



YAP1 Suppression by ZDHHC7 Is Associated with Ferroptosis Resistance and Poor Prognosis in Ovarian Clear Cell Carcinoma

Yoko Furutake¹, Ken Yamaguchi¹, Koji Yamanoi¹, Sachiko Kitamura¹, Shiro Takamatsu¹, Mana Taki¹, Masayo Ukita², Yuko Hosoe¹, Ryusuke Murakami¹, Kaoru Abiko³, Akihito Horie¹, Junzo Hamanishi¹, Tsukasa Baba⁴, Noriomi Matsumura⁵, and Masaki Mandai¹

ABSTRACT

Ovarian clear cell carcinoma (OCCC), which has unique clinical characteristics, arises from benign endometriotic cysts, forming an oxidative stress environment because of excess iron accumulation, and exhibits poor prognosis, particularly in advanced stages owing to resistance to conventional therapeutics. Ferroptosis is an iron-dependent form of programmed cell death induced by lipid peroxidation and controlled by Hippo signaling. We hypothesized that overcoming ferroptosis resistance is an attractive strategy because OCCC acquires oxidative stress resistance during its development and exhibits chemoresistant features indicative of ferroptosis resistance. This study aimed to determine whether OCCC is resistant to ferroptosis and clarify the mechanism underlying resistance. Unlike ovarian high-grade serous carcinoma cells, OCCC cells were exposed to oxidative stress. However, OCCC cells remained unaffected by lipid

peroxidation. Cell viability assays revealed that OCCC cells exhibited resistance to the ferroptosis inducer erastin. Moreover, Samroc analysis showed that the Hippo signaling pathway was enriched in OCCC cell lines and clinical samples. Furthermore, patients with low expression of nuclear yes-associated protein 1 (YAP1) exhibited a significantly poor prognosis of OCCC. Moreover, YAP1 activation enhanced ferroptosis in OCCC cell lines. Furthermore, suppression of zinc finger DHHC-type palmitoyltransferase 7 (ZDHHC7) enhanced ferroptosis by activating YAP1 in OCCC cell lines. Mouse xenograft models demonstrated that ZDHHC7 inhibition suppressed tumor growth via YAP1 activation by erastin treatment. In conclusion, YAP1 activation regulated by ZDHHC7 enhanced ferroptosis in OCCC. Thus, overcoming ferroptosis resistance is a potential therapeutic strategy for OCCC.

Introduction

Ovarian cancer is the fifth leading cause of female mortality in the United States and the most severe gynecologic malignancy (1). Compared with other histologic subtypes, including high-grade serous carcinoma (HGSC), advanced stage ovarian clear cell carcinoma (OCCC) exhibits a poor prognosis because of chemoresistance (2). We previously demonstrated that exposure to iron-rich components within endometriotic cysts leads to the development of resistance to oxidative stress during carcinogenesis (3–5). Although the dysfunction of mitochondrial metabolism and redox homeostasis is known to cause chemoresistance (6), the mechanism underlying resistance to oxidative stress in OCCC remains unclear. Therefore, it is necessary to elucidate the biological mechanisms

underlying resistance to stressful environments to overcome the unfavorable behaviors of OCCC.

Ferroptosis is an iron-dependent form of cell death characterized by lipid peroxidation, which differs from apoptosis (7). The process of ferroptosis is controlled directly by glutathione peroxidase 4 (GPX4), which neutralizes lipid peroxides, and indirectly by the depletion of its substrate glutathione (GSH; ref. 8). Ferroptosis has been reported to be a novel therapeutic strategy for malignancies that exhibit resistance to current therapies (9). The depletion of GSH and GPX4 induces ferroptosis in cells with clear cell morphology (10, 11). OCCC develops in oxidative stress environments containing iron-rich components, indicating the proliferation of OCCC cells in ferroptotic environments. Nevertheless, the mechanism underlying the survival of OCCC cells in iron-rich environments remains unclear.

Some studies have indicated the role of yes-associated protein 1 (YAP1) and transcriptional coactivator with PDZ-binding motif (TAZ) in inducing ferroptosis in cancer cells (12, 13). YAP1, a tumor promoter, is activated in various cancers and plays a critical role in excessive cell proliferation (14). It is related to poor prognosis in several malignancies, including ovarian cancer (15–17). However, the role of YAP1 in OCCC has not yet been elucidated. In addition to TAZ, YAP1 is one of the major downstream effectors of the Hippo signaling pathway (18, 19). Furthermore, scribble planar cell polarity protein (SCRIB) is a major protein involved in cell polarity (20) and affects YAP1 activity. A recent study demonstrated that zinc finger DHHC-type palmitoyltransferase 7 (ZDHHC7) regulates SCRIB through S-palmitoylation, and ZDHHC7 knockout leads to SCRIB mislocalization and YAP1 activation (21).

¹Department of Gynecology and Obstetrics, Graduate School of Medicine and Faculty of Medicine, Kyoto University, Kyoto, Japan. ²Department of Obstetrics and Gynecology, Shizuoka General Hospital, Shizuoka, Japan. ³Department of Obstetrics and Gynecology, National Hospital Organization Kyoto Medical Center, Kyoto, Japan. ⁴Department of Obstetrics and Gynecology, School of Medicine, Iwate Medical University, Iwate, Japan. ⁵Department of Obstetrics and Gynecology, Faculty of Medicine, Kindai University, Osaka, Japan.

Corresponding Author: Ken Yamaguchi, Department of Gynecology and Obstetrics, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: soulken@kuhp.kyoto-u.ac.jp

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This study aimed to determine whether OCCC exhibits resistance to ferroptosis and to clarify the underlying mechanism of resistance, which would be a potential therapeutic target.

Materials and Methods

Study approval

This study was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (approval number: G1322) and complied with the Declaration of Helsinki. Written informed consent was obtained from all participants via an opt-in approach (wherein participants were given a printed informed consent form) or an opt-out approach (wherein participants were informed about the study through the website). All animal studies were approved by the Kyoto University Animal Research Committee.

Cell culture

A total of 12 ovarian cancer cell lines were used for the cell viability assay: RMG-I (RRID: CVCL_1662), RMG-V (RRID: CVCL_M716), KOC-5C (RRID: CVCL_5306), KOC-7C (RRID: CVCL_5307), JHOC-7 (RRID: CVCL_4641), JHOC-8 (RRID: CVCL_4642), SK-OV-3 (RRID: CVCL_0532), HEY (RRID: CVCL_0297), OVCA420 (RRID: CVCL_3935), OVCA433 (RRID: CVCL_0475), A2780 (RRID: CVCL_0134), and TYK-nu (RRID: CVCL_1776). RMG-I and RMG-V cell lines were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (RRID: SCR_023187; RMG-I, JCRB1072; RMG-V, JCRB1729). JHOC-7 and JHOC-8 were purchased from RIKEN BioResource Center (RRID: SCR_003250; JHOC-7; RCB1688, JHOC-8; RCB1723). KOC-5C, KOC-7C, SK-OV-3, HEY, OVCA420, OVCA433, A2780, and TYK-nu were kindly provided by Dr. Susan K Murphy of Duke University in June 2007. RMG-I cells were cultured in F-12 (#11765, Thermo Fisher Scientific, Waltham, MA, USA), RMG-V, KOC-5C, KOC-7C, SK-OV-3, HEY, OVCA420, OVCA433, A2780, and TYK-nu cells were cultured in RPMI1640 (#R8758, Sigma-Aldrich, St. Louis, MO, USA). JHOC-7 and JHOC-8 cells were cultured in DMEM/F-12 (#D8437, Sigma-Aldrich). All cells were supplemented with penicillin and streptomycin (100 IU/mL penicillin and 100 µg/mL streptomycin; #26253-84, Nacalai Tesque, Kyoto, Japan) and 10% (v/v) heat-inactivated FBS (#S1810, Bio-West, San Marcos, TX, USA) at 37°C under 5% CO₂. Cell line authentication was performed using short tandem repeat analysis (BEX Co., Ltd, Tokyo, Japan) for all cell lines obtained within the past 3 years. All experiments were performed using *Mycoplasma*-free cells, which were tested using Mycoplasma Detection Kit (#G238, Applied Biological Materials, Richmond, BC, Canada) after thawing a new stock. Cell lines within 20 passages were used for further analysis.

Cell viability assay

An equal number of cells were seeded into 96-well plates. At 72 hours after treatment, cell viability was assessed in response to erastin (#E7781, Sigma-Aldrich), ML210 (#SML0521, Sigma-Aldrich), DMSO (#031-24051, Wako, Osaka, Japan), and ferrostatin-1 (#SML0583, Sigma-Aldrich) using the WST-8 assay via Cell Count Reagent SF (#07553-44, Nacalai Tesque) according to the manufacturer's instructions. Absorbance at 450 nm was measured using an iMark microplate reader (#168-1130J, Bio-Rad, Hercules, CA, USA). Furthermore, after treatment with erastin and

ML210, IC₅₀ values were calculated using GraphPad Prism 9 software (RRID: SCR_002798, GraphPad, San Diego, CA, USA).

IHC analysis of human ovarian cancer specimens

Surgical specimens were collected from patients with ovarian cancer who underwent primary surgery at Kyoto University Hospital between 1998 and 2017. Patient clinical characteristics, including age, stage, histology, progression-free survival (PFS), and overall survival (OS), were extracted from the medical records.

Immunostaining of formalin-fixed, paraffin-embedded clinical specimens was performed using the following antibodies: 8-hydroxy-deoxyguanosine (8-OHdG, #MOG-100P, Japan Institute for the Control of Aging, Shizuoka, Japan; 1:10 dilution in PBS), malondialdehyde {MDA, #MMD-030n, Japan Institute for the Control of Aging; 1:200 dilution in PBS-B [PBS containing 1% (v/v) BSA (#A7284, Sigma)]}, YAP1 (#sc-101199, Santa Cruz, Dallas, TX, USA; 1:200 dilution in PBS-T [PBS containing 0.1% (v/v) Tween-20 (#35624-15, Nacalai Tesque)]}, and ZDHHC7 (#ab254954, Abcam, Cambridge, UK; 1:80 dilution in PBS). The conditions for each antigen retrieval step are described in Supplementary Table S1. All tissue sections with primary antibodies were incubated overnight at 4°C and stained with horseradish peroxidase (HRP)-conjugated secondary antibodies (#424022, #424032, Nichirei Biosciences Inc., Tokyo, Japan). Peroxidase activity was visualized via staining with 3,3'-diaminobenzidine (#D4418, Sigma). Hematoxylin was used as a counterstain.

