

Erianin facilitates pyroptosis in endometrial cancer via targeting m6A reader YTHDF1

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Abstract

Background

Endometrial cancer (EC) is a gynecological malignancy that originates from the endometrial epithelium and has a poor prognosis when advanced, recurrent, or metastatic. The limited therapeutic efficacy and severe adverse effects of conventional chemotherapy in advanced EC highlight the urgent need to develop more effective therapeutic drugs. Accumulating clinical evidence has revealed that natural compounds possess pharmacological advantages, including low toxicity and multi-target mechanisms. Erianin is a natural small-molecule compound isolated from *Dendrobium chrysotoxum Lindl* that has multiple pharmacological effects. However, the effects of erianin on EC have not been confirmed and its anticancer mechanisms remain unclear.

Methods

Erianin was identified as a potent natural compound against EC through compound library screening. The CCK-8 assays, colony formation assays, Edu experiments, and Live/Dead cell staining assays were utilized to analyze the anti-proliferative activity of erianin. Morphological characteristics, transmission electron microscopy, LDH release assays, and Western blot assays were used to evaluate the activation of pyroptosis. A transcriptome sequencing analysis was performed to identify potential mechanism of erianin. We also synthesized biotin-erianin and used a 20k human proteome microarray to identify its direct targets. Molecular docking and CETSA were used to investigate whether erianin binds to YTHDF1. To evaluate the in vivo therapeutic potential of erianin, we established an EC xenograft model and performed mechanistic investigations incorporating H&E staining, IHC staining, and Western blot assays.

Results

Our results demonstrated that erianin inhibited the cell proliferation of EC cells and promoted pyroptosis through the caspase-3/GSDME pathway. Mechanistically, we established a crucial role for FOXM1/RRM2-mediated DNA damage in erianin-induced pyroptosis. Protein microarray revealed that erianin-biotin directly targeted m6A reader YTHDF1. We further confirmed that erianin bound to YTHDF1 by molecular docking and CETSA. Molecular studies indicated erianin inhibited YTHDF1 recognized m6A-modified FOXM1 and promoted FOXM1 mRNA degradation, which leads to DNA damage and caspase-3-mediated GSDME cleavage. Erianin also significantly inhibited EC tumor growth in EC models.

Conclusion

Erianin directly targets YTHDF1 to suppress the FOXM1/RRM2 axis and consequently promotes caspase-3/GSDME-dependent pyroptosis in EC cells. Our findings provide a new strategy for further

Graphical abstract

1. Background

Endometrial cancer (EC) comprises a group of malignant epithelial tumors that arise in the endometrium and mostly consists of adenocarcinomas originating from the endometrial glands(1, 2). The global morbidity and mortality of EC is increasing rapidly(3). While the outcomes of early-stage EC can be favorable after surgical intervention, the patients with stage III and IV EC have low five-year survival rates(4). Although adjuvant chemotherapy is typically prescribed to manage recurrent and late-stage EC, its efficacy is limited by side effects and resistance. Therefore, novel targeted drugs are essential for improving patient outcomes.

Pyroptosis is an inflammatory mode of programmed cell death characterized by cellular membrane perforation and continuous swelling until membranes rupture and release cellular contents, which triggers a potent inflammatory response(5, 6). The significant roles of pyroptosis in antitumor treatments, including chemotherapy, targeted therapy, and immunotherapy, have been previously verified(7–9). Some natural low-toxicity small-molecule compounds can trigger pyroptosis by activating the caspase-3-dependent cleavage of gasdermin E (GSDME)(10). The organic compound triptolide induces GSDME-dependent pyroptosis by inhibiting mitochondrial hexokinase II in head and neck cancer(11). Therefore, exploring natural compounds that target pyroptosis is a novel anticancer strategy.

The most prevalent RNA modification in eukaryotes is N6-methyladenosine (m6A), which regulates all stages of RNA metabolism to modulate mRNA transcript levels(12, 13). A “writer” protein complex consisting of methyltransferase-like 3/14 (METTL3/14) and WTAP17 catalyzes m6A, and then the demethylases, fat mass and obesity-associated protein (FTO) and alkylation repair homolog protein 5 (ALKBH5), erase m6A from mRNA(14). The m6A “readers”, YTH domain family proteins (YTHDF1-3) and insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs), recognize and regulate m6A-modified transcripts(15). These readers are implicated in several biological processes and are closely associated with tumor progression. Ovarian cancer progression is promoted by YTHDF1, which enhances eukaryotic translation EIF3C(16). Cervical cancer proliferation is promoted by METTL3 via YTHDF1/HK2-driven aerobic glycolysis(17). These findings suggest that YTHDF1 has potential as a therapeutic target in cancer.

Erianin, which is isolated from *Dendrobium chrysotoxum* Lindl., has anticancer effects. For example, erianin inhibits tumor progression by suppressing PI3K/Akt/mTOR and JNK/c-Jun signaling(18, 19) and directly inhibits c-Raf and MEK2 activity to suppress MAPK signaling(20). These findings indicate that erianin could be an effective anticancer drug with significant development prospects. However, its effects on EC tumor growth and the underlying mechanism and targets remain elusive. Our findings showed that erianin directly binds to YTHDF1 and induces the ubiquitination and degradation of YTHDF1,

inhibits YTHDF1 from recognizing m6A-modified FOXM1, promotes the degradation of FOXM1 mRNA, inhibits RRM2-regulated nucleotide metabolism, and leads to DNA damage, which activates caspase3/GSDME-dependent pyroptosis.

2. MATERIALS AND METHODS

2.1. Cells and reagents

HEC-1-B, Ishikawa, and KLE cells (Zhongqiao, Shanghai, China) authenticated by short tandem repeat (STR) analysis were grown in a temperature-controlled incubator at 37°C under a 5% CO₂ atmosphere. We cultured these cells in F12 and MEM media (Servicebio, Wuhan, China) supplemented with 10% serum (Gibco, MD, USA) and 1% streptomycin and penicillin (Servicebio, Wuhan, China). Natural compounds and erianin was obtained from TargetMol (Boston, MA, USA). The caspase-3 inhibitors cell-permeable fluoromethyl ketone (Z-DEVD-FMK), cycloheximide (CHX), and chloroquine (CQ) and the proteasome inhibitor MG132 were obtained from Selleck Chemicals (Houston, TX, USA).

2.2. Cell viability assay

Cell viability was assayed via a cell counting kit-8 (C0005; TargetMol, Boston, USA). We seeded 5×10^3 cells/well into 96-well plates and incubated them with or without erianin followed by incubation with 10% CCK-8 reagent for 2 h. Cell viability was determined by measuring the absorbance at 450 nm using a microplate reader.

2.3. Transmission electron microscopy (TEM)

The culture medium was removed, and the cells were fixed in electron microscope fixative (Servicebio, Wuhan, China) for 5 min at room temperature in the dark and then dehydrated. The embedded cell pellets were solidified into blocks at 60°C for 48 h. Ultrathin sections were stained, and ultrastructural morphology was analyzed using TEM images.

2.4. EdU assay

Cell proliferation was assayed via an EdU kit (C0075S, Beyotime Biotechnology, Shanghai, China) and analyzed using fluorescence microscopy.

2.5. Live/Dead Cell Staining Assay

We analyzed the viability of EC cells with a live/dead cell kit (Yeasen, China). After incubation with calcein-AM/PI for 0.5 h, fluorescence images were acquired by a fluorescence microscope (Nikon Corporation, Japan). Green fluorescence (calcein-AM) represents viable cells, and red fluorescence (propidium iodide) represents dead cells.

2.6. Lactate dehydrogenase assays

We used LDH Cytotoxicity Detection Kits (C0016, Beyotime Biotechnology) and then determined the LDH release rates at 490 nm. To provide a more intuitive representation of the LDH release rate, the release rate for each group was increased by 100% to standardize the baseline data.

2.7. Colony formation

The cells (5×10^2) were cultured for 14 days in 6-well plates containing medium refreshed every 3 days, fixed in 4% paraformaldehyde, stained with 0.1% crystal violet (Servicebio), photographed, and counted.

2.8. Western blot

Cells or xenograft tumors (20–40 mg) were lysed in RIPA buffer (Beyotime Biotechnology) supplemented with protease inhibitor and phosphatase inhibitor. Protein concentration was quantified using BCA protein assay kits (Beyotime Biotechnology). Equal amounts of protein were denatured in 5X loading buffer (Servicebio), resolved by SDS–PAGE, and transferred to polyvinylidene difluoride membranes. Nonspecific antigen binding was blocked with 5% fat-free milk in TBST for 2 h at room temperature, after which the samples were incubated overnight at 4°C with primary antibodies against target proteins. The following antibodies were obtained from the respective suppliers: GSDME and GSDMD (Abcam, Cambridge, UK); caspase-3 and cleaved caspase-3 (ABclonal, Wuhan, China); PARP, FOXM1, RRM2, YTHDF1, β -actin, and GAPDH (Proteintech, Wuhan, China); and phospho-histone H2A.X (Ser139) (Abmart, Shanghai, China). The membranes were incubated overnight with HRP-conjugated secondary antibodies (ABclonal, Wuhan, China) for 2 h at room temperature. The protein bands were visualized via an enhanced chemiluminescence (ECL) substrate (Bio-Rad, CA, USA) and imaged via a ChemiDoc system. Band density was quantified using ImageJ software.

2.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA extracted via TRIzol reagent (Yeasen, Shanghai, China) was reverse transcribed using Vazyme RT Premix (Vazyme, Nanjing, China), after which qRT–PCR was performed via SYBR® Green PCR Master Mix (ABclonal) on a CFX Connect Real-Time PCR System (Bio-Rad, CA, USA). GAPDH was used as a housekeeping gene. Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method. Table 1 shows the sequences of primers used for PCR.

Table 1
The list of primers used in this study.

