. None of the patients received radiotherapy or chemotherapy before surgery, and all tissues were stored at liquid nitrogen. This study was approved by the Ethics Committee of the Renmin Hospital of Wuhan University [approval number: 2012LKSZ (010) H], and all the patients signed the informed consent.

Antibodies and reagents

CHX (S7418), MG132 (S2619), Compound C (S7306), CQ (S6999) and 3-MA (HY-19312) were purchased from Selleck. Anti-E-cadherin (20874-1-AP), N-cadherin (22018-1-AP), Vimentin (10366-1-AP), SNAIL (13099-1-AP), P62 (18420-1-AP), Beclin1 (11306-1-AP), AMPK (66536-1-Ig) and ULK1 (20986-1-AP) antibodies were obtained from Proteintech (Wuhan, China). Anti-Flag (ANT301) and β-actin (ANT321) were purchased from AntGene (Wuhan, China). Anti-phospho-AMPKα (Thr172) (#2535), anti-phospho-ULK1 (Ser317) (#12753) and Anti-phospho-mTOR (Ser2448) (#5536) were obtained from Cell Signaling Technology (USA). Anti-LC3 (GB11124) was purchased from Servicebio (Wuhan, China).

Cell culture and transfection

U87 and U251 cell lines were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO2. Flag-TMBIM1 was obtained from Miaolingbio (Wuhan, China). Cells were transfected with 2.0 ug plasmid per well in 6-well plates using Hieff Trans™ liposomal transfection reagent (Yeasen Biotechnology, China) according to the manufacturer's instruction.

Stable cell lines establishment

The knockdown lentivirus vector of TMBIM1 (rLV-shRNA-TMBIM1) was obtained from Huamengbio (Wuhan, China) and all lentiviral vectors are verified by DNA sequencing. Cells were cultured in 6-well plates and infected by lentivirus according to the manufacturer's instruction for 24 hours. Subsequently, cells were treated using puromycin (2 μg/ml) for 72 hours and fresh medium was added after washed with PBS buffer. The knockdown efficiency of TMBIM1 was verified by real-time qPCR and WB. The TMBIM1 shRNA sequence was as follows (shTMBIM1-1-F:5'GATCCGGAGAGAGCGGTGAGTGATAGCTCGAGCTATCACTCACCGCTCTCTCCTTTTTTG-3', shTMBIM1-1-R:5'-AATTCAAAAAAGGAGAGAGCGGTGAGTGATAGCTCGAGCTATCACTCACCGCTCTCTCCG-3', shTMBIM1-2-F: 5'-GATCCGCCGTTTCCCATGGAACATCACTCGAGTGATGTTCCATGGGAAACGGCTTTTTTG-3', shTMBIM1-2-R: 5'-AATTCAAAAAAGCCGTTTCCCATGGAACATCACTCGAGTGATGTTCCATGGGAAACGGCG-3')

Transwell assay

Cell invasion and migration were detected using the Matrigel (R&D, USA)-coated transwells and no Matrigel -coated tarnswells, respectively. 3 × 10^5 cells in serum-free medium were added to upper chamber, and 600 ul DMEM supplemented with 10% FBS was added to lower chamber. The cells were fixed with 4% paraformaldehyde for 30 minutes after incubated in an incubator (37 °C, 5% CO2) for 24 hours. Then cells were stained with 0.5% crystal violet for 15 minutes and observed under a microscope (Olympus, BX51, Japan). In each experimental group, we randomly selected 6 fields to calculate the average cell count, and we repeated all the experiments three times.

Western blot

Cells were lysed in RIPA lysis buffer (Beyotime, China) on ice for about 30 minutes after washed 3 times with PBS buffer. After centrifugation, protein was added SDS-PAGE sample loading buffer and heated at 100 °C for 10 minutes. Equal amount protein was added to SDS-PAGE gels and then transferred to PVDF membrane. After blocking in 5% skim milk powder for 60 minutes, the membranes were incubated with primary antibody at 4 °C overnight. The next day, the bands were incubated with incubated secondary antibody after washing with PBST, and then visualized using ChemiDoc™ Touch Imaging System (BIO RAD, China)

RNA isolation and RT-PCR

Total RNA was extracted from cells by using TRIzol reagent (Invitrogen, USA). PrimeScript RT Reagent Kit with gDNA Eraser (RR047A, Japan) was used to synthesize cDNA. Quantitative RT-PCR was performed using SYBR® Premix Ex Taq™ II (RR820A, Takara). The specific primer pairs were as follows: GAPDH Forward:5'-TGCCAAATATGATGACATCAAGAA-3', GAPDH Reverse: 5'-GGAGTGGGTGTCGCTGTTG-3'. TMBIM1 Forward: 5'-CACCCGATGCCCATGAACTA-3', GAPDH Reverse: 5'-CACTTTCCGGTCATCCCACT-3', E-cadherin Forward: 5'-GCGAACTGTTTGCAGAGG-3', E-cadherin Reverse:5'-CAGTGCGTGTCGTGGAGT-3'

Immunohistochemistry

The normal brain and glioma tissues were embedded in paraffin and sectioned. The sections were deparaffinized with xylene for 15 minutes, and then dehydrated with 100%, 95% and 75% ethanol. After washing with PBS buffer for three times, Tris-EDTA antigen repair solution (Servicebio, China) was used for antigen retrieval, and the endogenous peroxidase was removed with 3% H_2O_2. Subsequently, the sections were incubated with primary antibody overnight and HRP-labelled secondary antibody (Service bio, China) for 1 hour the next day. DAB staining solution (Service bio, China) was added to the slices, and the sections were also stained with hematoxylin. The intensity of IHC was divided into: 0, 1, 2, 3 points which represented background staining, faint staining, moderate staining and strong staining respectively. Two independent pathologists examined and scored. If they have different opinions, a third pathologist will be added for scoring. IHC score of 0-1 was defined as low expression group, and score of 2-3 was divided into high expression group.

Autophagosome and autophagic flux detection

For electron microscopic analysis, cells were collected in a 1.5ml EP tube. The cells were fixed with cold fixative solution (Servicebio, China) overnight at 4°C, then embedded, sectioned and observed with transmission electron microscope (TEM) (Hitachi, Japan). Lentivirus lenti-mCherry-EGFP-LC3B (Beyotime, China) was used to monitor the autophagic flux in U251 cells transferred vector or Flag-TMBIM1 plasmid for 24 hours. After infected for 24 hours, cells were fixed with 4% paraformaldehyde, observed and photographed with a confocal fluorescence microscope (FV1200, Olympus).

Intracranial Xenograft model

U87 cell line transfected with sh-TMBIM1 or the control shRNA were digested with trypsin and washed with PBS to a concentration of 1×10^5 cells/μl, and then 4×10^5 cells were injected into the right striatum of 6-week-old Balb/c nude mice. The sh-TMBIM1 mice were randomly divided into two groups (n=10), and CQ (50 mg/kg) or DMSO was injected every 2 days for 10 consecutive times after cell inoculation for 10 days. Tumor volume was monitored by bioluminescence using IVIS 200 Spectrum Imaging System (Caliper Life Sciences, USA) after retroorbital injection of luciferin (150 mg/kg). For survival analysis, we observed the nude mice on a regular basis, and sacrificed the mice when there are severe neurological symptoms and/or significant weight loss (over 20% of body weight). Then the brain tissue of nude mouse was taken out, fixed with polyoxymethylene, and embedded in paraffin. The animal experiment was reviewed and approved by the Animal Ethics Committee of Renmin Hospital of Wuhan University

Statistical analysis

The results were presented as the mean ± standard. Student's t-test was used to test differences between two groups. For three or more groups, one-way analysis of variance (ANOVA) was used, and Tukey's multiple comparisons test was performed to test the differences between groups when analysis of variance was significant. Patients were divided into high and low groups according to upper quartile cutoff ,and Kaplan–Meier survival analysis was used to interstate significance between groups. This analysis was performed through GraphPad prism 8.0 software. SP33.23 software was used to perform univariate and multivariate Cox regression analysis. A p value < 0.05 was considered significant.