The IHC score was calculated as follows: staining intensity score × percentage of tumor cells stained. The staining intensity was classified as negative 0, weak 1, or strong 2. For YAP1, the stained area increased from 0% to 100% in increments of 10%. Based on the YAP1 expression score, patients with OCCC were categorized into two groups: YAP1-high, score ≥ 1 (*n* = 49) and YAP1-low, score < 1 (*n* = 23). Images were captured using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Transient transfection with YAP4SA

YAP1-activated type of RMG-I cells were obtained via transient transfection with YAP4SA (S109; RRID: Addgene_33081, provided by Kunliang Guan of University of California, San Diego, CA, USA; ref. 22). An empty vector was constructed based on YAP4SA vector using Q5 Hot Start High-Fidelity (#M0494S, New England Biolabs, Hitchin, Hertfordshire, UK) and KLD Enzyme Mix (#M0554, BioLabs) according to the manufacturer's instructions. Cells were transfected with Lipofectamine 3000 transfection reagent (#L3000-015, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 48 hours. The transfection efficacy was demonstrated by the overexpression of YAP1 target genes [connective tissue growth factor (*CTGF*), cysteine-rich angiogenic inducer 61 (*CYR61*), and ankyrin repeat domain 1 (*ANKRD1*)] confirmed using RT-PCR as well as YAP1 nuclear translocation confirmed using immunofluorescence. The primer sequences are listed in Supplementary Table S2.

Stable knockdown using short hairpin RNAs

RMG-I and RMG-V stable knockdown cells of ZDHHC7, LATS1, and LATS2; RMG-V stable knockdown cells of HNF1 homeobox B (*HNF1B*); and control cells were established via transduction using lentiviral particles of short hairpin RNAs (shRNA) or a nonsilencing control (#SHC002, Sigma-Aldrich) in the presence of polybrene (#12996-81, Nacalai Tesque). The cell lines were named as follows: RMG-I-shZDHHC7_1 and RMG-I-shZDHHC7_2; RMG-V-shZDHHC7_1 and RMG-V-shZDHHC7_2; RMG-I-shLATS1_1

and RMG-I-shLATS1_2; RMG-I-shLATS2; RMG-V-shLATS1_1 and RMG-I-shLATS1_2; RMG-V-shLATS2; RMG-V-shHNF1B_1 and RMG-V-shHNF1B_2; RMG-I-control; and RMG-V-control. The shRNA sequences are listed in Supplementary Table S3. For lentivirus production, in addition to the target gene plasmids, HEK293-FT (RRID: CVCL_6642) cells were transfected with the lentiviral packaging plasmid psPAX2 (RRID: Addgene_12260, provided by Didier Trono of École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) and the VSV-G envelope-expressing plasmid pMD2.G (RRID: Addgene_12259, provided by Didier Trono) using polyethylenimine (#24765, Polysciences, Warrington, PA, USA). The medium was replaced with fresh medium 6 hours after transfection. Viral particles were collected and filtered through a 0.45- μ m Millex filter (#SLHVR33RS, Merck Millipore, Burlington, MA, USA) 48 hours after transfection. Stable knockdown cells were established using puromycin (#ant-pr-1, InvivoGen, San Diego, CA, USA). The transduction efficacy of the target genes was confirmed using RT-PCR and immunoblotting.

RNA extraction and qRT-PCR

Total RNA was extracted from cells using the RNeasy micro kit (#74104, Qiagen, Hilden, Germany). cDNA synthesis was performed using the ReverTra Ace qPCR RT kit (#FSQ-101, TOYOBO, Osaka, Japan) according to the manufacturer's instructions. In addition, gene amplification was performed using PowerUp SYBR Green Master Mix (#A25742, Thermo Fisher) via the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The primer sequences used in this experiment are listed in Supplementary Table S2.

Immunoblotting

Whole-cell lysates were prepared using RIPA lysis buffer (#89900, Thermo Fisher Scientific) supplemented with a phosphatase inhibitor cocktail (#07575-51, Nacalai Tesque) and protease inhibitor cocktail (#03969-21, Nacalai Tesque) and were sonicated for 15 minutes. Then, protein concentrations were determined using the BCA Protein Assay kit (#23227, Thermo Fisher Scientific). The proteins were denatured in 2 $\times$ Laemmli sample buffer (#1610737, Bio-Rad) and heated to 95°C for 5 minutes. Equal amounts of the samples were loaded onto mPAGE 4% to 12% Bis-Tris Gel (#MP41G12, Merck, Darmstadt, Germany) for the detection of LATS1, LATS2, and actin beta (ACTB) or onto NuPAGE 4% to 12% Bis-Tris Gel (#NP0322BOX, Invitrogen) for detecting ZDHHC7 and tubulin alpha 1b (TUBA1B). The samples were electrophoresed and transferred onto polyvinylidene fluoride membranes (#1620177, Bio-Rad). After blocking with a blocking buffer (#03953-95, Nacalai Tesque), the membranes were washed twice with TBS-T. Then, they were probed overnight at 4°C with the following primary antibodies: LATS1 rabbit mAb [#3477S, Cell Signaling Technology, MA, USA; 1:1,000 dilution in solution 1 (#NKB-201, TOYOBO)], LATS2 rabbit polyclonal antibody (#20276-1-AP, Proteintech, Rosemont, IL, USA; 1:500 dilution in solution 1), ACTB rabbit polyclonal antibody (#4967, Cell Signaling Technology; 1:1,000 dilution in solution 1), ZDHHC7 rabbit polyclonal antibody (#ab138210, Abcam; 1:500 dilution in solution 1), and TUBA1B mouse mAb (#66031-1-Ig, Proteintech; 1:2,000 dilution in solution 1). The membranes were washed three times with TBS-T and incubated for 1 hour with the indicated secondary antibodies. Subsequently, the HRP-linked anti-rabbit IgG antibody (#7074, CST, 1:3,000) was used for the detection of LATS1, LATS2, ACTB, and ZDHHC7, whereas HRP-linked anti-mouse IgG antibody (#7076, CST, 1:

3,000) was used for the detection of TUBA1B. The blots were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (#34580, Thermo Fisher Scientific) and ChemiDox XRS+ System (Bio-Rad).

Immunofluorescence assay

A total of 4×10^4 mock- and YAP4SA-transfected cells or shZDHHC7 RMG-I and RMG-V cells were seeded into eight-well chambers (#SCS-N08, Matsunami Glass, Osaka, Japan) on the day before the experiment. The cells were fixed with 4% paraformaldehyde (#163-20145, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), permeabilized with PBS and Tween-20 for 20 minutes, and blocked with PBS-B (4% BSA in PBS solution) for 30 minutes. The cells were incubated for 2 hours at room temperature with the primary YAP1 antibody (#sc-101199, Santa Cruz, 1:100 dilution). Then, the cells were incubated for 1 hour at room temperature with the Alexa Fluor 647-conjugated secondary antibody (#ab150115, Abcam), and 4',6-diamidino-2-phenylindole (DAPI) counterstaining was performed (#P36935, Thermo Fisher Scientific). All images were captured using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