Primer name	Forward Primer (5'-3')	Reverse Primer (5'-3')
Caspase3	CTCTGGTTTTTCGGTGGGTGT	CTTCCATGTATGATCTTTGGTTCC
GSDME	ACGACGATGCAGAAGTGTGT	CGGAGAAGGCAGAACTCGAA
YTHDF1	GGGGACAAGTGGGTCTCAAG	AGGGTGTCGCTGTGAAAGC
FOXM1	GGAGCAGCGACAGGTTAAGG	GTTGATGGCGAATTGTATCATGG
RRM2	CACGGAGCCGAAAACAAAGC	TCTGCCTTCTTATACATCTGCCA
CENPA	TTCCTCCCATCAACACAGTCG	CACACCACGAGTGAATTTAACAC
PLK1	CACCAGCACGTCGTAGGATTC	CCGTAGGTAGTATCGGGCCTC
GAPDH	AGATCCCTCCAAAATCAAGTGG	GGCAGAGATGATGACCCTTTT

2.10. Small interfering (si) RNA transfection

The siRNA oligos (GenePharma, Jiangsu, China) shown in Table 2 or nonspecific siControl (negative control) oligos were transfected into cells using LipoRNAi™ (C0535; Beyotime Biotechnology, Shanghai, China).

Table 2
The sequences of siRNAs used in this study.

si RNA	Sense (5'-3')
GSDME siRNA 1	GGUGACCUGAUUGCAGUAUTT
GSDME siRNA 2	GCAGCAAGCAGCUGUUUAUTT
CASP3 siRNA 1	GGAACCAAAGAUCAUACAUTT
CASP3 siRNA 2	GCAGCAAACCUCAGGGAAATT
FOXM1 siRNA1	GCUGGGAUCAAGAUUAUUATT
FOXM1 siRNA2	GCCAACCGCUACUUGACAUTT

2.11. Plasmid transfection

The coding sequence of human FOXM1 was cloned and inserted into the CMV enhancer-MCS-3FLAG-SV40-Puro vector (GeneChem, Shanghai, China). The coding sequence of human YTHDF1 was cloned and inserted into the penter-CMV-YTHDF1-SV40-Puro vector. The YTHDF1 short hairpin RNA (shRNA) expression vector (GeneChem, Shanghai, China) was packaged in the hU6-MCS-Ubiquitin-EGFP-IRES-puro vector. The YTHDF1 shRNA sequences used were 5'-CGCCGTCCATTGGATTTCTT-3' (shRNA-1) and 5'-TACCTGCTCTTCAGCGTCAAT-3' (shRNA-2).

2.12. RNA library construction and sequencing

Total RNA was extracted with TRIzol reagent (Invitrogen, CA, USA). Then, RNA-Seq was performed by CapitalBio Technology on an MGISEQ2000 sequencer. Library preparation was performed via an Optimal Dual-mode mRNA Library Prep Kit (BGI-Shenzhen, China).

2.13. Human proteome microarray

We used a 20k HuProt™ human proteome microarray to screen for erianin targets. The microarray was immersed in blocking buffer at room temperature for 1 h and then incubated with either 10 μM biotinylated erianin (Ruixi Biological Technology, Xian, China) or free biotin (control) for 1 h. Free proteins were removed, and the microarray was incubated with fluorescence-labeled streptavidin (Cy5-SA) to detect and quantify the amount and affinity of erianin binding. Raw data were generated using GenePix® Pro 6 Microarray Acquisition and Analysis software (<https://axon-genepix-pro.software.informer.com/6.0/>) and background-corrected. Sample signals were normalized by Z score standardization before comparisons. Proteins with Z scores > 2 in the Erianin group and Z scores < 2 in the control (D-biotin) group were considered positive hits.

2.14. Molecular docking

Protein structures were downloaded from the Protein Data Bank (PDB) database (PDB ID: 7QKN). The 3D structure file of erianin was obtained from the PubChem database. We analyzed the molecular docking data via the Glide module integrated in Schrödinger software.

2.15. Cellular thermal shift assays (CETSA)

We determined target engagement using CETSA as previously described (21). The cells were incubated without erianin (control) or with erianin (100 nM) for 2 h, and 50 μL portions of the cells suspended in PBS containing 1 mM phenylmethylsulfonyl fluoride protease inhibitor were distributed into tubes and heated for 3 min at 4°C intervals from 36–68°C. After three freeze–thaw cycles in liquid nitrogen, soluble proteins were separated via centrifugation at 12,000 × g for 30 min. Thermal stability was analyzed by immunoblotting.

2.16. Immunofluorescence

Cells cultured on coverslips were incubated with erianin for 24 h, fixed with paraformaldehyde, and permeabilized with Triton X-100 (P0096; Beyotime Biotechnology, Shanghai, China). Nonspecific antigen binding was blocked with bovine serum albumin (BSA), and the cells were incubated overnight with 1:200-diluted anti-phospho-histone H2A.X (Ser139) (Abmart, Shanghai, China), followed by secondary antibodies. The cells were stained with DAPI and visualized using fluorescence microscopy.

2.17. Xenograft tumor model and immunohistochemistry (IHC)

Ishikawa cells (1×10^6) were suspended in 100 μL of PBS and then subcutaneously injected into the right scapular region of 5-week-old female BALB/c-nu nude mice. When the tumors reached 50–100 mm³, the mice were randomly assigned to groups that were intraperitoneally injected with normal saline

(control) or 25 or 50 mg/kg erianin daily. The tumor volume was calculated every 3 days as length $\times$ width²/2. The mice were euthanized by cervical dislocation 15 days later, and the xenograft tumors were excised and weighed. Parts of the tumors were fixed in formalin and stained with hematoxylin and eosin (HE) for histopathological analysis or for immunohistochemical (IHC) analysis using YTHDF1 primary antibodies (Proteintech, Wuhan, China). The Ethics Committee at Huazhong University of Science and Technology Union Hospital approved all the animal procedures in this study (Approval ID: 2022–3060).

2.18. RNA immunoprecipitation (RIP)

We detected YTHDF1-FOXM1 mRNA interaction using Megna RNA Binding Protein Kits (MilliporeSigma, Burlington, MA, USA). Cells were lysed with RIP lysis buffer and then rotary incubated with 5 μ L of a YTHDF1 or IgG antibody (Cell Signaling Technology [CST], Danvers, MA, USA) at 4°C overnight, followed by washing with magnetic beads at 4°C overnight. We extracted RNA via TRIzol reagent, and FOXM1 expression was detected via RT–qPCR.

2.19. Messenger RNA stability assay

Ishikawa and HEC-1-B cells were transfected with shYTHDF1 and incubated with 5 μ g/mL actinomycin D. Then, RNA was extracted to detect FOXM1 mRNA expression at the indicated time points.

2.20. Statistical analysis

All the data were statistically analyzed using GraphPad Prism software v. 8.0. The means of three independent replicates between paired groups were compared using unpaired Student's t tests, and those of three or more groups were compared using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. The results are presented as the means $\pm$ standard deviations (SDs), and values with $P < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Erianin inhibited EC cell proliferation

Compound library screening is a novel tool for discovering potential anticancer drugs. We screened small molecule compounds with anticancer efficacy from 60 compounds, and the CCK-8 assays demonstrated that erianin is a promising anti-cancer drug for EC (Fig. 1A). We explored the effects of erianin on the growth of EC cell lines. We incubated HEC-1-B, Ishikawa, and KLE cells with 50, 100, and 200 nM erianin for 24, 48, and 72 h. The results of the CCK-8 assays revealed that erianin decreased the viability of these cells in a time- and concentration-dependent manner (Fig. 1B). The IC₅₀ values of erianin in HEC-1-B and Ishikawa cells at 24 h were 174.1 and 113.0 nM, respectively (Figure S1A). We selected 100 nM erianin, which can significantly inhibit cell proliferation, for the follow-up studies. Erianin significantly reduced the numbers of Ishikawa, HEC-1-B, and KLE colonies compared with those of the controls (Fig. 1C). The EdU findings also revealed that erianin inhibited EC cell proliferation (Fig. 1D). Live/Dead cell staining assays revealed that erianin obviously increased the ratio of PI-positive cells

(Fig. 1E). Microscopy findings of swollen and rounded cells revealed that erianin inhibited EC cell progression at the nanomolar level (Fig. 1F). Collectively, these data show that erianin inhibits EC cell progression.

3.2. Erianin induced pyroptosis in EC cells via GSDME signaling

Pyroptosis is usually accompanied by cell swelling and membrane rupture. We found that erianin induced cell swelling and increased the proportion of PI-positive cells. Therefore, we speculated that erianin induced EC cell pyroptosis. The swollen cells had characteristic large bubbles extruding from the plasma membrane according to the TEM images. Significant membrane disruption was accompanied by mild swelling of mitochondria and irregular shape of cell nucleus (Fig. 2A). The phenomena closely matched the typical morphological features associated with pyroptosis. Cell rupture is usually accompanied by LDH release, and erianin increased the percentage of LDH release from EC cells (Fig. 2B). GSDM proteins are key effectors of pyroptosis. Western blotting analysis further revealed that erianin induced the cleavage of GSDME rather than GSDMD, thus yielding a N-terminal fragment that formed pores in the HEC-1-B and Ishikawa cell membranes (Fig. 2C). These findings suggest that erianin induces GSDME-dependent pyroptosis in EC cells. After silencing GSDME by siRNA-mediated knockdown, qRT-PCR and Western blotting analysis indicated that siGSDME-2 reduced GSDME mRNA and protein levels in HEC-1-B cells (Figure S2A). The CCK-8 results suggested that GSDME knockdown increased the viability of EC cells incubated with erianin (Fig. 2D). Erianin-induced LDH release (Fig. 2E) and GSDME cleavage (Fig. 2F) were reduced and inhibited, respectively, in Ishikawa and HEC-1-B cells. GSDME knockdown resulted in reduced colony formation (Fig. 2G) and EC cell swelling (Fig. 2H). Therefore, silencing GSDME impedes erianin-induced pyroptosis in EC cells, suggesting that GSDME mediates the mechanism through which erianin exerts anticancer effects.