Results

TMBIM1 is overexpressed and predicts poor prognosis in GBM

Normalized RNA-Seq data from TCGA and Rembrandt indicated that TMBIM1 expression was elevated in GBM compared to normal brain tissues (NBTs) (**Fig.1A**). Western blot and IHC analysis confirmed that the expression level of TMBIM1 in GBM was higher than that in NBTs (**Fig.1B-1D, Fig.S1A**). Moreover, the level of TMBIM1 was significantly correlated with WHO grade (**Fig.1E**), and TMBIM1 expression was higher inthan LGG (**Fig.1F, Fig.S1B**). Then we tried to explore association between TMBIM1 expression and clinical prognosis. Our independent queue showed that TMBIM1 high expression has a shorter overall survival time in 111 glioma samples by immunohistochemical score (**Fig.S1C**). We obtained the same results in TGCA, CGGA, Rembrandt and Gravendeel databases (Fig,1G). We also found that both

GBM and LGG patients with lower TMBIM1 expression have better overall survival than patients with high TMBIM1 in TCGA, CGGA, Rembrandt and Gravendeel datasets (**Fig.S1D**). We also confirmed these findings using our in-house cohort. Glioma patients with high TMBIM1 expression had worse cancer-specific survival than patients with low TMBIM1(**Fig.S1**). In addition, results of Cox regression analysis showed that TMBIM1 was an independent risk factor for prognosis in glioma both in TCGA and CGGA datasets (**Table S2, S3**). These results suggested that TMBIM1 might be a novel oncogene and prognostic marker in glioma.

TMBIM1 promotes EMT of GBM cells

GBM was subclassified into four subtypes by Verhaak et al, including classical, mesenchymal, neural and proneural(15). GBM patients with a mesenchymal subtype were considered to have significant metastatic ability and poor prognosis. We found that TMBIM1 expression was higher in mesenchymal subtype (**Fig.S2A**). The loss of E-cadherin protein is considered to be the foundation of EMT, which is related to the increase of cell metastasis(6). As we expected, E-cadherin was negatively correlated with TMBIM1 by IHC staining. (**Fig.2A,2B**). and TMBIM1 mRNA expression was positively correlated with mesenchymal markers of SNAIL and VIM based on CGGA dataset (**Fig.2SB**). Then, we investigated whether TMBIM1 could induce the metastatic potential of GBM cells by regulating EMT. The results revealed that knockdown of TMBIM1 expression inhibited cell migration and invasion of U251 and U87 cells (**Fig.2C,2E; Fig.S2C, S2E**), and overexpression of TMBIM1 facilitated cell migration and invasion (**Fig.2D,2F; Fig.S2D, S2F**), as observed by transwell assay. To further confirm that high TMBIM1 expression promotes metastatic potential of glioma cells. We detected the markers of EMT in GBM tissues by Western blot. The results demonstrated that the protein level of TMBIM1 was negatively correlated with E-cadherin, and positively correlated with N-cadherin and SNAIL (**Fig.2G**). We also found that TMBIM1 knockdown increased the expression of epithelial marker E-cadherin and decreased the expression of mesenchymal markers, such as Vimentin, N-cadherin and SNAIL. Furthermore, overexpression of TMBIM1 resulted in an increase in Vimentin, N-cadherin and SNAIL, and a slight decrease of E-cadherin (**Fig.2H**). These results reveal that TMBIM1 can induce EMT and promote metastasis of glioma cells.

TMBIM1 stimulates cell autophagy in GBM cells

Previous studies have shown that TMBIM1 is a lysosomal transmembrane protein(11), and we speculate that TMBIM1 may be involved in the regulation of autophagy. We found that TMBIM1 expression positively correlated with Beclin1 by IHC staining of tissue microarray **(Fig.3A,3B**). Western blot analysis indicated that TMBIM1 expression positively correlated with Beclin1, and negatively correlated with P62 in GBM tissues (**Fig.3C**). In addition, TMBIM1 overexpression enhanced the expression of Beclin1 and LC3-II in U87 and U251 cells, and decreased the level of P62. We also found that TMBIM1 knockdown decreased the expression of Beclin1 and LC3-II, and increased the expression of P62 in U87 and U251clles (**Fig.3D**). We observed increase in the number of autophagosomes with the features of double-membraned vacuolar structures containing cellular components under electron microscopy (**Fig.3E,3F**). In order to investigate and monitor the autophagic flux, we transfected EGFP-mCherry-LC3B

lentivirus into U251 cells which was transfected with vector or Flag-TMBIM1 plasmid. The yellow and red puncta represented autophagosomes and autolysosomes, respectively. The intensity of EGFP in cells transfected with vector was similar to that of mCherry, most of the punctates were yellow. However, in TMBIM1 overexpression cells, only weak signals of EGFP were observed and most of the punctates were nearly red (**Fig.3G,3H**), indicating that autophagy flux was unobstructed. These data suggest that that TMBIM1 stimulates cell autophagy in GBM cells.

Inhibition of autophagy reverses EMT induced by TMBIM1 in GBM cells

In order to investigate the underlying mechanism of autophagy regulating EMT, we used 3-Methyladenine (3-MA) and CQ to treat GBM cells. Transwell assays showed that inhibition of autophagy by 3-MA and chloroquine (CQ) reduced cell migration and invasion in TMBIM1 overexpression cells (**Fig4A-4D, Fig.S3A-3D**). Moreover, inhibition of autophagy also affected the expression of EMT-related proteins in TMBIM1 knockdown and overexpression cells. Treatment with 3-MA and CQ enhanced the E-cadherin expression, and reduced the expression of Vimentin, SNAIL and N-cadherin in TMBIM1 knockdown and overexpression in U251 cells (**Fig.4E**). These results revelated that suppression of autophagy reverses EMT induced by TMBIM1 in GBM cells.

TMBIM1 accelerates the degradation of E-cadherin through lysosomal pathway

Previous studies have suggested that the selective degradation of specific EMT proteins seems to be the main molecular mechanism for autophagy to mediate EMT(16).Since the loss of E-cadherin expression is considered the foundation of EMT, we hypothesized that TMBIM1 may mediate the EMT process in GBM by affecting the synthesis or degradation of E-cadherin. Therefore, we investigated the protein and mRNA expression of TMBIM1 after transfection with Flag-TMBIM1 plasmid at a specific concentration, and found that TMBIM1 overexpression reduced the protein expression of E-cadherin but did not affect the mRNA level (**Fig.5A-5C**). Therefore, we speculated that TMBIM1 may reduce the protein expression of E-cadherin by promoting its degradation. Cycloheximide (CHX), a protein synthesis inhibitor, was added to U251 cells, and it was showed that the expression of E-cadherin decreased after treated with CHX for a certain time. However, the expression of E-cadherin was restored in TMBIM1 knockdown cells, indicating that TMBIM1 knockdown can inhibit the degradation of E-cadherin (**Fig.5D,5E**). Lysosomal pathway and proteasome pathway are the main pathways for protein degradation(6). We then investigated the pathway of E-cadherin degradation induced by TMBIM1. Our results demonstrated that chloroquine (CQ), which inhibits the function of lysosome, delayed the degradation of E-cadherin in TMBIM1 knockdown cells, but MG132, which inhibits the proteasome, did not affected E-cadherin's degradation (**Fig.5F-5I**). Therefore, our results reveal that TMBIM1 accelerates the degradation of E-cadherin by lysosomal pathway.