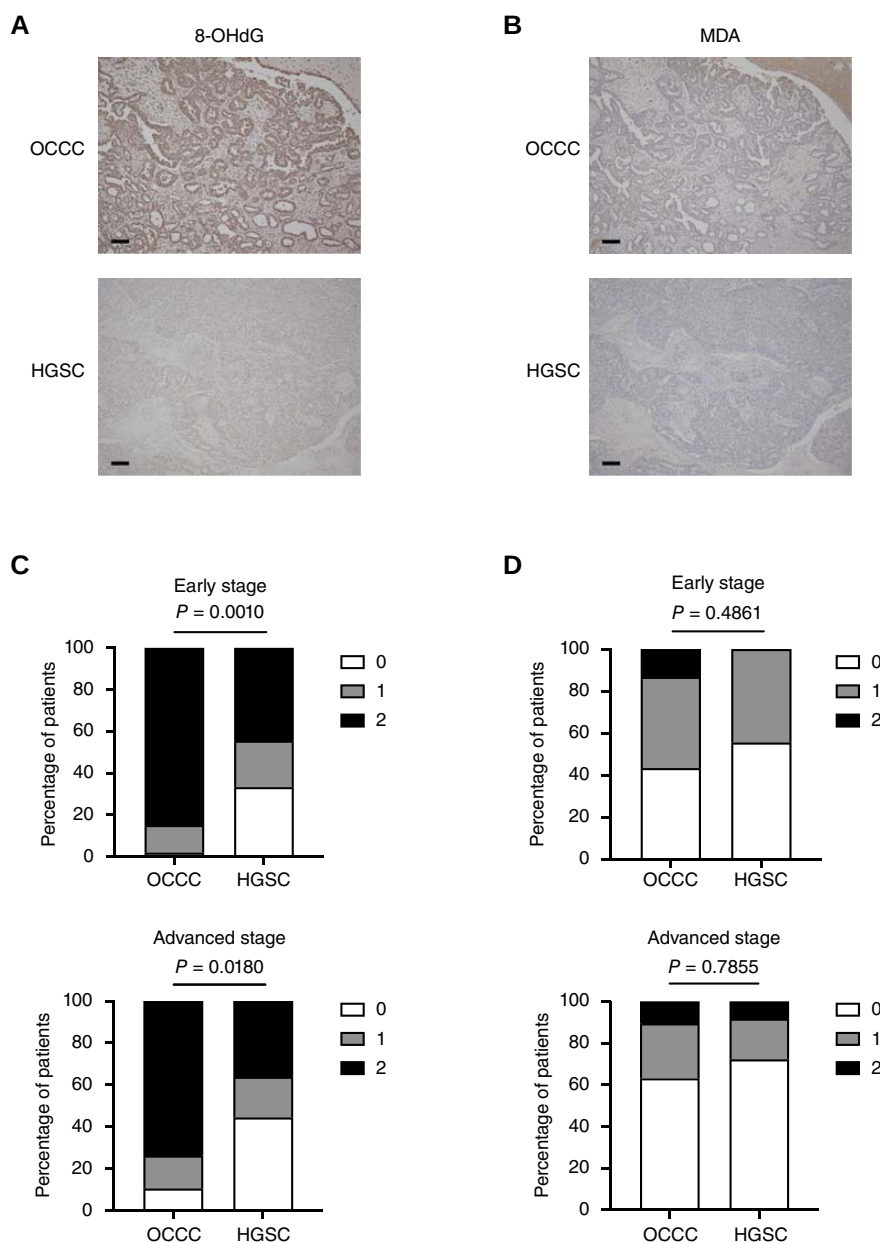
Mouse xenograft model

Six-week-old female athymic BALB/cAJcl-nu/nu mice (RRID: IMSR_JCL:MID-0001) were purchased from CLEA Japan, Inc. A total of 1.5×10^6 shZDHHC7 or control RMG-I cells were subcutaneously inoculated into the flanks of mice. At 3 days after inoculation, the mice were intraperitoneally injected with vehicle [65% D5W [5% dextrose (#049-31165, FUJIFILM Wako) in water]], 5% Tween-80 (#161-21621, FUJIFILM Wako), and 30% polyethylene glycol 400 (#161-09065, FUJIFILM Wako; ref. 12) or 25 mg/kg imidazole ketone erastin (IKE, #HY-115581, MedChemExpress, Monmouth Junction, NJ, USA) once daily. Tumor volume was measured using calipers every second day using the formula tumor volume (mm^3) = length $\times$ width $\times$ height $\times$ 1/2. The mice were sacrificed via carbon dioxide inhalation 3 weeks after inoculation based on ethical considerations.

Gene expression microarray analysis

Significantly differentially expressed genes (DEG) were detected using our previously published gene expression microarray data, GSE29175 (HG-U133A with 22,215 gene probes; ref. 23), which contains expression data from ovarian cancer cell lines, and GSE37290 (HG-U133plus2.0 with 54,613 gene probes; ref. 24), which contains expression data from HNF1B-knockdown OCCC cells. Moreover, gene expression microarray data from GSE39204 and GSE55512 (both HG-U133plus2.0; refs. 25, 26), which contain data about clinical ovarian cancer specimens obtained from 18 patients with clear cell carcinoma (CCC) and 25 patients with HGSC who underwent primary surgery at our institution, were assessed through the Gene Expression Omnibus website (RRID: SCR_005012).

Furthermore, microarray analysis was performed using R software (RRID: SCR_001905, version 4.0.4). Using the robust multi-array average method with the R package "affy" (RRID: SCR_012835), normalization of Affymetrix microarray datasets was performed. The gene symbol names of all datasets were mapped to the HG-U133A and HG-U133plus2 gene probe names. The top 50% of the probes were retained based on the highest average expression in each dataset used for gene set selection (GSE39204 and GSE55512). This subset of probes was then filtered based on the highest SD, resulting in the retention of 5,553 and 13,653 probes,

**Figure 1.**

Immunostaining for 8-OHdG and MDA in patients with clinical OCCC ($n = 72$) and HGSC ($n = 45$). **A**, Representative images of 8-OHdG IHC in clinical OCCC and HGSC samples. Scale bar, 100 μm . **B**, Representative images of MDA IHC in clinical OCCC and HGSC samples. Scale bar, 100 μm . **C**, Distribution of 8-OHdG IHC scores in patients with OCCC and HGSC in early (top) and advanced (bottom) stages. **D**, Distribution of MDA IHC scores in patients with OCCC and HGSC in early (top) and advanced (bottom) stages. Early stage: stages I and II; advanced stage: stages III and IV. P values were calculated using the χ^2 test.

respectively (27). DEG analysis was conducted between the histologic subtypes of CCC and HGSC cell lines or clinical samples using the Bioconductor package “SAGx” (RRID: SCR_006442). FDR q values were calculated based on Samroc P values using the R library “ P adjust.” Upregulated DEG probes in CCC that fulfilled the requirement of an FDR q value of < 0.05 remained as signatures of CCC DEGs. Commonly upregulated CCC DEGs (Supplementary Table S4) between GSE29175, GSE39204, and GSE55512 datasets were analyzed via Metascape (RRID: SCR_016620; ref. 28). Furthermore, enrichment analyses of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were performed using Metascape.

RNA sequencing analysis

Clinical information and bulk RNA sequencing data of 213 previously reported OCCC cases were kindly provided by the authors (29). FASTQ files were aligned with the human reference genome (GRCh38) using STAR (RRID: SCR_004463) v2.7.1a (30). Then, transcript abundances were normalized as transcripts per million values using RSEM (RRID: SCR_000262) v1.2.28 (31) based on gene annotations of Ensemble GRCh38.v99. The GOBP_REGULATION_OF_HIPPO_SIGNALING gene set was obtained from MSigDB (RRID: SCR_016863). The sample enrichment scores were calculated with ssGSEA using GenePattern software (RRID: SCR_003201; ref. 32).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (RRID: SCR_002798) and Rcmdr software (RRID: SCR_001905). The Mann–Whitney *U* test was used to assess the difference between two groups. A Spearman correlation coefficient was calculated to quantify the statistical dependence between the rankings of two variables. Prognostic factors for PFS and OS in patients with OCCC were assessed using log-rank tests with Kaplan–Meier curves. Univariate and multivariate analyses were performed using the Cox proportional hazards regression model. A *P* value of < 0.05 was considered to indicate statistical significance.

Data availability

The data generated and/or analyzed during this study are available within this article and its Supplementary Files. More detailed methods and materials generated in this study can be made available from the corresponding author upon reasonable request.

Results

OCCC cells are less affected by lipid peroxidation despite being exposed to oxidative stress

Compared with HGSC, the IHC scores of 8-OHdG, a marker of DNA damage induced by reactive oxygen species (ROS; ref. 33), were significantly higher in OCCC ($P < 0.0001$; **Fig. 1A**; Supplementary Fig. S1A). However, no significant difference was observed between the IHC scores of MDA, which is a product of lipid peroxidation and has been reported to represent ferroptosis (34), in OCCC and HGSC ($P = 0.0968$; **Fig. 1B**; Supplementary Fig. S1B). As the distribution of stages varied between OCCC and HGSC ($P < 0.0001$; Supplementary Table S5), IHC scores in OCCC and HGSC were assessed separately in early (I and II) and advanced stages (III and IV; Supplementary Table S6). In both early and advanced stages, the IHC scores of 8-OHdG were significantly higher in OCCC than in HGSC ($P = 0.0010$, early stage; $P = 0.0180$, advanced stage; **Fig. 1C**), whereas no significant differences were noted in the IHC scores of MDA between OCCC and HGSC ($P = 0.4861$, early stage; $P = 0.7855$, advanced stage; **Fig. 1D**). These results indicate that OCCC cells are less affected by lipid peroxidation despite exposure to an oxidative stress environment.

OCCC cells exhibit resistance to the ferroptosis inducer erastin through the Hippo signaling pathway

In response to IHC results of clinical samples, the tolerability of ferroptosis induction was assessed using six OCCC and six HGSC cell lines (Supplementary Table S7). The ferroptosis inducer erastin exhibited significantly higher IC_{50} values in OCCC than in HGSC ($P = 0.0152$; Supplementary Fig. S2A). This finding indicates the resistance of OCCC cells to the ferroptosis inducer.