3.3. Erianin induced pyroptosis via caspase-3/GSDME signaling

Caspase-3 cleaves GSDME during pyroptosis. The levels of cleaved caspase-3 and cleaved PARP were significantly increased in HEC-1-B and Ishikawa cells (Fig. 3A). We therefore investigated whether erianin induces GSDME cleavage through caspase-3 activation. The caspase-3 inhibitor Z-DEVD-FMK increased the viability of EC cells incubated with erianin (Fig. 3B) and significantly inhibited erianin-induced caspase-3/GSDME expression (Fig. 3C) as well as LDH release (Fig. 3D). We confirmed these effects by knocking down caspase-3 via qRT-PCR and Western blotting (Figure S2B). The results of the CCK-8 assays revealed that si-CAS3 increased EC cell survival (Fig. 3E). Western blotting revealed that caspase-3 knockdown inhibited erianin-induced caspase-3 activation and GSDME cleavage (Fig. 3F), notably, phase contrast microscopy revealed reduced numbers of pyroptotic cells (Fig. 3G). Collectively, these data suggest that erianin-induced GSDME cleavage and subsequent pyroptosis induction require caspase-3 activation.

3.4. Erianin induced DNA damage *via* the FOXM1/RRM2 axis in EC cells

We sequenced the transcriptome to further elucidate the mechanism through which erianin induces pyroptosis. Gene Ontology (GO) analysis revealed significantly enriched DNA damage pathways (Fig. 4A). Erianin significantly reduced the expression of the DNA repair factors CENPA, FOXM1, RRM2, PLK1, TOP2A, AURKB, and AURKA (Fig. 4B). The qPCR findings verified that erianin suppressed the expression of FOXM1, RRM2, CENPA, and PLK1 in EC cells (Fig. 4C). Analysis of the Animal Transcription Factor Database (AnimalTFDB3) revealed that the promoter regions of the genes associated with DNA repair described above share a binding site for FOXM1, which is upregulated in various tumors. It also acts as a transcription factor that drives RRM2 expression, and the FOXM1/RRM2 axis regulates the cell cycle and DNA repair(22). The Gene Expression Profiling Interactive Analysis (GEPIA) database revealed a positive correlation between FOXM1 and RRM2 expression in EC (Figure S2C). The Western blotting results showed that erianin downregulated the FOXM1/RRM2 axis and increased the expression of the damage marker H2A.X variant histone (γ H2AX) (Fig. 4D). The immunofluorescence staining also showed that erianin increased the number of fluorescent γ H2AX foci, indicating substantial DNA damage (Fig. 4E). Western blotting results also revealed that FOXM1 knockdown suppressed RRM2 expression while increasing γ H2AX and caspase-3 cleavage (Fig. 4F). A close association between GSDME-mediated pyroptosis and DNA damage has been suggested(23). Therefore, our results suggest that erianin induces DNA damage by inhibiting the FOXM1/RRM2 axis.

3.5. Erianin induced pyroptosis via FOXM1

We used plasmids overexpressing FOXM1, qRT–PCR and western blotting assays to further explore whether erianin induces pyroptosis through FOXM1 suppression (Fig. 5A). FOXM1 overexpression increased EC cell viability (Fig. 5B), suppressed LDH release induced by erianin (Fig. 5C), enhanced colony formation (Fig. 5D), and inhibited the proportion of swollen HEC-1-B and Ishikawa cells (Fig. 5E). FOXM1 overexpression also increased caspase-3/GSDME cleavage (Fig. 5F). These results indicate that erianin promotes pyroptosis in EC cells by inhibiting FOXM1.

3.6. Erianin directly targets YTHDF1 and induces YTHDF1 ubiquitination

We investigated the targets of erianin that are responsible for DNA damage and pyroptosis. Conjugation with biotin (Fig. 6A-6B) did not impact the anticancer activity of erianin (Fig. 6C). We then screened targets using the 20k HuProt™ human proteome microarray (Fig. 6D), which identified the m6A reader YTHDF1 as a direct target of erianin (Fig. 6E). We compared the crystal structures of YTHDF1 and erianin using molecular docking and found that erianin formed bonds with Trp-411, Ser-413, Asp-401, and Tyr-397 of YTHDF1 (Fig. 6F). We then further investigated whether erianin directly targets YTHDF1 using CETSA to quantify the binding affinity of small molecules for specific proteins on the basis of changes in

protein thermal stability(24). The CESTA results revealed that incubation with erianin resulted in detectable protein bands of YTHDF1 even at 56°C compared with those of the control (Fig. 6G-6H). Thus, erianin improved the thermal stability of YTHDF1, which supported direct binding interactions. YTHDF1 was abundantly expressed in EC cells according to The Cancer Genome Analysis (TCGA) data (Fig. 6I). We then explored the role of YTHDF1 in EC cell proliferation via the use of a shRNA targeting YTHDF1. Western blotting and qRT-PCR confirmed that YTHDF1 was silenced in HEC-1-B cells (Figure S2D). Knockdown of YTHDF1 suppressed cell proliferation (Figure S2E) and colony formation (Figure S2F), suggesting that YTHDF1 plays a vital role in EC cell growth. We then assessed the effect of erianin on YTHDF1 expression. Erianin reduced YTHDF1 protein expression but did not significantly affect YTHDF1 mRNA in Ishikawa and HEC-1-B cells (Fig. 6J-6K). Therefore, we speculated that erianin degrades YTHDF1 mainly at the posttranslational level. Cycloheximide (CHX) assays revealed a shorter half-life of YTHDF1 in cells treated with both erianin and CHX than in those treated with CHX alone (Fig. 6L). To determine the pathway of YTHDF1 degradation caused by erianin, we incubated Ishikawa cells with erianin and the lysosomal inhibitor chloroquine (CQ) and the proteasomal inhibitor MG132. Western blot analysis revealed that MG132, but not CQ, effectively rescued YTHDF1 degradation (Fig. 6M). These findings further confirmed that the ubiquitin-proteasome pathway is involved in the degradation of YTHDF1 by erianin. Therefore, we concluded that erianin acts directly on YTHDF1 and promotes its degradation via ubiquitination. Therefore, we postulated that erianin directly targets and promotes YTHDF1 ubiquitination and degradation.

3.6. Erianin induced EC pyroptosis depends on YTHDF1

We investigated the role of YTHDF1 in erianin-induced pyroptosis and discovered that YTHDF1 overexpression increased cell viability and reduced LDH release. Conversely, YTHDF1 knockdown decreased cell viability and increased LDH release (Fig. 7A-7B). Western blot analysis revealed that YTHDF1 knockdown increased the levels of cleaved caspase-3 and GSDME (Fig. 7C), while YTHDF1 overexpression suppressed their expression (Fig. 7D). Silencing YTHDF1 impaired the colony formation ability of EC cells, whereas YTHDF1 overexpression restored it (Fig. 7E and Figure S3A). Combining YTHDF1 knockdown with erianin treatment significantly increased the proportion of swollen EC cells (Fig. 7F and Figure S3B) and γH2AX foci formation (Fig. 7G). Conversely, YTHDF1 overexpression reduced both pyroptotic cells (Fig. 7F and Figure S3B) and DNA damage foci (Fig. 7G). These findings suggest that erianin exerts anti-cancer effects by directly targeting YTHDF1.

3.8. The stability of FOXM1 mRNA in EC cells is regulated by YTHDF1

YTHDF1 recognizes m6A-modified FOXM1 and affects its stability(25). We identified a significant positive correlation between YTHDF1 and FOXM1 mRNA levels in the GEPIA database in EC (Fig. 8A). We further investigated whether erianin regulates FOXM1 expression by targeting YTHDF1. We analyzed FOXM1 expression after YTHDF1 knockdown and overexpression via qRT-PCR and Western blotting. The knockdown and overexpression of YTHDF1 inhibited (Fig. 8B) and promoted (Fig. 8C) FOXM1 RNA

and protein expression, respectively. Inhibiting transcription with actinomycin D showed that YTHDF1 knockdown promoted FOXM1 degradation in EC cells (Fig. 8D and 8E). Ribonucleic acid immunoprecipitation (RIP) assays revealed that YTHDF1 knockdown (Fig. 8F) and erianin impaired YTHDF1-FOXM1 mRNA binding (Fig. 8G). These results further suggest that YTHDF1 plays an important role in regulating FOXM1 expression.

3.9. Effects of erianin on EC xenograft tumors *in vivo*

We investigated the anticancer effects of erianin in an EC xenograft model. Ishikawa cells were inoculated into the scapulae of female BALB/c-nu nude mice. The mice were then assigned to three groups that were intraperitoneally injected with 25 or 50 mg/kg erianin or normal saline daily (Fig. 9A). The results showed that erianin markedly inhibited tumor growth and tumor weight by > 50% compared with the control (Fig. 9B–D). The weights of the mice treated with erianin did not significantly change (Fig. 9E). We further analyzed the expression of YTHDF1 and caspase3/GSDME axis in tumor tissues (Fig. 9F–9G). The results showed that erianin downregulated the levels of YTHDF1 in tumor tissues (Fig. 9F–9G). The upregulated levels of cleaved caspase-3 and N-GSDME were observed in erianin-treated tumors (Fig. 9G). No signs of toxicity were detected in various HE-stained organ tissues from the model mice, suggesting that these doses were safe (Fig. 9H). These findings highlight the safety and effectiveness of erianin in treating EC.

Discussion

In our study, we demonstrated that erianin induces pyroptosis in EC cells through the caspase-3/GSDME axis. Using the 20k HuProt™ human proteome microarray, we first identified YTHDF1, an m6A reader, as the primary target of erianin. We further elucidated the critical role of YTHDF1 in erianin-mediated pyroptosis. RNA-seq analysis revealed that erianin regulates the FOXM1/RRM2-mediated DNA damage pathway. Inhibition of FOXM1 led to the activation of cleaved caspase-3, while overexpression of FOXM1 partially rescued erianin-induced pyroptosis. Mechanistically, erianin directly targets and ubiquitinates YTHDF1 to regulate the stability of FOXM1 mRNA, thereby activating the caspase-3/GSDME-dependent pyroptosis.