TMBIM1 stimulates cell autophagy by AMPK/mTOR/ULK1 axis in GBM cells

AMPK, a regulator of cellular and organismal metabolism, is also involved in the regulation of autophagy(17).In order to investigated whether AMPK pathway is related to TMBIM1 induced autophagy

in GBM cells. We first detected P-AMPKα Thr172 and P-ULK1 Ser317 by in GBM tissues by Western blot. The results showed that P-AMPKα Thr172 and P-ULK1 Ser317 were positively correlated with TMBIM1 expression (**Fig.6A**). In addition, TMBIM1 knockdown decreased the expression of P-AMPKα Thr172 and P-ULK1 Ser317 in U87 and U251 cells, and upregulated the expression of P-mTOR at Ser2448. We also found that TMBIM1 overexpression enhanced P-AMPKα Thr172 and P-ULK1 Ser317 expression, and reduced the level of P-mTOR at Ser2448, in U87 and U251 cells. However, TMBIM1 had no effect on AMPK and mTOR (**Fig.6B**). These results suggest that TMBIM1 stimulates cell autophagy by activating AMPK pathways in GBM cells. To further confirm our hypothesis, Compound C, also known as BML-275, which is an effective AMPK inhibitor, was employed to treat TMBIM1 knockdown /overexpression cells. Consistent with our preconception, Western blot analysis showed that autophagy was suppressed after inhibition of AMPK pathway by Compound C (**Fig.6C**). Furthermore, we observed that the number of autophagosomes and autolysosomes in TMBIM1 overexpression cells was decreased after Compound C treatment (**Fig.6D-6G**).

TMBIM1 knockdown suppresses autophagy and EMT in intracranial xenograft model

In order to further investigate the effect of TMBIM1 on autophagy and EMT, we constructed an intracranial xenograft model. It demonstrated that the tumor volume in TMBIM1 knockdown mice was smaller than of control mice by whole-body bioluminescence and HE staining. Interestingly, the tumor volume of TMBIM1 knockdown mice decreased after CQ treatment (**Fig 7B,7C**). As expected, the survival time of TMBIM1 knockdown mice was longer than that of control group. Surprisingly, the survival time of mice in the TMBIM1 knockdown group was further prolonged after CQ treatment (**Fig.7A**). IHC staining showed that TMBIM1 knockdown resulted in decreased expression of P-AMPKα, P-ULK1 and Beclin1, and increased expression of P-mTOR and P62 (**Fig 7D**). Moreover, TMBIM1 knockdown decreased the expression of N-cadherin, Vimentin and SNAIL, and enhanced E-cadherin expression. We also found that CQ treated mice in TMBIM1 knockdown group further increased E-cadherin expression, and decreased the expression of N-cadherin, Vimentin and SNAIL (**Fig.7E**), which was consistent with in vitro results. As shown in figure 8, TMBIM1 serves as lysosomal transmembrane protein to promote EMT by stimulating autophagic degradation of E-cadherin via AMPK/mTOR/ULK1 in GBM cells. These results suggested that inhibition of TMBIM1 induced autophagy may be an underlying strategy for the prevention and treatment of glioma.

Discussion

TMBIM1 is identified as a lysosomal transmembrane protein which inhibits adipogenesis and improves obesity-related metabolic disease by reducing the stability of PPARγ(12). In addition, TMBIM1 is involved in the prevention of non-alcoholic steatohepatitis, metabolic syndrome and cardiomyopathy (11, 12). However, few studies have linked TMBIM1 with the formation and progression of human tumors. Our study found for the first time that the expression of TMBIM1 in GBM tissues was significantly higher than that in normal brain tissues. The prognosis of GBM and LGG patients with low TMBIM1 expression was better. Furthermore, TMBIM1 knockdown mice had a longer survival time in intracranial xenograft model.

Cox regression analysis based on TCGA and CGGA datasets indicated that TMBIM1 was an independent risk factor for human glioma.

Tumor metastasis is a critical step in cancer progression, indicating a more advanced stage and worse prognosis, which involved the essential factor of EMT (18). In our research, we demonstrated that TMBIM1 induced EMT and autophagy in GBM cells. Recent research has shown that there is a complex link between EMT and autophagy. More and more evidence indicate that autophagy plays a dual role in the regulation of EMT (5). It has been reported that autophagy deficiency inhibits TWIST1 degradation through autophagosomes and promotes EMT, tumor growth and metastasis in mice(19). Sahib Zada found that autophagy-dependent degradation of SNAIL suppressed EMT and cell metastasis (20). Myriam Catalano reported that autophagy reversed EMT of glioblastoma cells and inhibited cell invasion and migration(21). These studies indicate that autophagy plays a negative role in EMT. However, autophagy seems to be an active regulator of EMT. A recent study showed that SPHK1 induced EMT by promoting autophagic degradation of E-cadherin in HepG2 cells (6). Similarly, another study revealed that SIRT1 accelerated tumor metastasis by stimulating autophagic degradation of E-cadherin (22). E-cadherin is identified as a key protein involved in EMT, and its expression level is closely related to EMT and tumor metastasis(23).In our study, we demonstrated that TMBIM1 promoted EMT by stimulating autophagic degradation of E-cadherin in GBM cells, while inhibition of autophagy by CQ and 3-MA reversed EMT and impaired the migration and invasion of GBM cells. Therefore, we speculated that the function of autophagy in regulating EMT mainly depends on the tissue/cell types and tumor development stage. E-cadherin loss is considered as the foundation of the EMT, and our research suggested that TMBIM1 induced the degradation of E-cadherin by promoting autophagy. Hong L and Ting Sun reported that E-cadherin was degraded by the lysosomal pathway (6, 22). Su Mi at al. demonstrated that autophagy was involved in the regulation of endocytic lysosomal pathway (24). Hongliang Li identified TMBIM1 as a previously unknown regulator of the multivesicular body (MVB)-lysosomal pathway, and prevented non-alcoholic steatohepatitis (NASH) in mice and monkeys by promoting lysosomal degradation of TLR4(11). Our finding showed that TMBIM1 decreased the protein expression of E-cadherin, but did not affect the mRNA level, and the suppression of autophagy by CQ delayed the lysosomal degradation of E-cadherin. These results reveal that TMBIM1 promotes EMT by stimulating autophagy-linked lysosomal degradation of E-cadherin.

AMPK is an important regulator involved in energy metabolism, cell growth and autophagy in cells. Previous finding have shown that AMPK mediates autophagy by regulating the phosphorylation of ULK1, and the isolation membrane formed by activated ULK1 is considered to be the first step of autophagosomes formation(25). AMPK activation of ULK1 mainly involves two mechanisms, one of which is the direct activation of phosphorylation of ULK1 by AMPK at Ser317, Ser777 and Ser555. The other is indirectly activated by suppression of mTOR phosphorylation (26). In this study, we found that the expression of P-AMPK and P-ULK1 in TMBIM1 overexpression group were increased, while the level of P-mTOR was dramatically decreased both in vivo and in vitro. In addition, our data demonstrated that cell autophagy was obviously inhibited by suppressing AMPK pathway with compound C treatment in

TMBIM1 knockdown and overexpression cells. These results suggest that TMBIM1 mediated cell autophagy via AMPK/ mTOR/ULK1 signaling pathway.