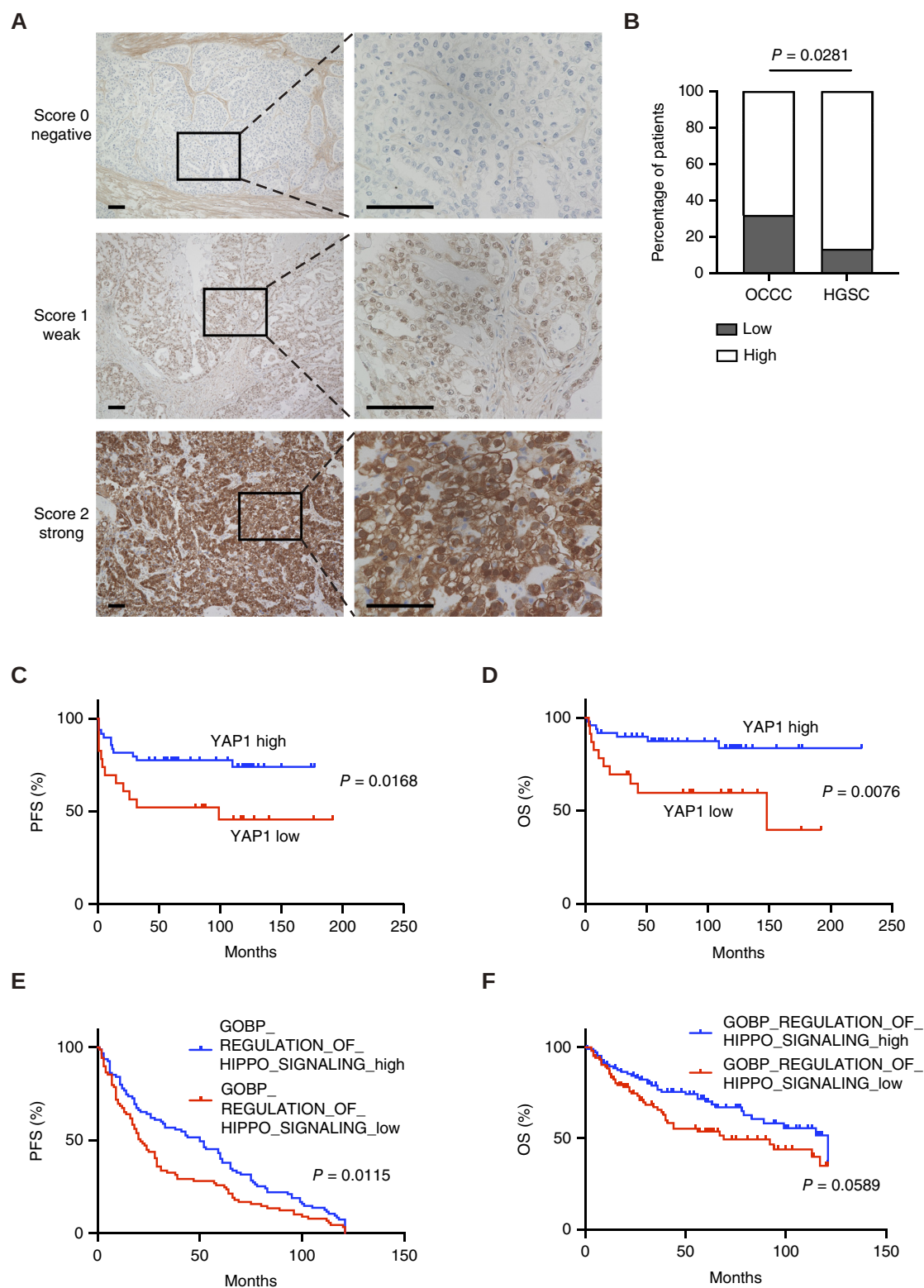
Samroc analysis was performed to determine the biological backgrounds of six OCCC and six HGSC cell lines using mRNA expression datasets (GSE29175; ref. 23). Compared with HGSC cell lines, 279 DEGs were detected in OCCC cell lines. Furthermore, Samroc analysis was performed using microarray datasets of ovarian cancer clinical samples from Kyoto University (GSE39204 and GSE55512; refs. 25, 26). A total of 3,137 DEGs were detected in clinical samples from Kyoto University, and 111 DEGs were commonly upregulated between OCCC cell lines and clinical samples from Kyoto University (Supplementary Fig. S2B; Supplementary Table S4). The KEGG pathway analysis of 111 DEGs using Meta-scape (28) revealed that 27 KEGG pathways were significantly

upregulated (Supplementary Table S8). The KEGG category of the Hippo signaling pathway was included in these 27 pathways.

Low nuclear YAP1 expression is a poor prognostic factor for OCCC

YAP1, a key molecule of the Hippo signaling pathway (18), is an inducer of ferroptosis (12, 13) and cell–cell adhesion (20, 35). Therefore, IHC analysis of OCCC and HGSC clinical samples was initially conducted to investigate the roles of YAP1 (**Fig. 2A**). Only 68.1% (49/72) of OCCC cases showed an IHC score of ≥ 1.0 , compared with 86.7% (39/45) of HGSC cases ($P = 0.0281$; **Fig. 2B**). In OCCC, patients with low expression of nuclear YAP1 exhibited a significantly higher recurrence rate than those with high expression ($P = 0.0389$; Supplementary Table S9). However, factors such as the International Federation of Gynecology and Obstetrics (FIGO) stage and the presence of residual tumor at primary surgery did not reveal any statistically significant differences between the high and low YAP1 expression groups ($P = 0.3898$ and $P = 0.7618$, respectively; Supplementary Table S9). Prognostic analyses of the Kaplan–Meier curves revealed that cases with low expression of nuclear YAP1 had significantly poorer PFS and OS than those with high expression ($P = 0.0168$, PFS; $P = 0.0076$, OS; **Fig. 2C and D**). Univariate Cox proportional regression analysis of the clinical samples revealed that FIGO stages III and IV, residual tumor at primary surgery, and low nuclear YAP1 expression were significantly poor prognostic factors for PFS [$P < 0.0001$, HR = 10.82, 95% confidence interval (CI): 4.540–25.79; $P < 0.0001$, HR = 13.40, 95% CI, 5.627–31.91; and $P = 0.0231$, HR = 2.533, 95% CI, 1.136–5.647, respectively; **Table 1**] and OS ($P < 0.0001$, HR = 14.25, 95% CI, 4.522–44.91; $P < 0.0001$, HR = 20.16, 95% CI, 6.333–64.19; and $P = 0.0122$, HR = 3.459, 95% CI, 1.311–9.127, respectively; **Table 1**). Multivariate analyses of the clinical samples revealed that residual tumor at primary surgery and low nuclear YAP1 expression were independent poor prognostic factors for PFS ($P = 0.0113$, HR = 6.826, 95% CI, 1.545–30.16; $P = 0.0379$, HR = 2.476, 95% CI, 1.052–5.829, respectively; **Table 1**) and OS ($P = 0.0137$, HR = 17.25, 95% CI, 1.792–166.0; $P = 0.0177$, HR = 3.609, 95% CI, 1.249–10.42, respectively; **Table 1**).

Because nuclear expression of YAP1 is suggested to be associated with regulation of Hippo signaling, activity of Hippo signaling is applied for the validation analysis. We consequently proceeded to use GOBP_REGULATION_OF_HIPPO_SIGNALING as the analytical variable because a positive correlation was observed between the IHC scores of YAP1 and GOBP_REGULATION_OF_HIPPO_SIGNALING scores ($R = 0.1957$, $P = 0.4813$; Supplementary Fig. S3), indicating that cases with high GOBP_REGULATION_OF_HIPPO_SIGNALING scores corresponded to those with high nuclear YAP1 expression. In the datasets with 213 previously reported OCCC cases (29), Kaplan–Meier analysis of the GOBP_REGULATION_OF_HIPPO_SIGNALING gene signature revealed that cases with low scores had significantly poorer PFS and tended to exhibit poorer OS than those with high scores ($P = 0.0115$, PFS; $P = 0.0589$, OS; **Fig. 2E and F**). Multivariate Cox proportional regression analysis of the previously reported cohort (29) revealed that advanced FIGO stage and low signature scores were significantly poor prognostic factors for PFS ($P = 0.0010$, HR = 2.094, 95% CI, 1.350–3.248; $P = 0.0079$, HR = 1.577, 95% CI, 1.127–2.208, respectively; Supplementary Table S10). Moreover, low age, advanced FIGO stage, residual tumor at primary surgery, and low signature scores were significantly poor prognostic factors for OS ($P = 0.0378$, HR = 0.5944, 95% CI, 0.3638–0.9711; $P < 0.0001$, HR = 4.436, 95% CI, 2.480–7.936; $P = 0.0376$, HR = 1.808, 95% CI, 1.034–3.159; and $P = 0.0371$, HR = 1.690, 95% CI, 1.032–

**Figure 2.**

Low nuclear YAP1 expression is associated with poor prognosis in patients with OCCC. **A**, Representative images of YAP1 IHC in clinical samples. Scale bar, 100 μm. **B**, Distribution of YAP1 IHC scores in patients with OCCC and HGSC. *P* values were calculated using the Fisher exact test. **C** and **D**, Kaplan-Meier analysis of PFS (**C**) and OS (**D**) curves according to YAP1 expression in patients with OCCC in Kyoto University Hospital. **E** and **F**, Kaplan-Meier analysis of PFS (**E**) and OS (**F**) curves according to GOBP_REGULATION_OF_HIPPO_SIGNALING signature score in OCCC patients with validation datasets. *P* values were calculated using log-rank tests.

Table 1. Multivariate analysis of prognostic factors associated with PFS and OS in patients with OCCC at Kyoto University.

PFS	Univariate			Multivariate		
Parameter	HR	95% CI	P	HR	95% CI	P
Age (years) (<60 vs. ≥60)	1.409	0.6153–3.228	0.4171	1.018	0.4146–2.502	0.9682
FIGO stage (I and II vs. III and IV)	10.82	4.540–25.79	<0.0001*	2.416	0.5298–11.02	0.2545
Residual tumor (no vs. yes)	13.40	5.627–31.91	<0.0001*	6.826	1.545–30.16	0.0113*
YAP1 expression (high vs. low)	2.533	1.136–5.647	0.0231*	2.476	1.052–5.829	0.0379*
OS	Univariate			Multivariate		
Parameter	HR	95% CI	P	HR	95% CI	P
Age (<60 vs. ≥60)	1.665	0.6298–4.400	0.3041	1.177	0.4148–3.3339	0.7595
FIGO stage (I and II vs. III and IV)	14.25	4.522–44.91	<0.0001*	1.312	0.1378–12.49	0.8134
Residual tumor (no vs. yes)	20.16	6.333–64.19	<0.0001*	17.25	1.792–166.0	0.0137*
YAP1 expression (high vs. low)	3.459	1.311–9.127	0.0122*	3.609	1.249–10.42	0.0177*

*Statistical significance.

2.769, respectively; Supplementary Table S10). These findings indicate that low nuclear YAP1 expression is a poor prognostic factor for OCCC.

YAP1 activation induces ferroptosis in OCCC

To evaluate whether LATS1 and LATS2 induce ferroptosis through YAP1 activation in OCCC, we focused on LATS1/LATS2, which are key molecules of Hippo signaling and are known to suppress YAP1 activation. First, OCCC cell lines with *LATS1/2* knockdown were established as follows: RMG-V-sh*LATS1* (two cell lines: sh*LATS1_1* and sh*LATS1_2*), RMG-V-sh*LATS2*, RMG-I-sh*LATS1* (two cell lines: sh*LATS1_1* and sh*LATS1_2*), and RMG-I-sh*LATS2* (Supplementary Fig. S4A and S4B). RMG-V-sh*LATS1* and RMG-V-sh*LATS2* cells exhibited higher nuclear YAP1 expression than control cells (Fig. 3A). The knockdown of *LATS1/2* in RMG-V cells induced the overexpression of *CTGF*, *CYR61*, and *ANKRD1*, which are YAP1 target genes (Fig. 3B). The IC₅₀ values of RMG-V-sh*LATS1* and RMG-V-sh*LATS2* cells were significantly lower than those of RMG-V-control cells [IC₅₀ values (μmol/L) = 7.850 in control, 1.189 in sh*LATS1_1*, 2.173 in sh*LATS1_2*, and 5.021 in sh*LATS2*; *P* < 0.0001, *P* < 0.0001, and *P* = 0.0001 at a concentration of 6 μmol/L erastin, respectively; Fig. 3C]. After the knockdown of *LATS1* and *LATS2*, RMG-V-sh*LATS1* and RMG-V-sh*LATS2* cells showed significant susceptibility to ML210 compared with RMG-V-control cells (*P* = 0.0002, *P* = 0.0001, and *P* = 0.0013 at a concentration of 1 μmol/L ML210, respectively; Supplementary Fig. S4C). In addition, the ferroptosis inhibitor ferrostatin-1 alleviated cell death (Supplementary Fig. S4D and S4E).