Low-toxicity natural compounds have potential as anticancer therapeutics(26, 27), and erianin has pharmacological properties and anticancer effects(28, 29). Erianin suppresses hepatocellular carcinoma by targeting pyruvate carboxylase-mediated metabolic reprogramming and inducing Ca²⁺/CaM-dependent ferroptosis(30). Here, we explored the potential targets and mechanisms of erianin in EC. Systemically administered erianin suppressed EC growth at nanomolar concentrations without adverse effects. We also found that erianin induces GSDME-mediated pyroptosis, which is characterized by swollen cells and ruptured cell membranes. Some chemotherapeutic and natural compounds induce tumor cell pyroptosis via the caspase-3/GSDME axis. For example, *Salvia miltiorrhiza* root extracts miltirone(31) and myricetin(32) induce cancer cell pyroptosis through the caspase-3/GSDME pathway. Our results also revealed that erianin induces GSDME cleavage through caspase-3 activation, which is impeded by the silencing and inhibition of caspase-3.

m6A modification play crucial roles in tumor progression(33, 34). For example, the m6A demethylase ALKBH5 promotes resistance to HER2-targeted therapy(35), and IGF2BP1 m6A-dependently stabilizes PEG10 mRNA to promote EC progression(36). The ability to target m6A has attracted considerable attention. Research reports indicate that natural compounds can target m6A to exert anticancer effects. Cucurbitacin B (CuB) targets IGF2BP1 (Cys253), which blocks c-mycelocytomatosis (c-MYC) recognition to induce apoptosis and antitumor immunity in hepatocellular carcinoma(37). Curcumin inhibits the expression of the m6A demethylase ALKBH5, leading to increased m6A methylation of TRAF4 and inhibition of adipogenesis(38). YTHDF1 has also been shown to exhibit anticancer effects in various tumors. For example, the m6A reader YTHDF1 regulates ARHGEF2 translation in an m6A-dependent manner, promoting colorectal Cancer (CRC) progression(39). YTHDF1 enhances m6A-dependent TINAGL1 translation, driving malignant progression in esophageal cancer(40). Furthermore, YTHDF1 mediates m6A modification of GLS1, contributing to oxaliplatin resistance in CRC(41). Thus, YTHDF1 is considered a promising therapeutic m6A target. However, the role of YTHDF1 in EC remains unclear, and there are no reports on natural compounds exerting anticancer effects through YTHDF1. Our study reveals that YTHDF1 is highly expressed in EC and that its overexpression exhibits oncogenic properties. Silencing YTHDF1 suppresses EC proliferation. Using the HuProt™ 20k proteome microarray, we identified erianin as a potential YTHDF1-targeting compound, a finding validated by molecular docking and CESTA experiments. Previous studies have shown that YTHDF1 can inhibit caspase-1-dependent pyroptosis through WWP1 upregulation(42). YTHDF1 inhibition promotes caspase-3-dependent apoptosis(43) and enhances DNA damage(44). Here, we hypothesize that YTHDF1 inhibition promotes DNA damage and mediates caspase-3 activation. Our study further elucidates that YTHDF1 inhibition enhances erianin-induced DNA damage response and promotes caspase-3/GSDME-mediated pyroptosis. Erianin-induced pyroptosis in EC cells is impeded by YTHDF1 overexpression, indicating that YTHDF1 plays a pivotal role in erianin-induced pyroptosis.

The transcription factor forkhead box M1 (FOXO1) regulates various signaling pathways (45) and is upregulated in breast(46), gastric(47), pancreatic(48), and hepatocellular(49) cancers. It functions in tumorigenesis through its role in cell cycle, DNA damage, cellular senescence, and metastasis. FOXO1 regulates ZIC2, which promotes the malignant phenotype of renal clear cell carcinoma(50). In lung cancer, FOXO1 directly binds to the promoter of RRM2, thus regulating cell proliferation and metastasis(51). FOXO1 has also been demonstrated to play a critical oncogenic role in EC(52). RNA-seq revealed that erianin modulates the DNA damage response and downregulates the expression of FOXO1, a key gene involved in DNA repair. The qRT-PCR and western blotting analysis confirmed that erianin significantly downregulates FOXO1. The most differentiated target in the regulatory network of FOXO1 is RRM2, which is abundantly expressed in many types of cancers. Ribonucleotide reductase regulatory subunit M2 catalyzes deoxyribonucleotide synthesis, regulates nucleotide metabolism (dNTP pools), and is closely associated with the cell cycle, chemotherapy resistance, and DNA repair mechanisms(53–55). Unstable dNTP pools can lead to DNA damage. Oxoglutarate dehydrogenase L (OGDHL) downregulates nucleotide metabolism in hepatocellular carcinoma, which leads to dNTP depletion; this is thought to be a major factor that mediates DNA damage(56). Thus, we further studied

RRM2 as a factor downstream of FOXM1. We found that erianin inhibited the FOXM1/RRM2 axis to regulate DNA damage in EC cells, and knockdown of FOXM1 suppresses RRM2 expression, promotes DNA damage, and activates cleaved caspase-3 in EC cells. Overexpression of FOXM1 reverses erianin-induced pyroptosis in EC. Thus, the FOXM1/RRM2 pathway is vital to the anticancer effect of erianin in EC. Additionally, YTHDF1 binds to m6A-modified FOXM1 and promotes its expression(57). Therefore, we hypothesize that during erianin-induced pyroptosis in EC cells, inhibition of YTHDF1 leads to decreased stability of FOXM1 mRNA. Knockdown of YTHDF1 suppresses the FOXM1/RRM2 signaling pathway, thereby inducing DNA damage and activating caspase-3/GSDME-mediated pyroptosis.

Conclusions

A low dose of erianin robustly inhibited the progression of EC via caspase-3/GSDME-dependent pyroptosis. Erianin directly targeted YTHDF1 and promoted its ubiquitination and degradation, which induced DNA damage mediated by FOXM1/RRM2, resulting in GSDME cleavage by cleaved caspase-3. We revealed the mechanism of the anticancer effects of erianin. Our findings provide a theoretical basis for the development of pyroptosis-targeted anticancer drugs and new strategies with which to treat EC.

Abbreviations

EC, endometrial cancer; CESTA, cellular thermal shift assays; H2AX, H2A histone family member X; GSDME, gasdermin E; OGDHL, Oxoglutarate dehydrogenase L; GEPIA, Gene Expression Profiling Interactive Analysis; METTL3/14, methyltransferase-like 3/14 ; FTO, fat mass and obesity-associated protein; ALKBH5, alkylation repair homolog protein 5; YTHDF, YTH domain family protein; IGF2BPs, insulin-like growth factor 2 mRNA-binding proteins; STR, short tandem repeat; CHX, cycloheximide; CQ, chloroquine; TCGA, the Cancer Genome Atlas; DEGs, differentially expressed genes; GO, Gene Ontology; RIP, Ribonucleic acid immunoprecipitation; IHC, immunohistochemistry; TEM, Transmission electron microscopy; c-MYC, c-myelocytomatosis; CRC, colorectal cancer; FOXM1, forkhead box M1.

Declarations

Competing interests

The authors declare no conflicts of interest.

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Author Contribution

H.W. and J.Z. designed the project. W.S. and X.Z. designed and supervised all experiments. R.Z., K.D., and X.S. have drafted the work or substantively revised it. G.C., S.C., Q.Z., and T.Z. contributed to the bioinformatics analysis. T.Z., S.Y., J.Z., and Y.L. participated in data interpretation and analysis. H.L. and W.Y. assisted with animal experiments. All authors have reviewed and approved the submitted manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