Conclusions

In conclusion, this study suggests that TMBIM1 promotes EMT by accelerating autophagic degradation of E-cadherin via AMPK/ mTOR/ULK1 signaling pathway in GBM. TMBIM1 is a novel prognostic factor and therapeutic target in GBM. Our findings reveal that blockage of TMBIM1 activity to suppression autophagy may be an underlying strategy for the prevention and treatment of human glioma.

Abbreviations

GBM: Glioblastoma

TMBIM1: Transmembrane BAX inhibitor motif-containing 1

EMT: Epithelial-mesenchymal transition

LGG: Lower-grade glioma

CQ: Chloroquine

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Renmin Hospital of Wuhan University and the Animal Ethics Committee of Renmin Hospital of Wuhan University.

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Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

Author contributions

LG, J-HL, GD and Q-XC conceived and designed the study. LG, J-HL, YL, S-QZ, Y-QT, J-AY, LW and F-EY performed experiments. LG, ZY, J-AY, J-YC, S-AT, B-HL, D-FT analyzed data. LG, DG and J-HL wrote the manuscript. All authors contributed to the article and approved the submitted version.

Consent for publication

All the listed authors have participated in the study, and approved the submitted manuscript.

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Figures

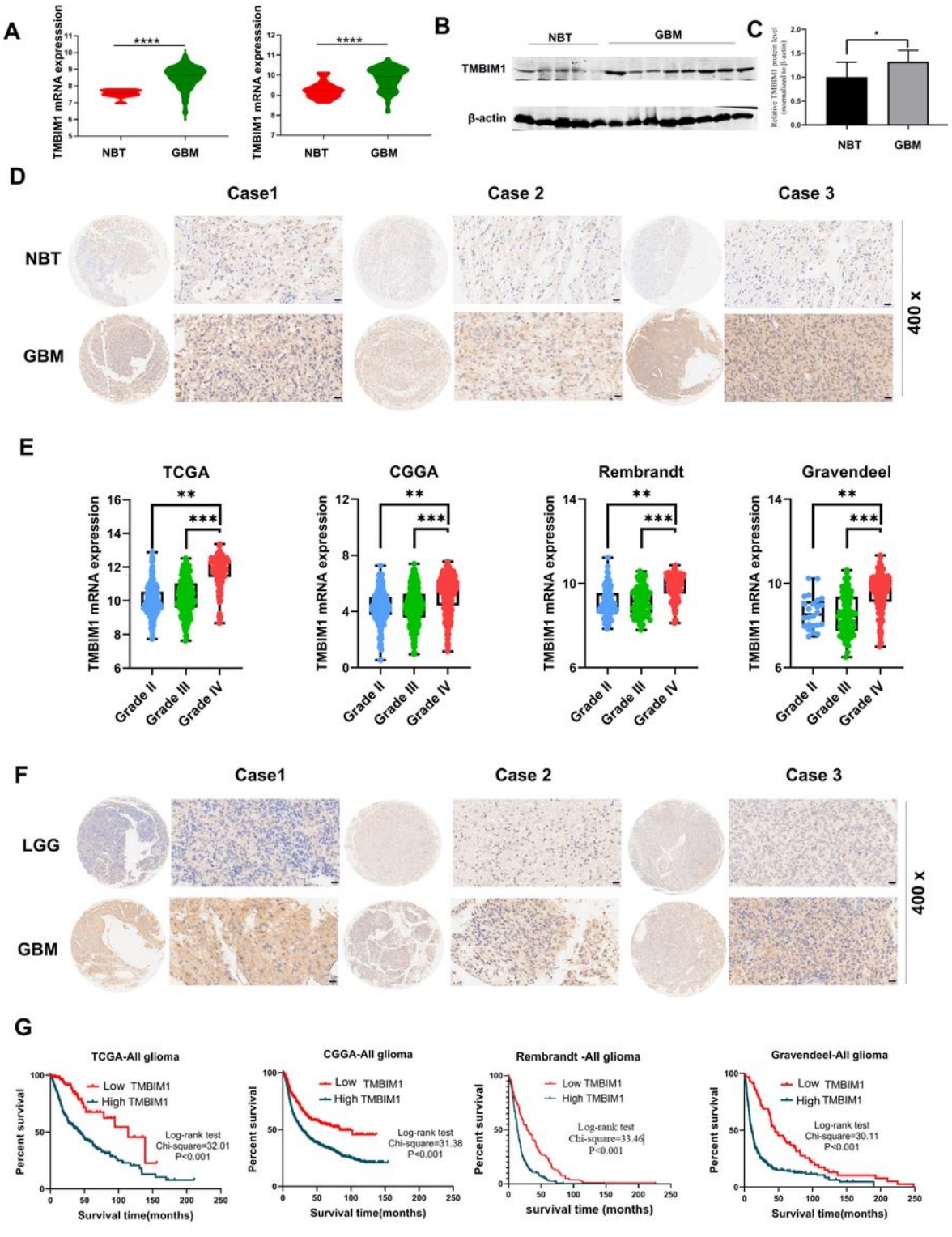

Figure 1

TMBIM1 is overexpressed and predicts poor prognosis in GBM. (A) An analysis of TMBIM1 mRNA expression in normal brain tissues (NBT) and GBM from TCGA and Rembrandt datasets. (B, C) TMBIM1 expression in normal brain tissues and GBM tissues. (D) Representative images of IHC staining of TMBIM1 in NBT and GBM tissues. (E) Expression level of TMBIM1 mRNA in gliomas of different WHO grades based on TCGA, CGGA, Rembrandt and Gravendeel datasets. (F) Representative images of IHC

staining of TMBIM1 in LGG and GBM tissues. (G) Kaplan-Meier survival analysis for TMBIM1 expression in all gliomas based on TCGA, CGGA, Gravendeel and Rembrandt datasets. * P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Ctrl: Control