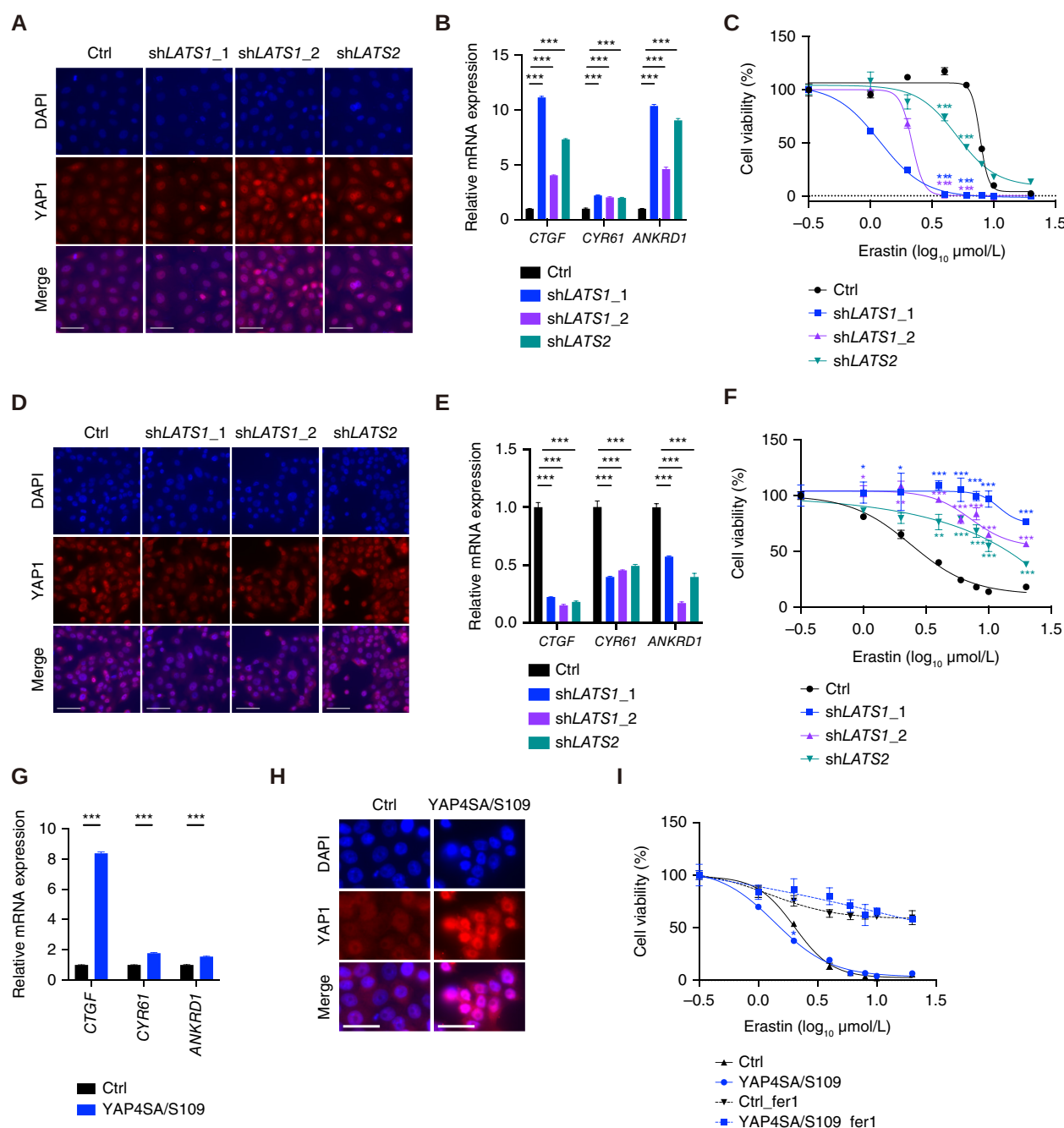
Contrarily, *LATS1/2* played a distinct role in YAP1 signaling in RMG-I cells. Compared with RMG-I-control cells, immunofluorescence assays showed no increased nuclear localization of YAP1 in RMG-I-sh*LATS1* and RMG-I-sh*LATS2* cells (Fig. 3D), indicating the suppression of YAP1 activity in both cell lines. The expression of *CTGF*, *CYR61*, and *ANKRD1* was suppressed in RMG-I-sh*LATS1* and RMG-I-sh*LATS2* cells (Fig. 3E). The IC₅₀ values of RMG-I-sh*LATS1* and RMG-I-sh*LATS2* cells were significantly higher than those of RMG-I-control cells [IC₅₀ values (μmol/L) = 2.421 in RMG-I-control, 12.10 in RMG-I-sh*LATS1_1*, 7.217 in RMG-I-sh*LATS1_2*, and incalculable in RMG-I-sh*LATS2*; *P* < 0.0001, *P* < 0.0001, and *P* = 0.0012 at a concentration of 10 μmol/L erastin,

respectively; Fig. 3F). Furthermore, after treatment with ML210, another ferroptosis inducer, the IC₅₀ values of RMG-I-sh*LATS1* and RMG-I-sh*LATS2* cells were significantly higher than those of RMG-I-control cells [IC₅₀ values (μmol/L) = 0.4881 in RMG-I-control, 0.6188 in RMG-I-sh*LATS1_1*, 0.6279 in RMG-I-sh*LATS1_2*, and 0.6011 in RMG-I-sh*LATS2*; *P* = 0.0030, *P* = 0.0084, and *P* = 0.0107 at a concentration of 0.6 μmol/L ML210, respectively; Supplementary Fig. S4F and S4G]. These findings indicate that ferroptosis is induced by YAP1 activation, although it is not regulated by *LATS1/2* in RMG-I cells.

We established constitutively active forms of YAP1 and RMG-I-YAP4SA cells by transfecting the cells with YAP4SA (S109) to determine whether YAP1 activation in RMG-I leads to enhanced ferroptosis. RMG-I-YAP4SA cells induced the overexpression of *CTGF*, *CYR61*, and *ANKRD1* (Fig. 3G) and increased nuclear localization of YAP1 (Fig. 3H). As expected, RMG-I-YAP4SA cells exhibited sensitivity to erastin [IC₅₀ values (μmol/L) = 2.025 in RMG-I-control and 1.388 in RMG-I-YAP4SA; *P* = 0.0004 at a concentration of 2 μmol/L; Fig. 3I] and ML210 [IC₅₀ values (μmol/L) = 0.4406 in RMG-I-control and 0.2796 in RMG-I-YAP4SA; *P* = 0.0037 at a concentration of 0.4 μmol/L; Supplementary Fig. S4H]. Furthermore, the ferroptosis inhibitor ferrostatin-1 alleviated cell death (Fig. 3I; Supplementary Fig. S4H). These findings indicate that YAP1 plays a robust role in inducing ferroptosis and that another mechanism other than *LATS1/2* exists for the activation of YAP1 in OCCC.

ZDHHC7 knockdown enhances ferroptosis through YAP1 activation in OCCC *in vitro*

We focused on ZDHHC7 to identify the molecule that regulates YAP1 signaling in OCCC. ZDHHC7 is a YAP1 regulator (21) and falls within the OCCC-specific gene set known as the OCCC signature (23). *ZDHHC7* expression was significantly higher in OCCC than in ovarian HGSC clinical samples (Fig. 4A). Furthermore, IHC analysis revealed significantly higher expression of ZDHHC7 in OCCC than in HGSC (*P* < 0.0001; Fig. 4B). In total, 21 of 31 cases with low ZDHHC7 showed low expression of nuclear YAP1 and 14 of 42 cases with high ZDHHC7 exhibited low expression of YAP1 (Supplementary Table S11), indicating that approximately half OCCC cases suppress YAP1 signaling by ZDHHC7. Owing to the

**Figure 3.**

YAP1 activation induces ferroptosis in OCCC. **A**, **D**, and **H**, Immunofluorescence staining of YAP1 in RMG-V-shLATS1/LATS2 (**A**), RMG-I-shLATS1/LATS2 (**D**), and RMG-I-YAP4SA (**H**) cells. Scale bar, 50 μm (**A** and **D**) and 25 μm (**H**). **B** and **E**, RT-PCR of YAP1 target genes in RMG-V-shLATS1/LATS2 (**B**) and RMG-I-shLATS1/LATS2 (**E**; $n = 3$). **C** and **F**, Growth inhibition curves of RMG-V-shLATS1/LATS2 (**C**) and RMG-I-shLATS1/LATS2 (**F**) cells treated with erastin for 72 hours ($n = 3$). **G**, RT-PCR of YAP1 target genes and immunoblotting of RMG-I-YAP4SA cells ($n = 3$). **I**, Growth inhibition curves of RMG-I-YAP4SA (S109) cells treated with erastin with or without 1 μmol/L ferrostatin-1 for 72 hours ($n = 3$). Cell viabilities were normalized to DMSO ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (unpaired t test). Averaged data are presented as means with SEM.

established role of HNF1B in OCCC carcinogenesis and chemoresistance, particularly its impact on glycolytic processes and oxidative phosphorylation (23, 36), RMG-V cell lines with *HNF1B* knockdown were established to assess its expression. We revealed that *HNF1B* knockdown downregulated the expression of *ZDHHC7*

($P < 0.0001$; **Fig. 4C**). Moreover, we assessed *ZDHHC7* expression between sh*HNF1B* and control strains of OCCC RMG-II cells (GSE37290; ref. 24). *HNF1B* knockdown downregulated the expression of *ZDHHC7* ($P = 0.0005$; **Fig. 4D**), indicating that *ZDHHC7* is a downstream gene of *HNF1B*.