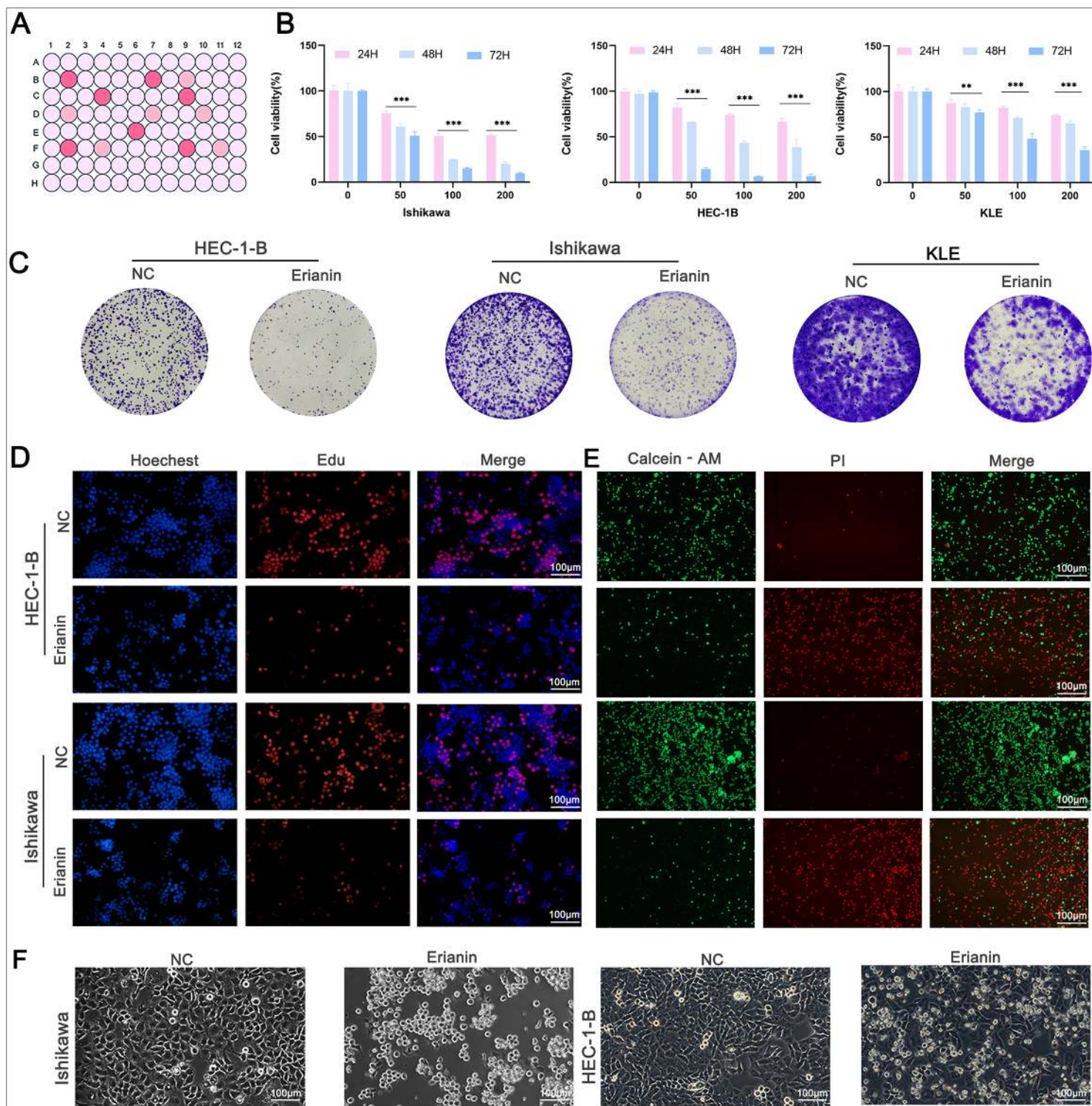


Figure 1

Erianin inhibits EC cell proliferation. (A) 60 natural compound library screening. (B) Viability of Ishikawa, HEC-1-B, and KLE cells incubated with 50, 100, or 200 nM erianin for 24, 48, or 72 h determined using CCK-8 assays. (C) Colony formation of Ishikawa, HEC-1-B, and KLE cells incubated with 100 nM erianin for 24 h. (D) EdU assays of proliferation after a 24 h incubation with 100 nM erianin. Scale bar = 100 μ m. (E) Fluorescence microscopy images showing the number of live and dead cells. Green represents live cells and red represents dead cells. Scale bar = 100 μ m. (F) Phase-contrast microscopy images showing

the morphological characteristics of Ishikawa and HEC-1-B cells. Scale bar = 100 μ m. The data are presented as the means $\pm$ SDs, n=3; **P<0.01 and ***P<0.001 compared with the NC (control group).

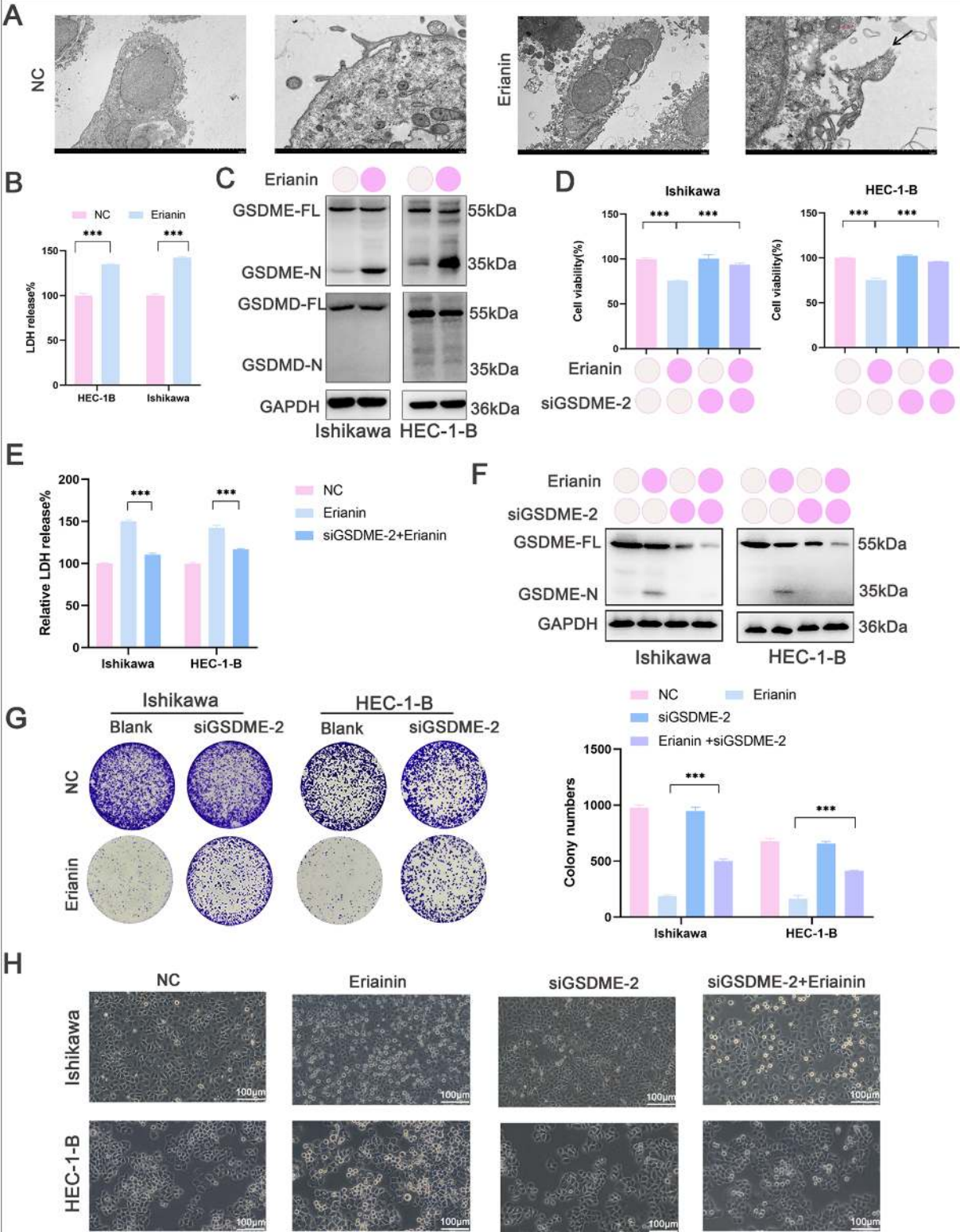


Figure 2

Eriainin induces GSDME-dependent EC cell pyroptosis.

Transmission electron microscopy (TEM) images of the cell membrane and organelles in HEC-1-B cells incubated with erianin (100 nM). Left and right scale bars, 5 and 1 μm , respectively. Black arrows indicate disruption of the cell membrane, while red arrows represent mitochondrial swelling. (B) Release of LDH in HEC-1-B and Ishikawa cells. (C) Western blots of GSDME and GSDMD in HEC-1-B and Ishikawa cells incubated with erianin (100 nM) for 24 h. (D–H) GSDME-silenced HEC-1-B and Ishikawa cells were incubated with erianin (100 nM) for 24 h. (D) CCK-8 assays. (E) LDH release assays. (F) Western blots of cleaved GSDME. (G) Colony formation assay. (H) Phase-contrast microscopy of EC cell morphological characteristics. Scale bar = 100 μm . The data are presented as the means $\pm$ SDs, $n=3$; *** $P<0.001$ compared with the NC (control group).

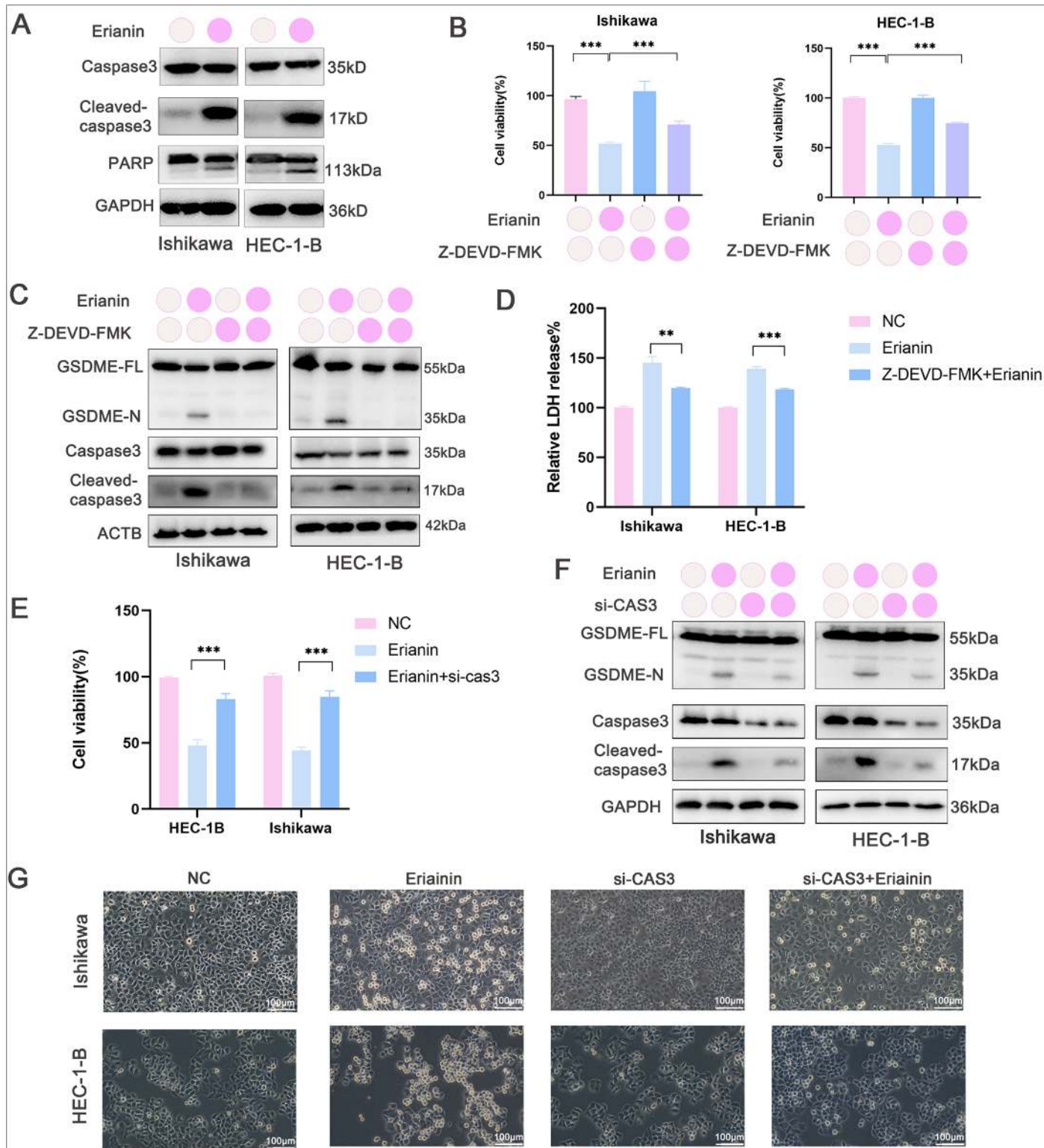


Figure 3

Erianin induces pyroptosis via caspase3/GSDME signaling in EC cells. (A) Western blotting analysis of caspase-3, cleaved caspase-3, and PARP in EC cells incubated with erianin (100 nM) for 24 h. (B–D) HEC-1-B and Ishikawa cells were incubated with or without the caspase-3 inhibitor z-DEVD-FMK (30 μ M) for 2 h, followed by erianin (100 nM) for 24 h. (B) CCK-8 assays. (C) Western blots. (D) LDH release. (E–G) HEC-1-B and Ishikawa cells transfected with si-CAS3 were incubated with erianin (100 nM). (E) CCK-8