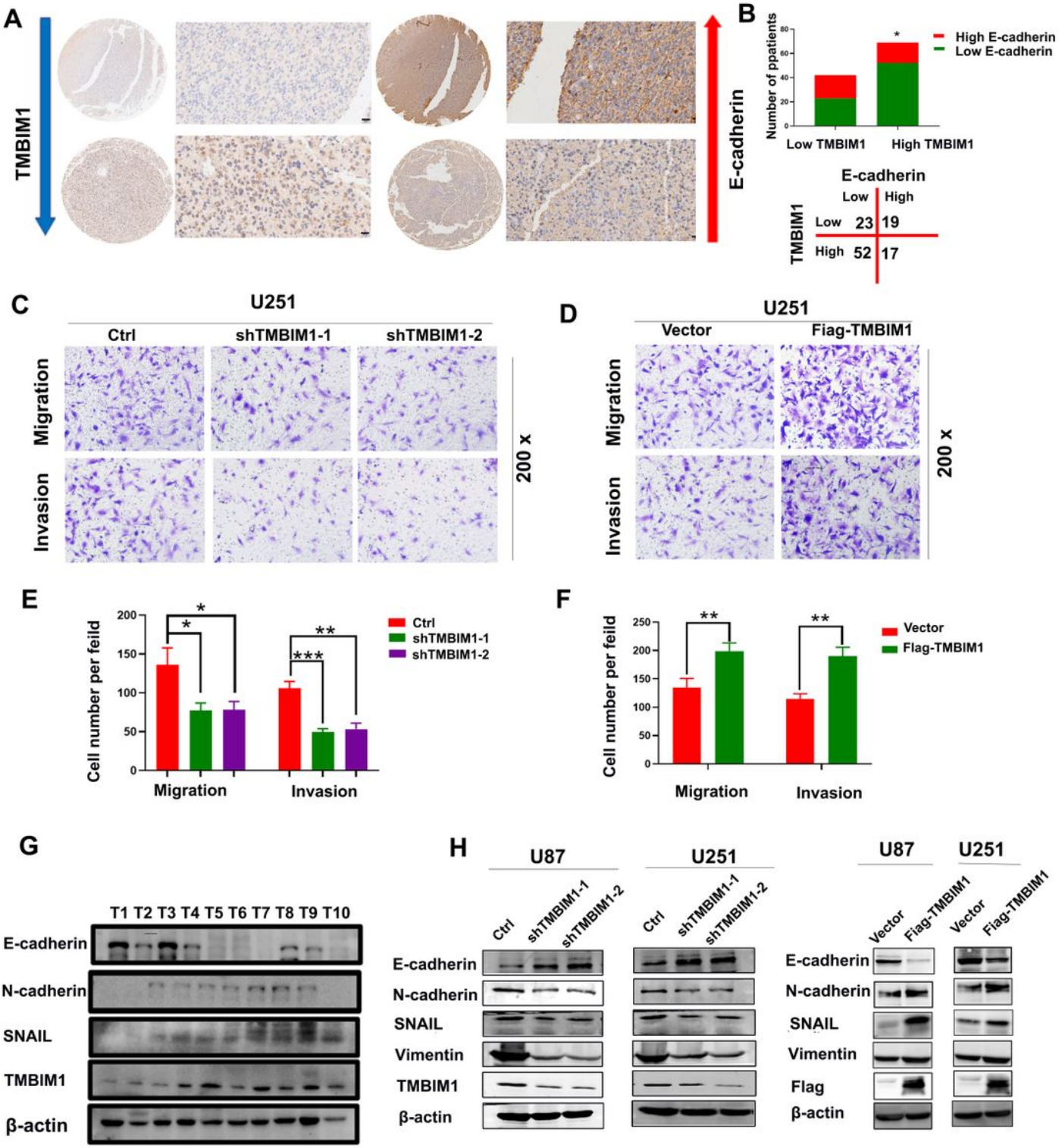

Figure 2

TMBIM1 promotes the EMT and metastatic potential in GBM cells. (A) Representative images of IHC staining showed TMBIM1 expression negatively correlates with E-cadherin in the same tissues. (B) The expression of TMBIM1 and E-cadherin in 111 glioma samples was analyzed. (C). TMBIM1 knockdown inhibited cell migration and invasion in U251 cells. (D) TMBIM1 overexpression enhanced cell migration and invasion in U251 cells. (E, F) Statistical analysis of cells per field in (C) and (D). (G) TMBIM1 expression was positively associated with N-cadherin and SNAIL expression, and negatively with E-cadherin expression in GBM tissues. (H) Western blot was used to detect the levels of epithelial and mesenchymal markers in U251 and U87 cells after TMBIM1 knockdown or overexpression. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Ctrl: Control

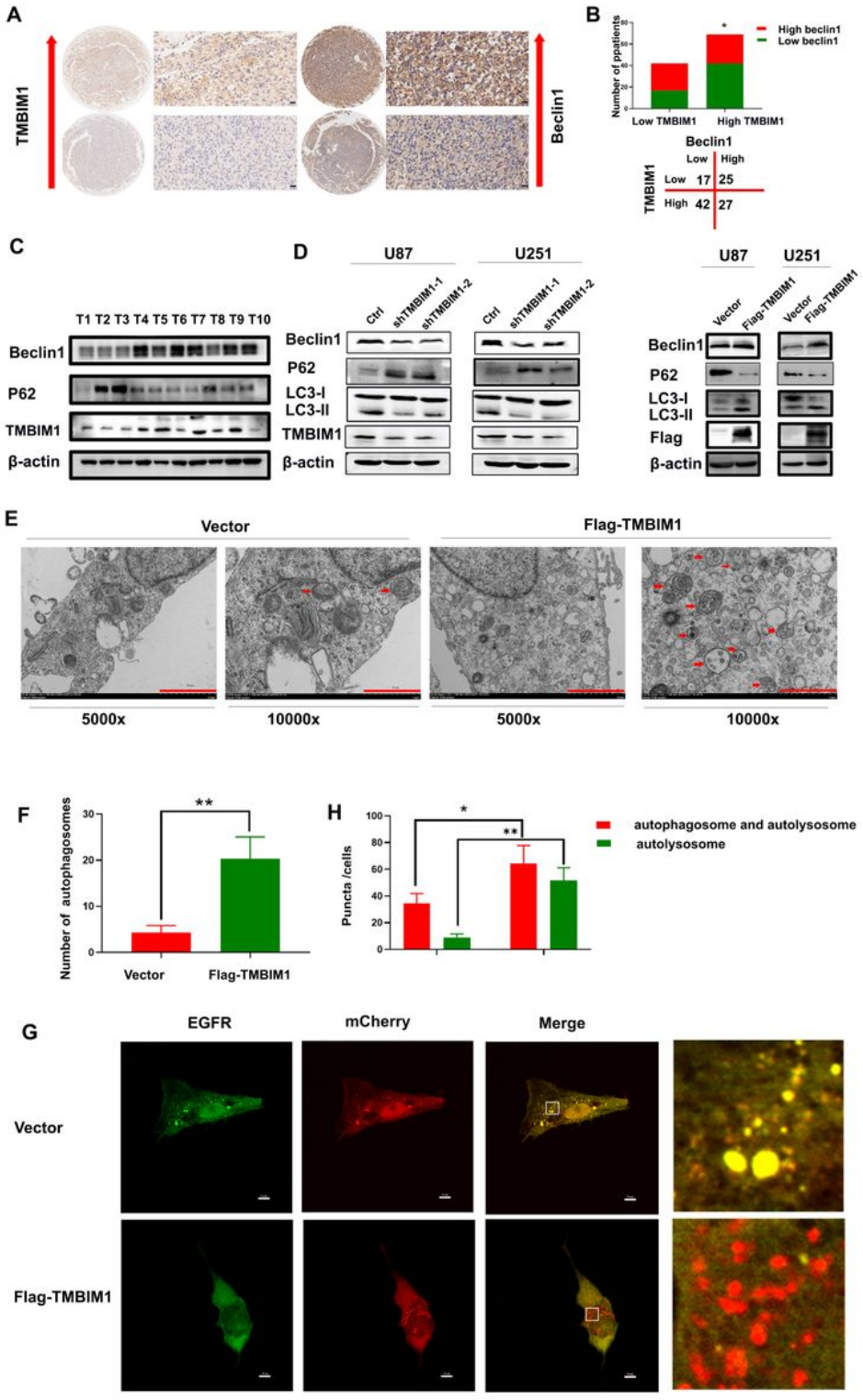

Figure 3

TMBIM1 stimulates cell autophagy in GBM cells. (A) Representative images of IHC staining showed TMBIM1 expression positively correlates with Beclin1 in the same tissues. (B) The expression of TMBIM1 and Beclin1 in 111 glioma samples was analyzed. (C)TMBIM1 expression was positively with Beclin1, and negatively with P62 in GBM tissues. (D) Western blot analysis showed that the expression of autophagy-related proteins was upregulated/downregulated in TMBIM1 overexpression/knockdown cells.