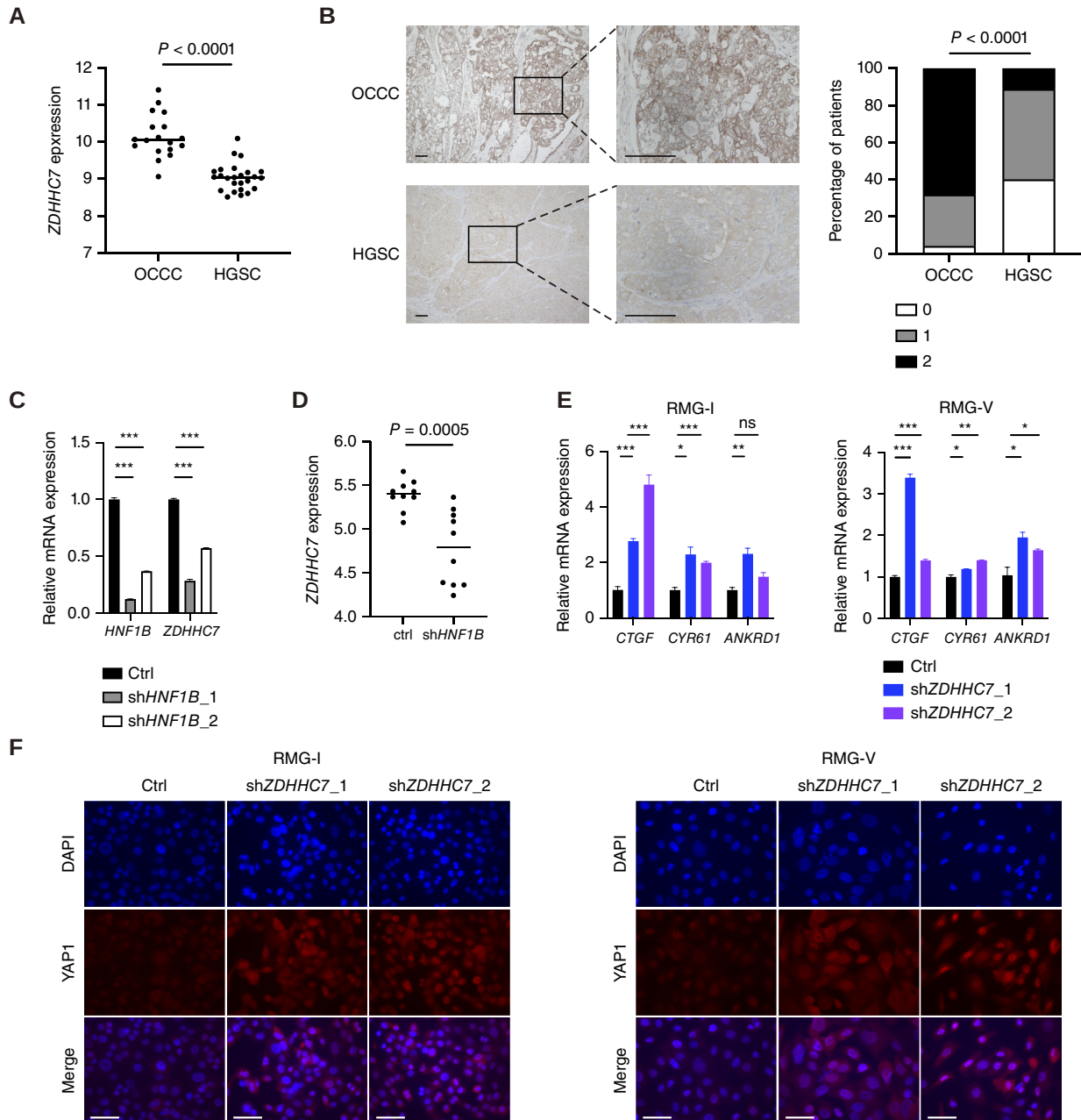
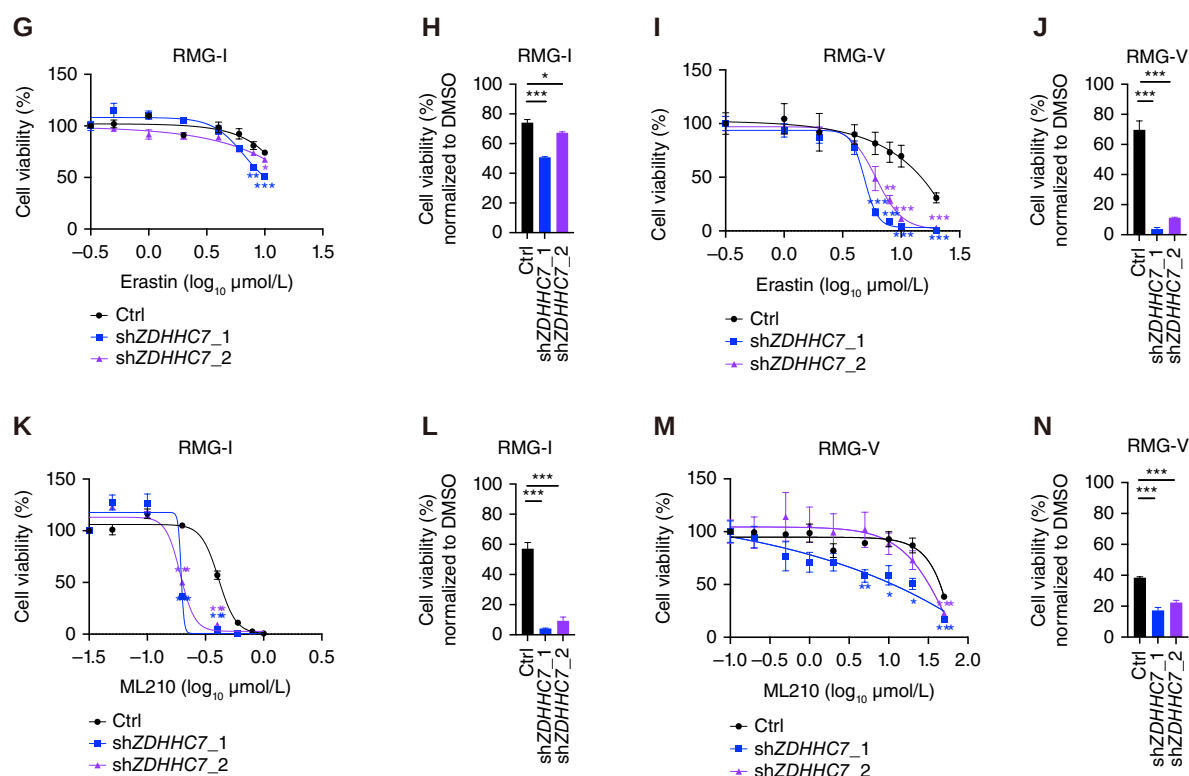


Figure 4.

ZDHHC7 knockdown enhances ferroptosis through YAP1 activation *in vitro*. **A**, Log₂-normalized values of *ZDHHC7* mRNA expression in clinical samples obtained from Kyoto University from 18 patients with OCCC and 25 patients with HGSC (GSE39204 and GSE55512). *P* values were calculated using the Mann-Whitney *U* test. **B**, Representative images of *ZDHHC7* immunostaining in clinical OCCC and HGSC samples (left). Distribution of IHC scores in patients with OCCC and HGSC at Kyoto University Hospital (right). *P* values were calculated using the χ^2 test. **C**, RT-PCR was performed to analyze the expression of *HNF1B* and *ZDHHC7* in RMG-V cell lines ($n = 3$). **D**, Log₂-normalized values of *ZDHHC7* mRNA expression in control and shHNF1B RMG-I cell lines (GSE37290). *P* values were calculated using the Mann-Whitney *U* test. **E**, RT-PCR of YAP1 target genes in RMG-I-sh*ZDHHC7* and RMG-V-sh*ZDHHC7* cells ($n = 3$). **F**, Immunofluorescence staining of YAP1 in RMG-I-sh*ZDHHC7* and RMG-V-sh*ZDHHC7* cells. Scale bar, 50 μ m. **G–J**, Growth inhibition curves of (Continued on the following page.)