assay. (F) Western blot analysis of cleaved caspase-3 and GSDME. (G) Phase-contrast microscopy images showing the morphological characteristics of EC cells. Scale bar = 100 μ m. The data are presented as the means $\pm$ SDs, n=3; **P<0.01 and ***P<0.001 compared with the NC (control group).

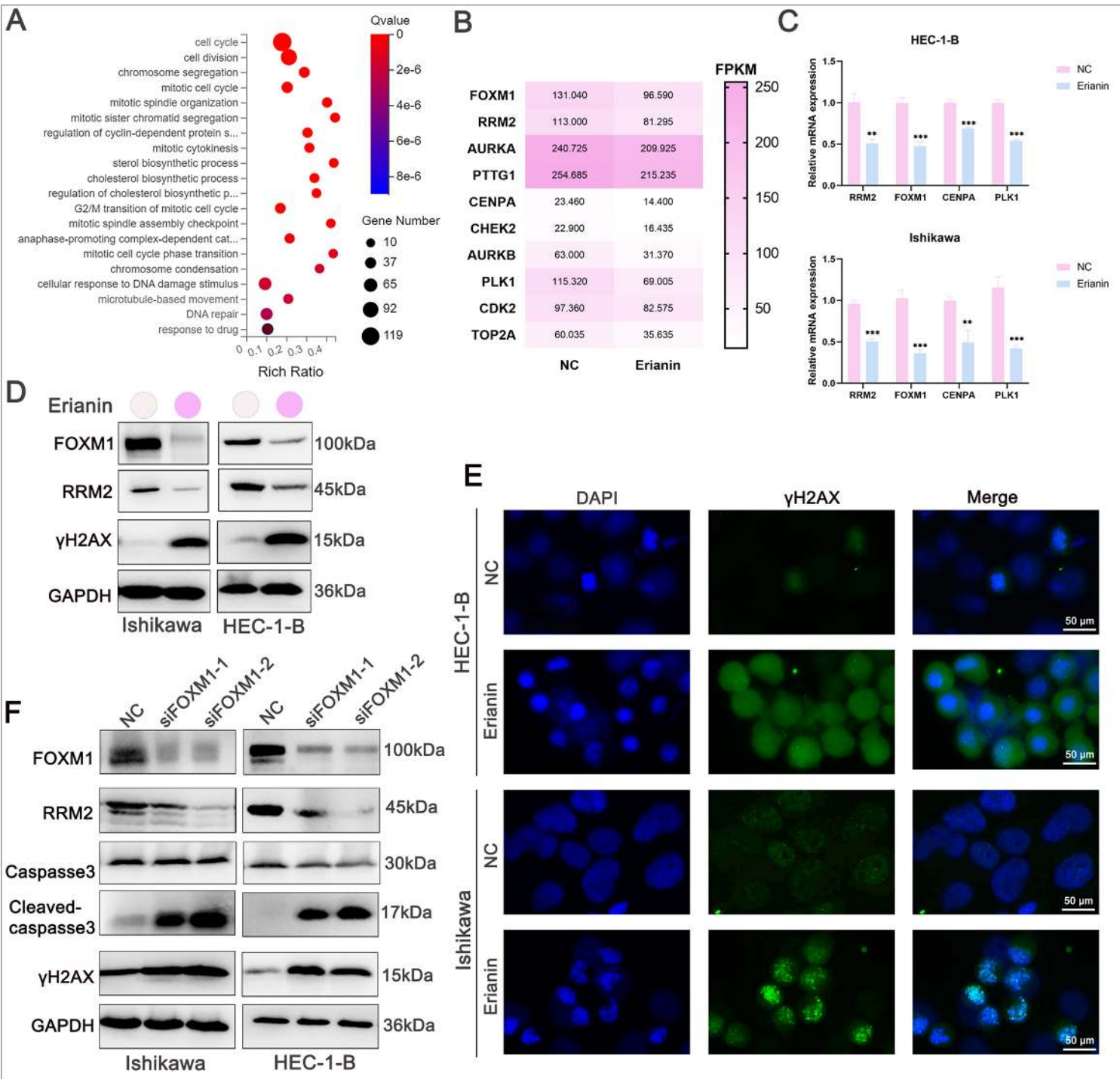


Figure 4

Erianin induces DNA damage via the FOXM1/RRM2 axis in EC cells.

(A) Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in EC cells incubated with erianin. (B) Heatmap of DEGs associated with DNA damage. (C) The expression of FOXM1, RRM2, CENPA, and PLK1 in EC cells incubated with erianin (100 nM) for 24 h was detected

using qPCR. (D) Western blots of FOXM1, RRM2, and γ H2AX in Ishikawa and HEC-1-B cells incubated with erianin (100 nM) for 24 h.

(E) Immunofluorescence staining of γ -H2AX in HEC-1-B and Ishikawa cells incubated with erianin (100 nM) for 24 h. Scale bar = 50 μ m. (F) Western blots of FOXM1, RRM2, cleaved caspase-3, and γ H2AX in Ishikawa and HEC-1-B cells with FOXM1 knockdown. The data are shown as the means $\pm$ SDs; n=3; **P<0.01 and ***P<0.001 compared with NC (control group).

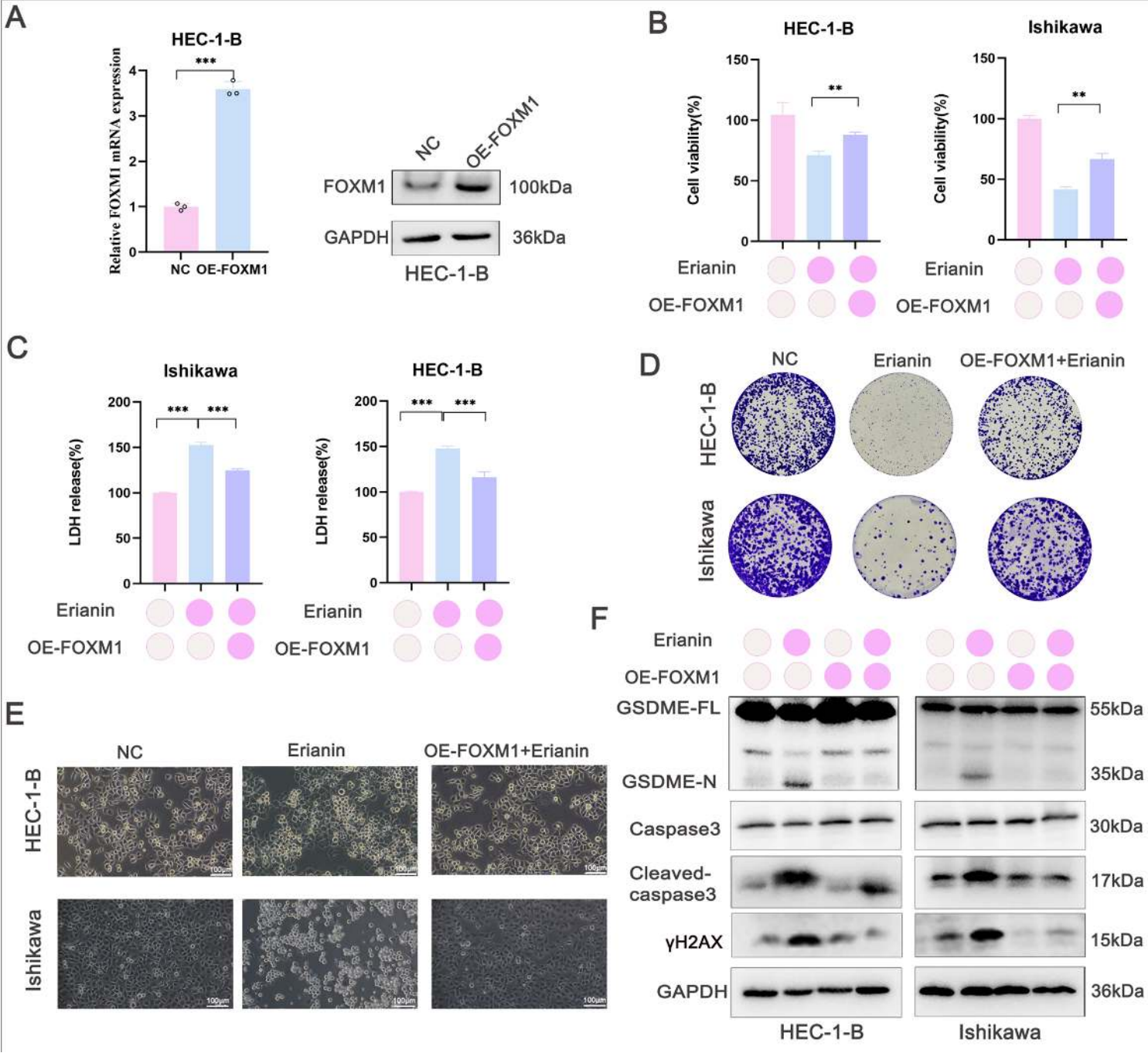


Figure 5

Erianin induces pyroptosis via FOXM1.