(E-F) TMBIM1 overexpression increased the number of f autophagosomes in U251 cells. Electron microscopy observed the typical autolysosomes (indicated by the red arrowhead). (G-H) Lenti-mCherry-EGFP-LC3B lentivirus was used to monitor the autophagic flux in U251 cells after transfected with vector or Flag-SIRT1 plasmid. The cells were observed autophagosome (yellow puncta) and autolysosome (red puncta) by confocal microscopy. * P<0.05, ** P<0.01, ***P<0.001

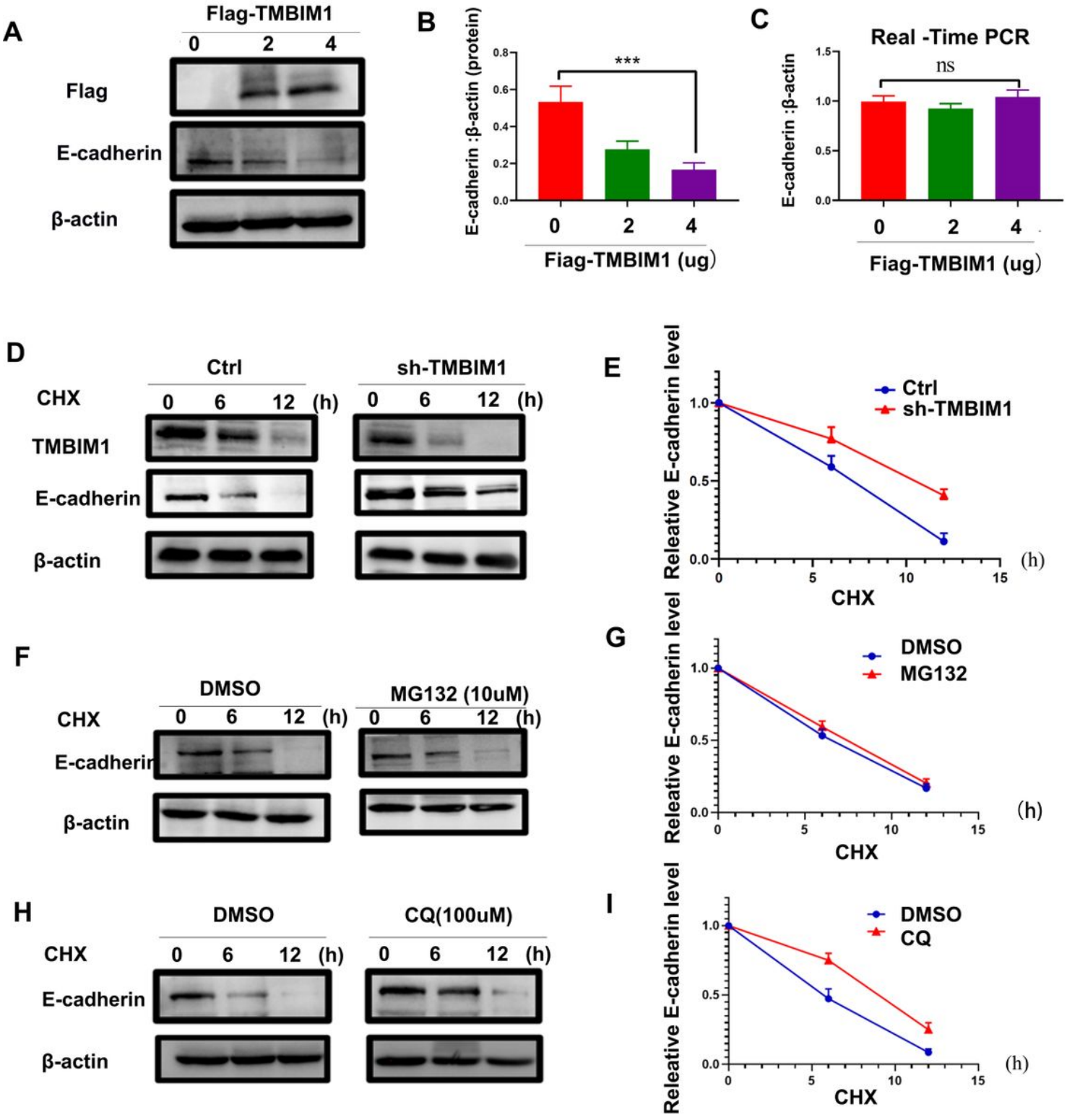

Figure 4

Inhibition of autophagy reverses EMT induced by TMBIM1 in GBM cells (A, C) Inhibition of autophagy by CQ and 3-MA reduced cell migration and invasion in TMBIM1 knockdown/overexpression U251 cells. (B, D) Statistical analysis of cells per field in (A) and (C). (E) The inhibition of autophagy recovered the expression of epithelial markers and mesenchymal markers in TMBIM1 overexpression cells, and further increased E-cadherin expression and decreased the expression of N-cadherin, vimentin and SNAIL in TMBIM1 knockdown cells. * P<0.05, ** P<0.01, ***P<0.001