We established *ZDHHC7*-knockdown cell lines using RMG-I and RMG-V to determine whether *ZDHHC7* induces YAP1 activation in OCCC (RMG-I-sh*ZDHHC7* and RMG-V-sh*ZDHHC7*, respectively; Supplementary Fig. S5A and S5B). Compared with control

cell lines, the YAP1 downstream genes, namely, *CTGF*, *CYR61*, and *ANKRD1*, were overexpressed in RMG-I-sh*ZDHHC7* and RMG-V-sh*ZDHHC7* cells (Fig. 4E). In RMG-I-sh*ZDHHC7* and RMG-V-sh*ZDHHC7* cells, the location of YAP1 shifted from the cytoplasm

**Figure 4.**

(Continued.) RMG-I-shZDHHC7 (G) and RMG-V-shZDHHC7 (I) cells treated with erastin, and cell viability of RMG-I-shZDHHC7 (H) and RMG-V-shZDHHC7 (J) cells treated with 10 $\mu\text{mol/L}$ erastin for 72 hours ($n = 3$). K–N, Growth inhibition curves of RMG-I-shZDHHC7 (K) and RMG-V-shZDHHC7 (M) cells treated with ML210, and cell viability of RMG-I-shZDHHC7 cells treated with 0.4 $\mu\text{mol/L}$ ML210 (L) and RMG-V-shZDHHC7 cells treated with 50 $\mu\text{mol/L}$ ML210 (N) for 72 hours ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, and ns, not significant (unpaired t test). Averaged data are presented as means with SEM.

to the nucleus (Fig. 4F). These findings indicate that YAP1 is activated by the suppression of ZDHHC7 in OCCC. The cell viability assay demonstrated that ZDHHC7-knockdown cells were more susceptible to erastin ($P = 0.0004$ and $P = 0.0382$ for RMG-I-shZDHHC7_1 and RMG-I-shZDHHC7_2 after 10 $\mu\text{mol/L}$ erastin treatment; Fig. 4G and H; $P = 0.0004$ and $P = 0.0006$ for RMG-V-shZDHHC7_1 and RMG-V-shZDHHC7_2 after 10 $\mu\text{mol/L}$ erastin treatment, respectively; Fig. 4I and J). In addition, ZDHHC7-knockdown cells were more sensitive to ML210 ($P = 0.0006$ and $P = 0.0002$ for RMG-I-shZDHHC7_1 and RMG-I-shZDHHC7_2 after 0.4 $\mu\text{mol/L}$ ML210 treatment; Fig. 4K and L; $P = 0.0005$ and $P = 0.0004$ for RMG-V-shZDHHC7_1 and RMG-V-shZDHHC7_2 after 50 $\mu\text{mol/L}$ ML210 treatment, respectively; Fig. 4M and N). These findings indicate that loss of ZDHHC7 sensitizes the cell lines to inducers of ferroptosis by activating YAP1 in OCCC.

ZDHHC7 inhibition enhances ferroptosis through YAP1 activation *in vivo*

We assessed the role of ZDHHC7 in ferroptosis using a mouse xenograft model (Fig. 5A; Supplementary Fig. S6A). No significant differences in mouse body weight were noted between the treated and nontreated groups with IKE, a ferroptosis inducer suitable for *in vivo* applications (Supplementary Fig. S6B; ref. 37). RMG-I-shZDHHC7 showed a significantly enhanced response to IKE

compared with control tumors ($P = 0.0099$; Fig. 5B). In addition, the IHC analysis of xenograft tumor cells showed enhanced expression of YAP1 and modestly elevated levels of MDA in ZDHHC7-knockdown tumors (Fig. 5C; Supplementary Fig. S6C). RT-PCR analysis of genes involved in RMG-I tumors revealed a higher expression of YAP1 downstream genes (*CTGF*, *CYR61*, and *ANKRD1*) in shZDHHC7 tumors than in control tumors ($P < 0.0001$ for ZDHHC7 treated with both DMSO and IKE, $P = 0.1131$ for DMSO-treated *CTGF*, $P = 0.5487$ for IKE-treated *CTGF*, $P = 0.0133$ for DMSO-treated *CYR61*, $P = 0.0906$ for IKE-treated *CYR61*, $P = 0.4245$ for DMSO-treated *ANKRD1*, and $P = 0.2563$ for IKE-treated *ANKRD1*; Fig. 5D). These findings indicate that ZDHHC7 suppression enhances ferroptosis through YAP1 activation in OCCC *in vivo*.

Discussion

The prognosis of OCCC, particularly in the advanced stage, is poor, primarily because of its intrinsic resistance to conventional chemotherapy (36). This resistance could be acquired during the carcinogenic process in an oxidative stress environment (3, 5). In this study, IHC staining of 8-OHdG, a marker of DNA damage induced by ROS (ref. 33), revealed increased levels of 8-OHdG in OCCC, corroborating our previous findings (5). MDA expression, which is indicative of lipid peroxidation and has

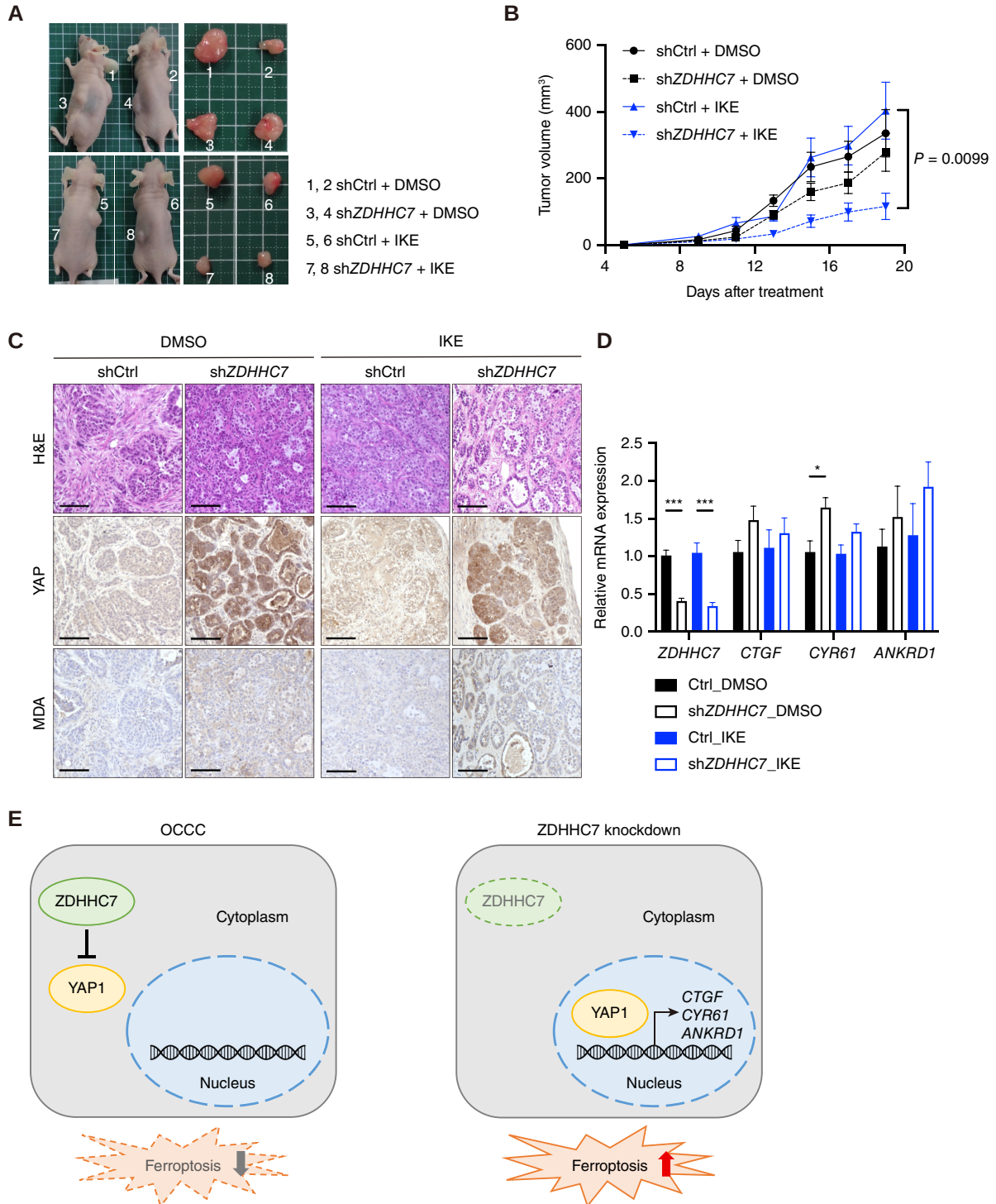


Figure 5.

ZDHHC7 knockdown enhances ferroptosis through YAP1 activation *in vivo*. **A** and **B**, Representative tumor images (**A**) and tumor volumes (**B**) of mouse xenograft models. Balb/c nude mice were inoculated subcutaneously with 1.5×10^6 RMG-I-control and RMG-I-sh*ZDHHC7* cells ($n = 7$ per group). Mice were treated with either DMSO or IKE (25 mg/kg intraperitoneally administered daily) 3 days after inoculation. *P* values represent the significant difference between control + IKE and sh*ZDHHC7* + IKE groups on day 19. **C**, Representative hematoxylin and eosin (H&E) staining and IHC staining images of YAP1 and MDA using RMG-I xenograft tumors. Scale bar, 100 μ m. **D**, RT-PCR of *ZDHHC7* and YAP1 target genes in RMG-I-control and RMG-I-sh*ZDHHC7* tumors ($n = 6$). *, $P < 0.05$; ***, $P < 0.001$ (unpaired *t* test). Averaged data are presented as means with SEM. **E**, Schematic illustration showing the role of *ZDHHC7* in activating YAP1 and promoting ferroptosis in OCC.

recently been identified as a ferroptosis marker (34, 37), was reduced in OCCC, suggesting that OCCC exhibits ferroptosis resistance. Previous studies have reported CCC susceptibility to ferroptosis (10, 11), which seems to be different from our findings. Miess and colleagues (10) reported that clear cell renal cell carcinoma (ccRCC) cells highly depend on the GSH pathway, which prevents lipid peroxidation and ferroptosis. These findings demonstrate the potential resistance of ccRCC to ferroptosis through the GSH pathway. However, in this study, we did not examine the GSH pathway, highlighting a reduction in ROS activity based on our previous results, which was attributed to increased GSH synthesis in OCCC, indicating ROS resistance (38). Moreover, KEGG pathway analysis, which assessed DEGs between OCCC and HGSC, showed no significant enrichment in GSH-related categories. Zou and colleagues (11) used 17 ccRCC and 9 OCCC cell lines from the Cancer Therapeutics Response Portal database and described a GPX4-dependent state in cancer cells, which confers sensitivity to ferroptosis. This finding indicates the role of GPX4 in suppressing ferroptosis and provides an alternative perspective on ferroptosis resistance. Notably, potential discrepancies are attributable to heterogeneity among the OCCC cell lines used in the current study and previous research (8, 11). RMG-I was the only cell line consistently used across studies. The current study established ferroptosis resistance in RMG-I cells; however, this resistance was attenuated by *ZDHHC7* knockdown both *in vitro* and *in vivo*. The Cancer Therapeutics Response Portal indicated a high AUC value for erastin in RMG-I cells, which aligns with our results. A sub-population of cells exhibiting ferroptosis resistance was identified in OCCC; moreover, *ZDHHC7* knockdown enhanced this resistance. The results suggest that OCCC exhibits a heightened resistance to ferroptosis compared with ovarian HGSC despite the noted variances in resistance levels within OCCC.