(A) FOXM1 expression in HEC-1-B cells transfected with OE-FOXM1 was validated via qRT-PCR and Western blotting. (B–F) FOXM1 was overexpressed in HEC-1-B and Ishikawa cells after incubation with erianin (100 nM) for 24 h. (B) CCK-8 assay. (C) LDH assay. (D) Colony formation. (E) Western blot analysis of cleaved GSDME, cleaved caspase-3, and γH2AX. The data are shown as the means ± SDs; n=3; **P<0.01 and ***P<0.001 compared with the NC (control group).

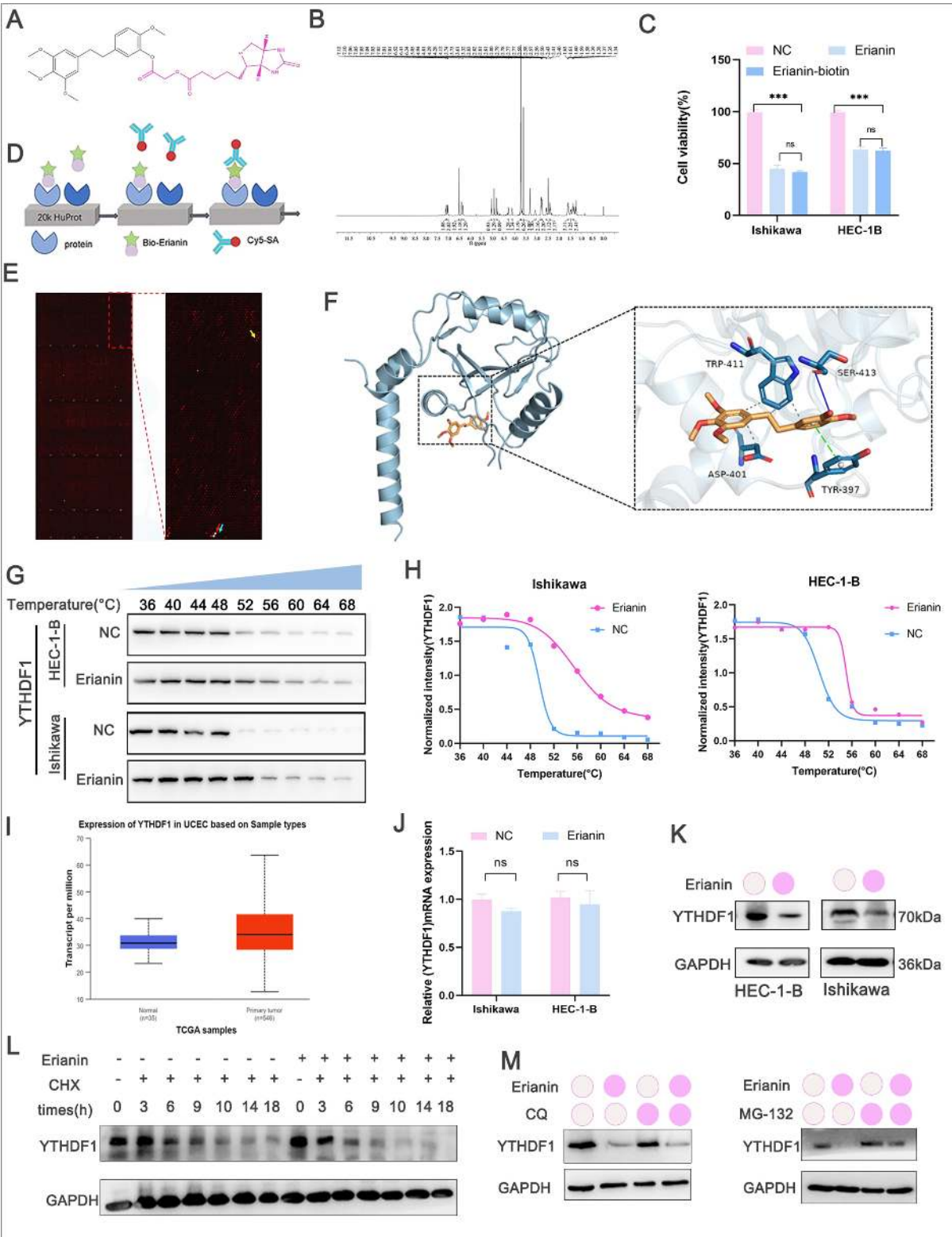


Figure 6

Erianin directly targets YTHDF1.

(A) Synthesis of erianin-biotin. (B) Erianin-biotin ^1H NMR spectrum. (C) CCK-8 results for Ishikawa and HEC-1-B cells incubated with erianin-biotin and erianin (50 and 100 nM) for 24 h. (D) Schematic workflow of protein array screening to identify erianin targets. (E) Representative images of the human proteomics chip. Blue, red, and yellow: negative control, positive control, and positive spot, respectively. (F) Molecular docking simulation of the binding between erianin and YTHDF1. (G) Thermal stability of erianin and YTHDF1 in EC cells detected using CESTA. (H) Quantification of YTHDF1 expression. (I) Expression of YTHDF1 in normal endometrium and EC tissues determined from the TCGA database. (J) qRT-PCR analysis of YTHDF1 expression.

(K-M) YTHDF1 expression in Ishikawa cells. (K) Ishikawa cells were incubated with erianin (100 nM). (L) Ishikawa cells were treated with erianin (100 nM) and cycloheximide (CHX) (50 mg/mL). (M) Ishikawa cells were treated with MG132 or CQ. The data are shown as the means $\pm$ SDs; $n=3$; *** $P<0.001$ compared with the NC (control group). ns, not significant.

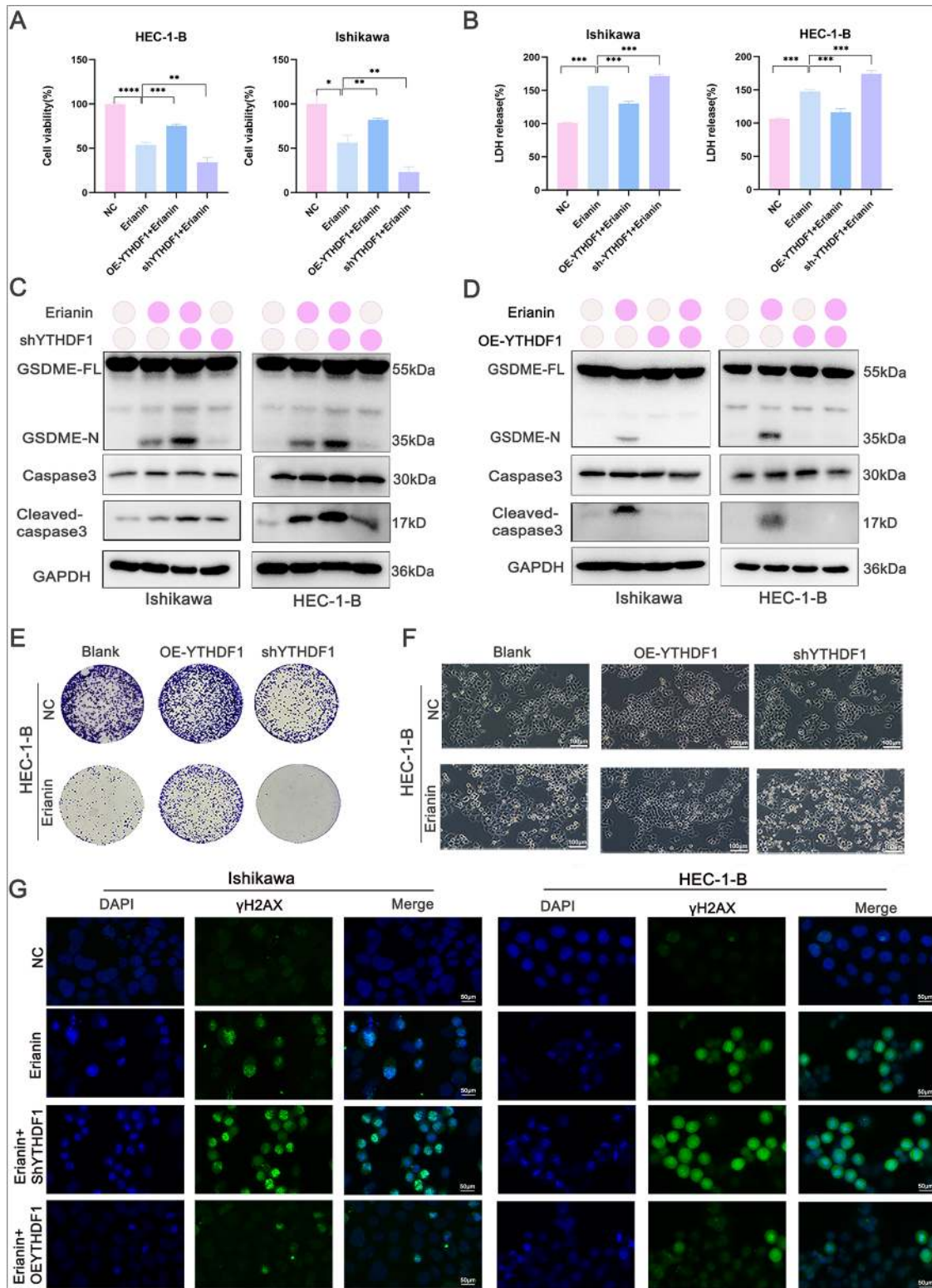


Figure 7

Erianin-induced EC pyroptosis depends on YTHDF1.