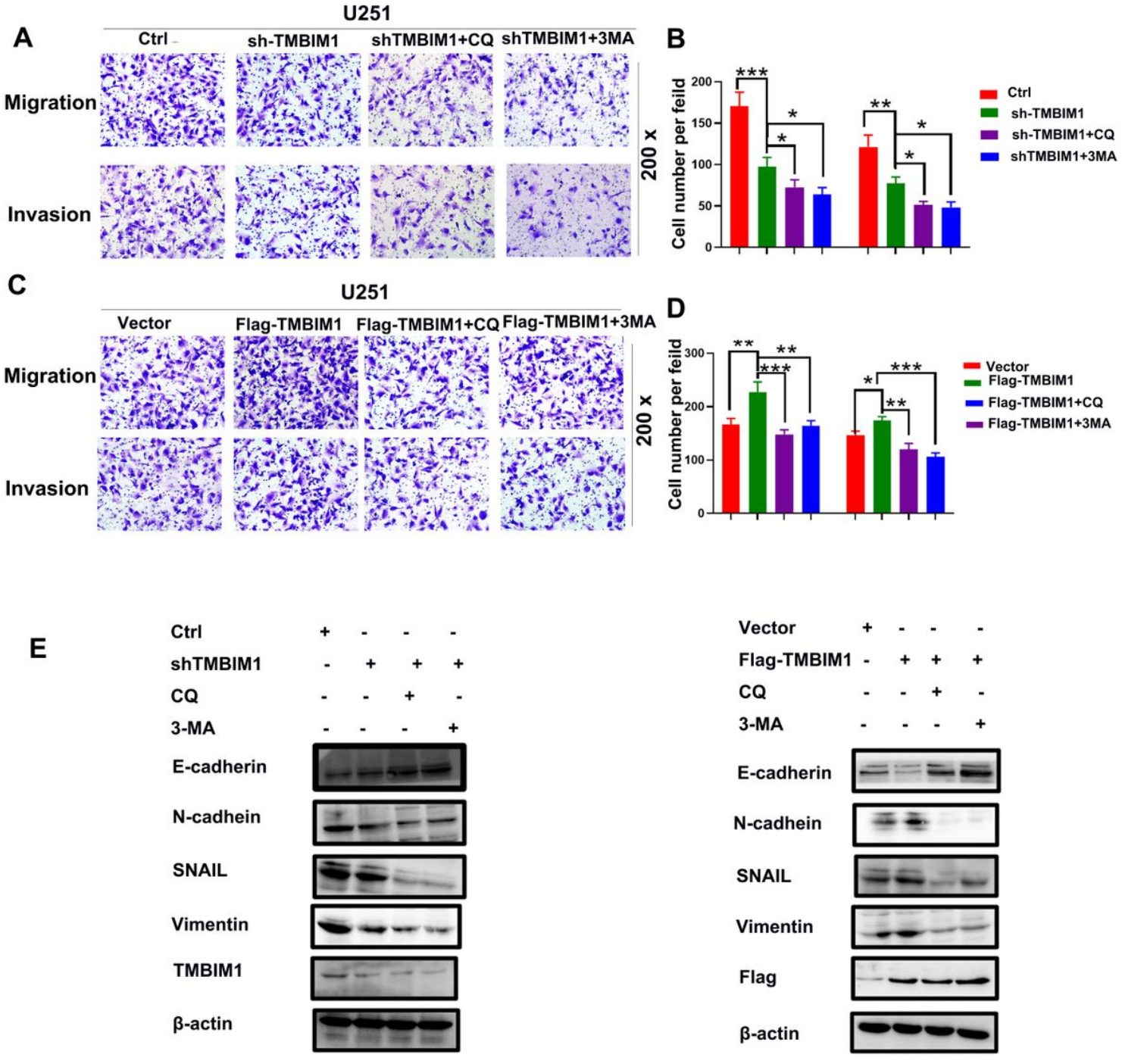

Figure 5

TMBIM1 accelerated the degradation of E-cadherin through the lysosomal pathway. (A-C) U251 cells were transfected with the indicated concentrations of Flag-TMBIM1 for 48h, and TMBIM1 decreased the expression of E-cadherin protein, but not affected the E-cadherin mRNA level. (D-E) U251-shTMBIM1 and control cells were treated with CHX (10 uM) for the indicated times, and TMBIM1 knockdown inhibited E-cadherin protein degradation. (F, G) CHX (10 uM) were added to U251-shTMBIM1 cells for the indicated times after treated with MG132 (10 uM) for 2 hours. Western blot suggested that TMBIM1 did not affect the proteasomal degradation of E-cadherin. (H, I) CHX (10 uM) were added to U251-shTMBIM1 cells for the indicated times after treated with CQ (100 uM) for 24 hours. Western blot indicated that TMBIM1 accelerated the lysosomal degradation of E-cadherin. ***P<0.001, Ctrl: Control, CHX cycloheximide; CQ chloroquine; ns nonsignificant

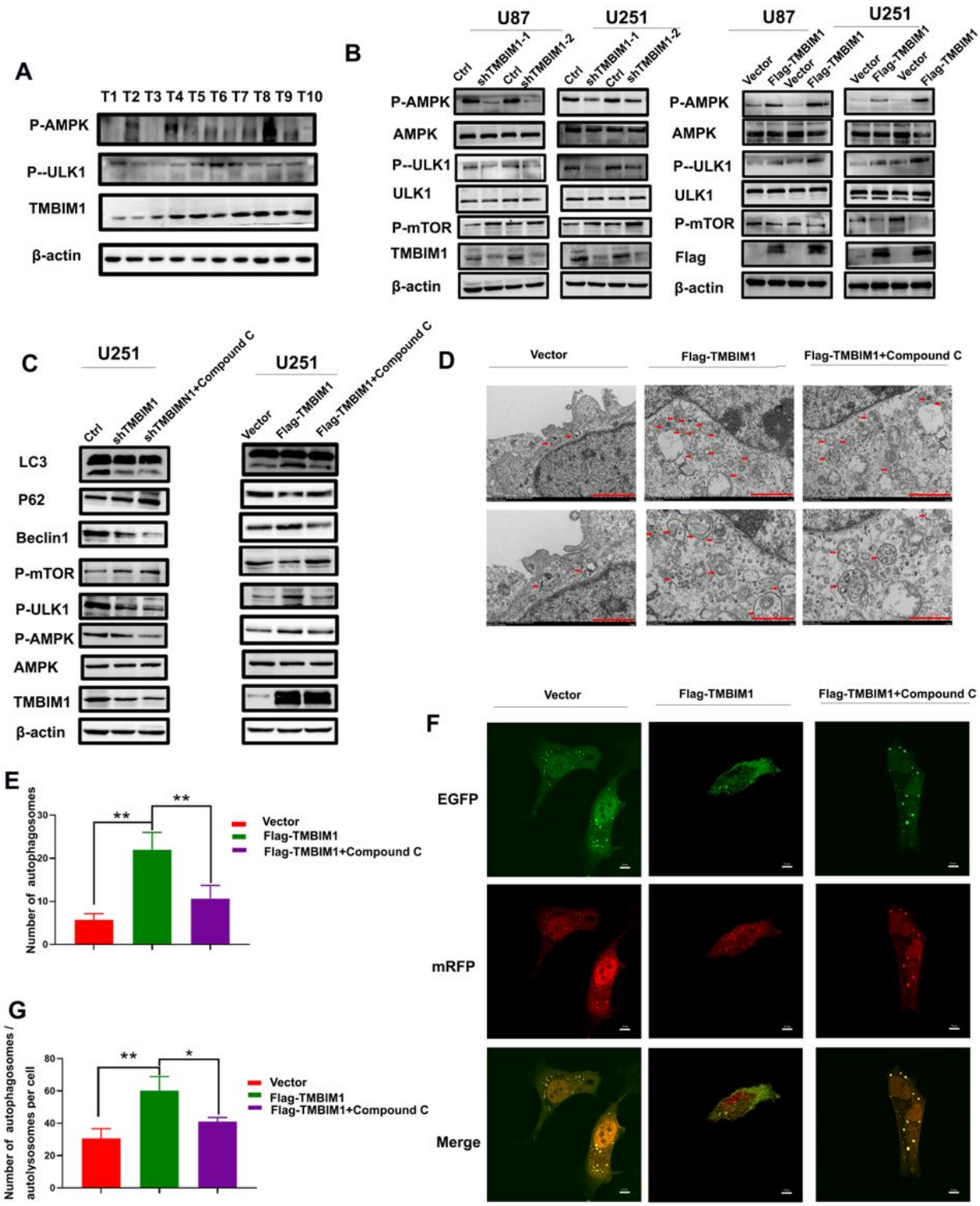

Figure 6

TMBIM1 stimulates cell autophagy by mediating AMPK/mTOR/ULK1 in GBM cells (A) TMBIM1 expression was positively with P-AMPK and P-ULK1 in human GBM tissues. (B) Western blot detected the expression of P-AMPKα Thr172, P-ULK1 Ser317, P-mTOR Ser2448, AMPK and ULK1 in TMBIM1 knockdown /overexpression GBM cells. (C) Inhibition of AMPK pathway by Compound C treatment, and western blot investigated autophagy-related protein and key protein of AMPK pathway. (D, E) Electron

microscopy observed that the number of autolysosomes after Compound C inhibited the AMPK pathway. (F, G) Confocal microscopy observation revealed that the number of autophagosome (yellow puncta) and autolysosome (red puncta) was decreased after Compound C treatment in TMBIM1 overexpression U251 cells. * P<0.05, ** P<0.01

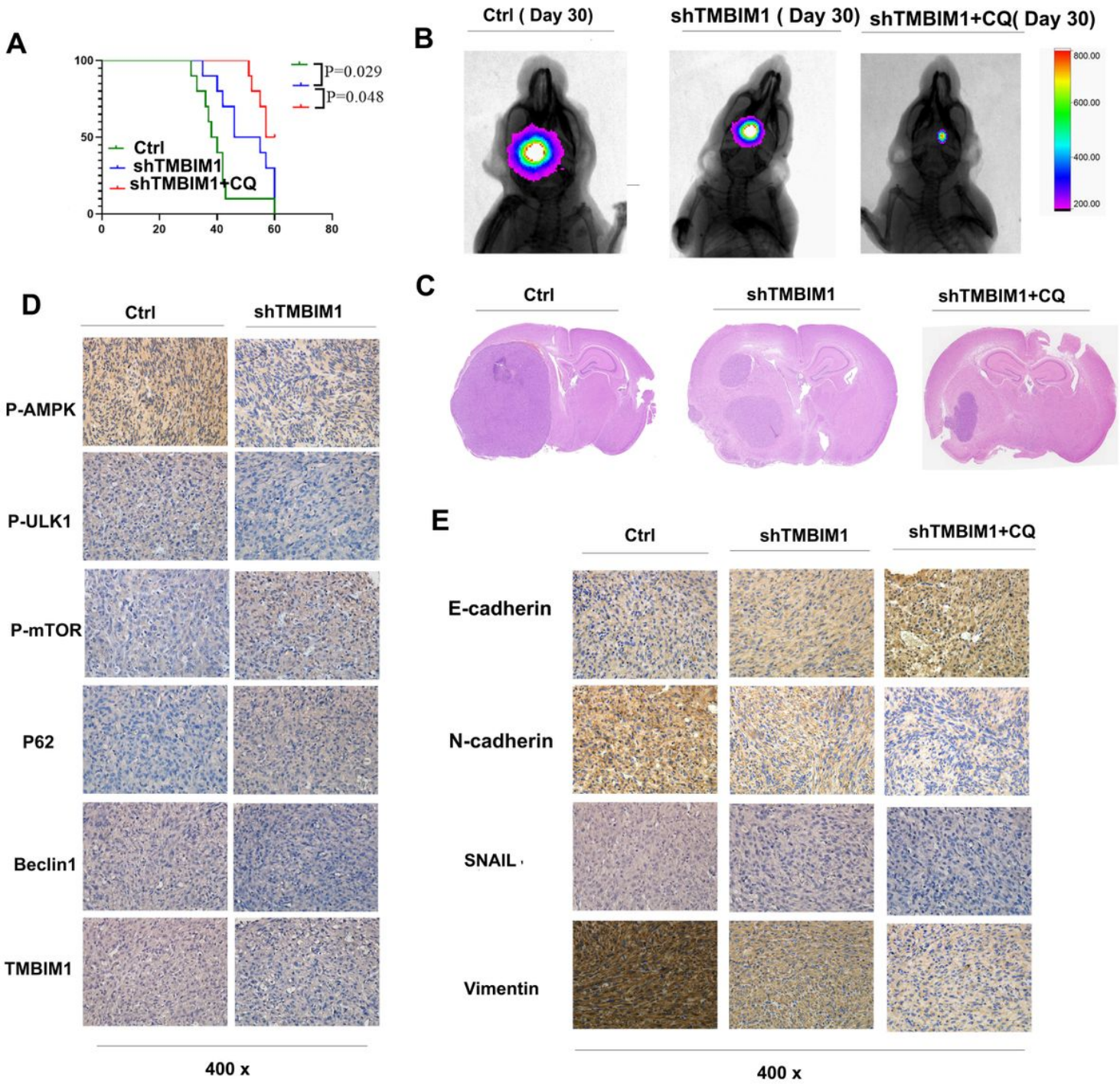

Figure 7

TMBIM1 knockdown suppresses autophagy and EMT in intracranial xenograft model (A)Mouse survival is shown by Kaplan-Meier curves. (B) In vivo bioluminescent imaging of nude mice at days of 30 cell

inoculation. (C) HE staining. (D) IHC staining of P-AMPK(Thr172), P-ULK1(Ser317), P-mTOR (Ser2448), Beclin1, P62 and TMBIM1. (E)IHC staining of E-cadherin, N-cadherin, Vimentin and SNAIL.

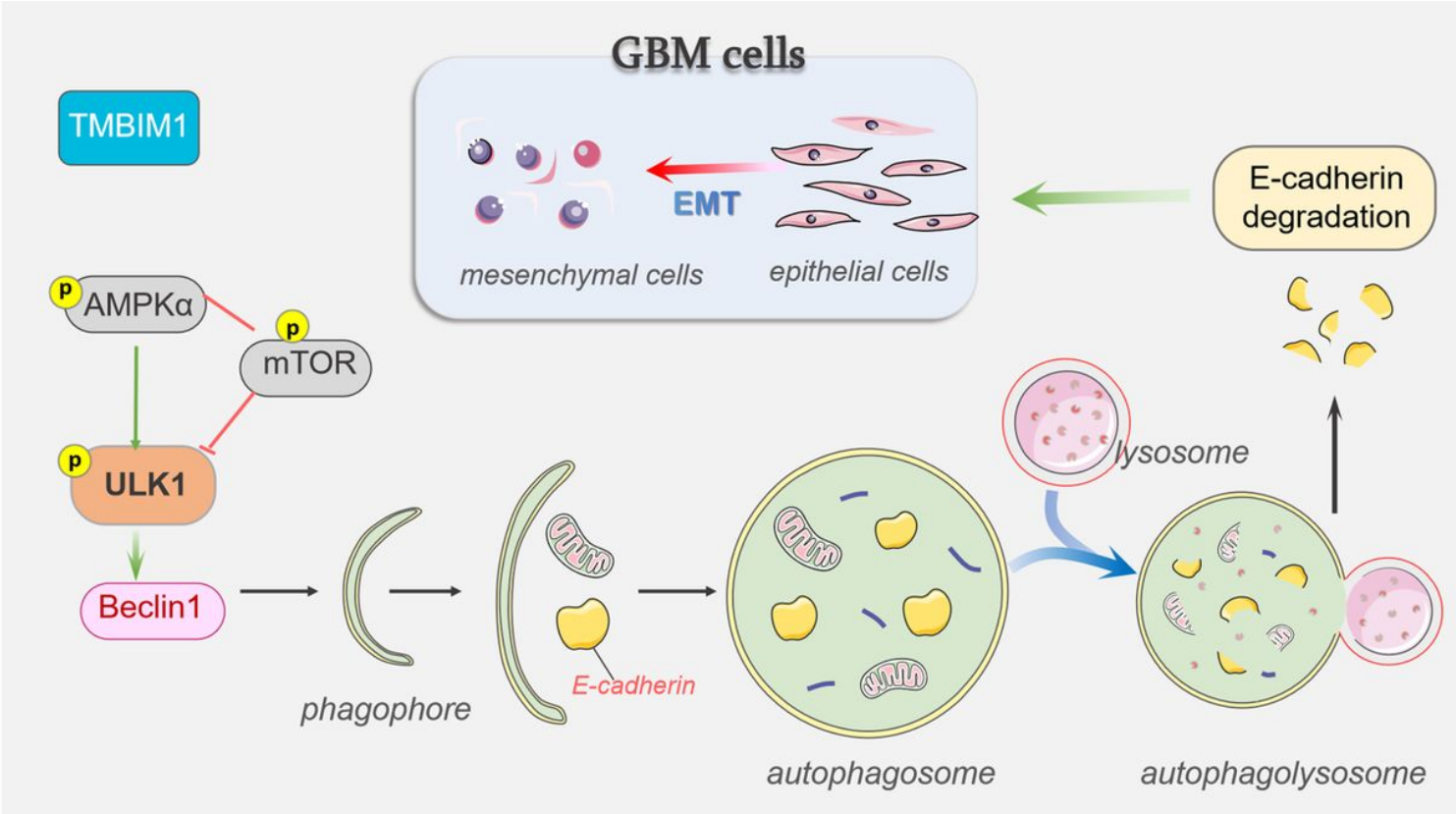

Figure 8

Schematic diagram of the mechanism of TMBIM1-mediated autophagic degradation of E-cadherin which stimulates the EMT in GBM cells

Supplementary Files

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