EC cells were transfected with shYTHDF1 and YTHDF1 vectors. (A) Survival of EC cells incubated with erianin (100 nM) for 24 h, as detected using CCK-8 assays. (B) LDH release assay. (C-D) Western blots of cleaved GSDME and cleaved caspase-3 in EC cells. (E) Colony formation assay. (F) Phase-contrast microscopy images of morphological features. Scale bar, 100 μ m. (G) Representative images of γ -H2AX

staining with immunofluorescence. Scale bar, 50 μ m. The data are presented as the means $\pm$ SDs; * P <0.05, ** P <0.01, and *** P <0.001 compared with the NC (control group).

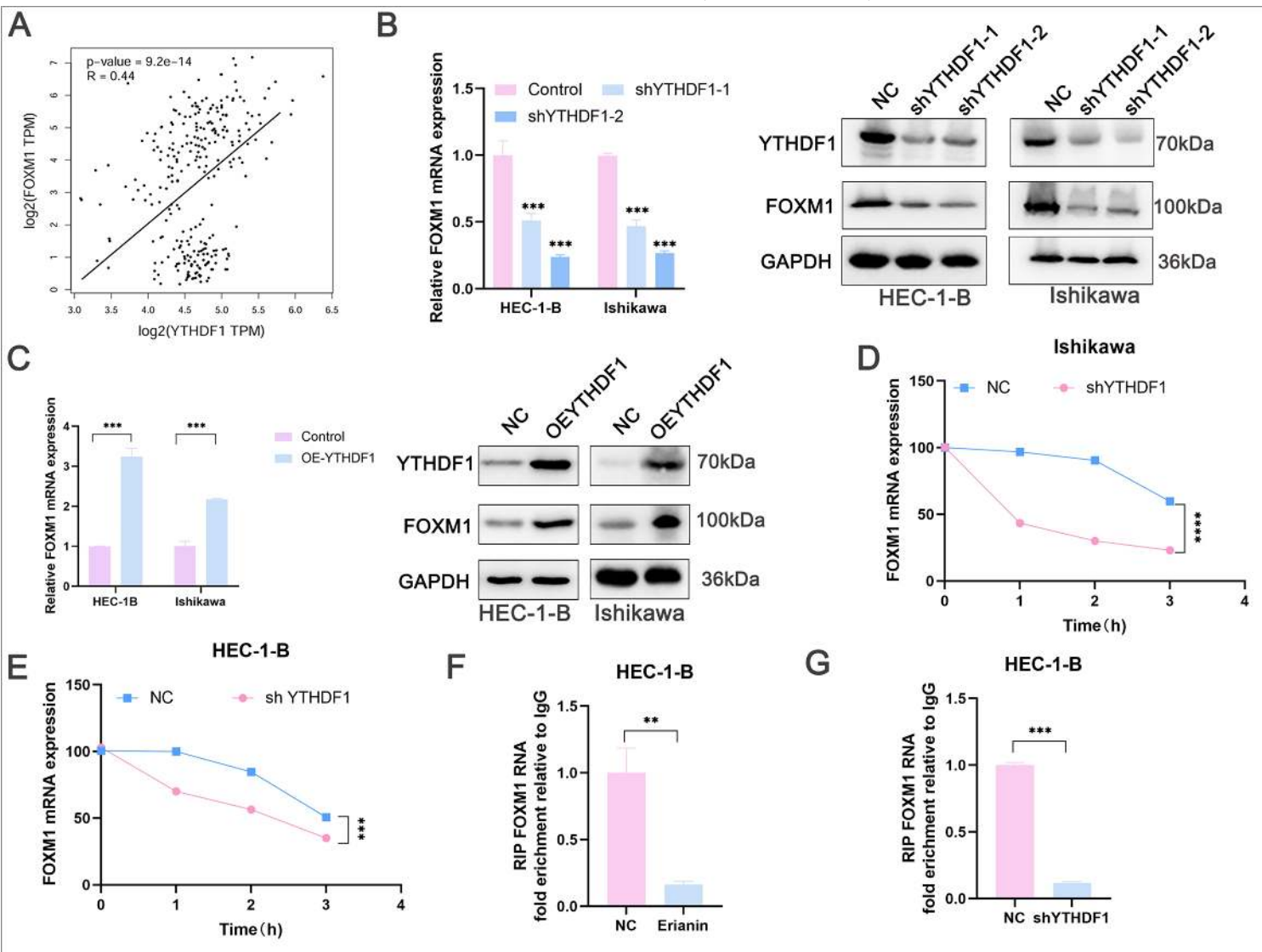


Figure 8

YTHDF1 regulates FOXM1 mRNA stability in EC. (A) Correlation between YTHDF1 mRNA and FOXM1 mRNA from the GEPIA database. (B) qRT–PCR and Western blotting experiments were used to validate FOXM1 expression in EC cells transfected with shYTHDF1. (C) qRT–PCR and Western blotting experiments were used to validate FOXM1 expression in EC cells transfected with shYTHDF1. (D–E) Relative expression of FOXM1 in Ishikawa and HEC-1-B cells with silenced YTHDF1 incubated with actinomycin D (5 μ g/mL). (F) Results of the RIP assays showing YTHDF1-FOXM1 mRNA binding in the HEC-1-B cells incubated with erianin. (G) Results of the RIP assays showing YTHDF1-FOXM1 mRNA binding in the HEC-1-B cells with silenced YTHDF1. The data are presented as the means $\pm$ SDs; * P <0.05, ** P <0.01, and *** P <0.001 compared with the NC (control group).

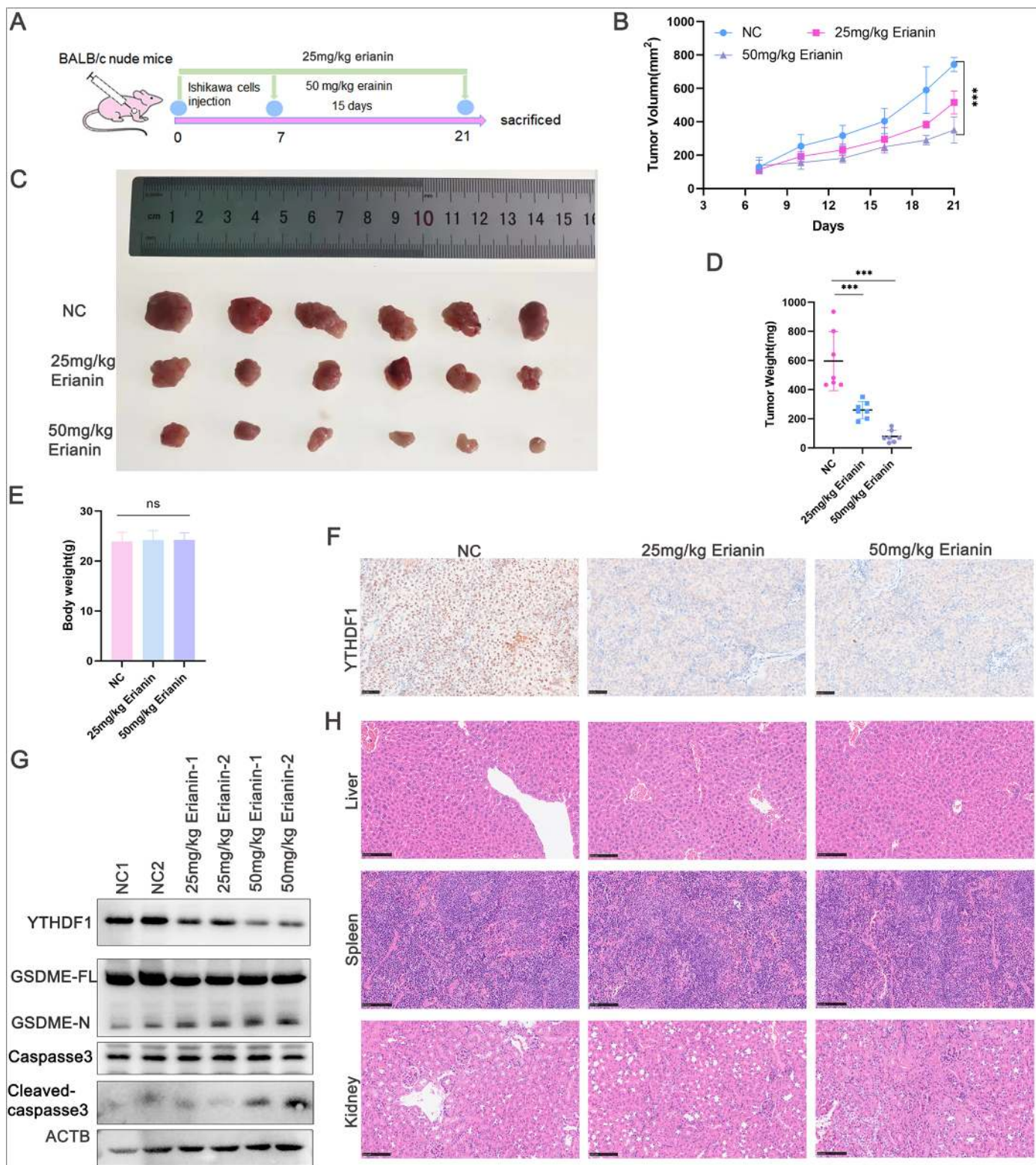


Figure 9

Erianin suppresses EC tumour growth *in vivo*. (A) Scheme of the animal experiments. (B) Tumor growth curves showing the effects of erianin on EC tumor xenograft growth (n = 6). (C) Erianin inhibits subcutaneous xenograft growth. (n = 6). (D) Tumor weight. (E) Body weights of the mice at the end of the study. (F) IHC staining of YTHDF1 in xenografts. Scale bar, 50 μ m. (G) The protein levels of YTHDF1, caspase-3, cleaved-caspase3, and GSDME in tumor tissues. (H) Representative images of livers, spleens,

and kidneys stained with H&E at the end of the study. Scale bar, 100 μm . The data are presented as the means $\pm$ SDs, n=6 ***P<0.001 compared with NC (control group). ns, not significant.

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