An intriguing finding is that low nuclear YAP1 expression is an unfavorable prognostic indicator in OCCC. The activation of YAP1, which promotes tumorigenesis, has been reported in various cancer types (14, 19, 39). YAP1 is activated upon its translocation into the nucleus, where it induces the expression of target genes, such as *CTGF*, *CYR61*, and *ANKRD1* (14). Several studies have reported that YAP1 is a tumor promoter associated with poor prognosis in ovarian cancer (15–17). These findings are not consistent with those of our study. Hall and colleagues (15) and Xia and colleagues (17) reported poor prognosis in patients with ovarian cancer with high nuclear YAP1 expression; however, their studies included various ovarian cancer subtypes, such as serous, endometrioid, mucinous, and transitional carcinomas. Zhang and colleagues (16) established a correlation between OCCC with increased nuclear YAP1 expression and a poor prognosis, but they only enrolled a cohort of 25 patients with OCCC. This study examined the prognostic implications of YAP1 expression in a more extensive sample of 72 patients with OCCC and validation datasets containing 213 OCCC cases (29). Several studies have reported a relationship between enhanced YAP1 expression and unfavorable prognosis in other cancers (40). Nevertheless, some evidence indicates that YAP1 is a tumor suppressor in cancers such as breast and colorectal cancers (41, 42). Our findings indicate that YAP1 downregulation, which results in ferroptosis resistance, offers insights into the adverse outcomes observed in patients with OCCC, indicating its unique behavior. YAP1 promotes cell proliferation through transcriptional activation of cell-cycle genes depending on TEAD and E2F

(43). Compared with ovarian HGSC, OCCC displays a more indolent growth phenotype (44). In this study, compared with ovarian HGSC, the nuclear expression of YAP1 was reduced in OCCC, which potentially explains the slower proliferation observed in OCCC. Furthermore, YAP1's influence on the cell cycle may clarify the poor prognosis noted in OCCC.

Another remarkable finding in this study is the biomolecular mechanism by which YAP1 activation enhances ferroptosis. Several studies have reported that YAP1 or its coactivator TAZ enhances ferroptosis in some cancer cells (12, 13), which is consistent with the finding of our study. However, the observed discrepancy in LATS inactivation that enhances YAP1 activity in OCCC persists. This finding is notable, considering the central role of YAP1 in the Hippo signaling pathway and its negative regulation by LATS (45). These findings indicate an alternative mechanism of YAP1 regulation in OCCC. Our previous study reported that *ZDHHC7* is a component of the OCCC signature (23). Compared with HGSC, this study revealed enhanced *ZDHHC7* expression in OCCC cell lines and clinical specimens. *ZDHHC7*, a major palmitoyl acyltransferase, is a downstream gene of *HNFB1B* that is predominantly overexpressed in OCCC, modulating YAP1 activity through scribble mislocalization (21). *ZDHHC7* suppression enhanced ferroptosis by YAP1 activation in OCCC. It is hypothesized that the unique features of OCCC develop during carcinogenesis, considering that its gene signature correlates with exposure to the carcinogenic environment (3, 23). The resistance of OCCC to ferroptosis could be acquired within an iron-abundant stressful environment (5, 7). These results indicate the potency of ferroptosis inducers against OCCC, both *in vitro* and *in vivo*, upon *ZDHHC7* suppression, suggesting a role of *ZDHHC7* in OCCC-associated ferroptosis. A previous study reported that OCCC and ccRCC exhibited high dependency on GSH and GPX4 to induce ferroptosis (11). This study demonstrated the potential role of *ZDHHC7* in conferring ferroptosis resistance in OCCC; it also revealed that *ZDHHC7* is a potential contributor to ferroptosis resistance, similar to GPX4 and GSH.

This study has several limitations. First, the mechanism underlying the association between low YAP1 expression and poor prognosis remains unclear. We primarily focused on the relationship between YAP1 expression and ferroptosis. Thus, future research should prioritize investigating the precise role of YAP1 in patient prognosis. Second, this study did not examine potential regulators of YAP1 beyond LATS and *ZDHHC7*. Wnt/ β -catenin signaling has been reported to be an alternative mechanism governing YAP1 expression (46). However, evidence has indicated decreased nuclear localization of β -catenin in OCCC (47). As *ZDHHC7* expression is markedly increased in OCCC compared with that in HGSC, it is plausible that *ZDHHC7* plays a role in conferring ferroptosis resistance in OCCC. Third, identifying ferroptotic cells using MDA is difficult because the cells undergo cell death as a result of ferroptosis. Unfortunately, no specific markers for ferroptosis exist. Although MDA is a product of lipid peroxidation and can indicate ferroptosis, it does not directly identify ferroptotic cells.

In conclusion, OCCC is associated with ferroptosis resistance because of decreased nuclear YAP1 expression, which correlates with a poor prognosis. In addition, YAP1 activation regulated by *ZDHHC7* enhances ferroptosis in OCCC (Fig. 5E). Hence, this study provides a novel perspective on the use of ferroptosis as a therapeutic strategy for OCCC.

Authors' Disclosures

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Authors' Contributions

Y. Furutake: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualization, writing—original draft. **K. Yamaguchi:** Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, writing—original draft. **K. Yamanoi:** Conceptualization, formal analysis, methodology, resources, writing—review and editing. **S. Kitamura:** Conceptualization, investigation, methodology, writing—review and editing. **S. Takamatsu:** Resources, software, validation, writing—review and editing. **M. Taki:** Methodology, writing—review and editing. **M. Ukita:** Methodology, writing—review and editing. **Y. Hosoe:**

Investigation, writing—review and editing. **R. Murakami:** Formal analysis, software, validation, writing—review and editing. **K. Abiko:** Conceptualization, writing—review and editing. **A. Horie:** Conceptualization, writing—review and editing. **J. Hamanishi:** Conceptualization, funding acquisition, project administration, resources, writing—review and editing. **T. Baba:** Conceptualization, supervision, validation, writing—review and editing. **N. Matsumura:** Conceptualization, supervision, validation, writing—review and editing. **M. Masaki:** Supervision, writing—review and editing.

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Note

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1 **Supplementary Tables**2 **Supplementary Table 1**

3 Conditions for each antigen retrieval step.

Antibody	Buffer	Antigen retrieval step
8-OHdG: anti-mouse mAb	Citrate (pH 6.0)	Incubation in microwave for 5 min
MDA: anti-mouse mAb	No antigen retrieval	No antigen retrieval
YAP: anti-mouse mAb	Citrate (pH 6.0)	Incubation in autoclave at 95°C for 10 min
ZDHHC7: anti-rabbit polyclonal Ab	Citrate (pH 6.0)	Incubation in autoclave at 110°C for 20 min
Scrib: anti-mouse mAb	Tris-EDTA (pH 9.0)	Incubation in autoclave at 110°C for 20 min

4 mAb, monoclonal antibody; MDA, malondialdehyde; 8-OHdG, 8-hydroxy-deoxyguanosine; YAP1,

5 Yes-associated protein 1; ZDHHC7, zinc finger DHHC-type palmitoyltransferase 7

6

7 **Supplementary Table 2**

8 Primers used for real-time polymerase chain reaction.

Gene (human)	Primer sequence
<i>CTGF</i>	Forward 5'-CCAATGACAACGCCTCCTG-3'
	Reverse 5'-TGGTGCAGCCAGAAAGCTC-3'
<i>CYR61</i>	Forward 5'-AGCCTCGCATCCTATACAACC-3'
	Reverse 5'-TTCTTTCACAAGGCGGCACTC-3'
<i>ANKRD1</i>	Forward 5'-AGTAGAGGAAGTGGTCACTGG-3'
	Reverse 5'-TGTTTCTCGCTTTTCCACTGTT-3'
<i>HNF1B</i>	Forward 5'-CCCAGCAAATCTTGTACCAGGC-3'
	Reverse 5'-ACCTCAGTGACCAAGTTGGAGC-3'
<i>ZDHHC7</i>	Forward 5'-GCTCTGTCTTCAGTCCATGC-3'
	Reverse 5'-TGGGTGCCAAACATAACTGC-3'
<i>ACTB</i>	Forward 5'-CACCTTCCAGCAGATGTCTGA-3'
	Reverse 5'-AGCATTTGCGGTGGACGATGG-3'

9

10

11 **Supplementary Table 3**

12 List of shRNAs.

Clone name	shRNA clone ID	Antisense sequence
sh <i>LATS1</i> _1	NM_004690.2-2375s1c1	5'-GATTACAACTTCACCTATTAC-3'
sh <i>LATS1</i> _2	NM_004690.x-4412s1c1	5'-GAGAAATTAAGCCATCGTGTT-3'
sh <i>LATS2</i>	NM_014572.x-2475s1c1	5'-GCCATGAAGACCCTAAGGAAA-3'
sh <i>HNF1B</i> _1	NM_000458.2-1811s21c1	5'-TGCAATGGTGGTCACAGATAC-3'
sh <i>HNF1B</i> _2	NM_000458.2-1457s21c1	5'-AGTCAGCACCTTGACGAATAT-3'
sh <i>ZDHHC7</i> _1	NM_017740.1-1557s21c1	5'-GGGTTATTACATGGGTTATTC-3'
sh <i>ZDHHC7</i> _2	NM_017740.1-995s1c1	5'-GATAACTGTAATCCTGTTGAT-3'
Control	#SHC002	

13 shRNAs, short hairpin RNAs

14

15 **Supplementary Table 4**

16 Commonly upregulated 111 DEGs in OCCC cell lines and Kyoto University clinical samples.

Gene symbol	Probe ID
<i>ABHD11</i>	221927_s_at
<i>ACSL1</i>	201963_at
<i>ACVR1B</i>	213198_at
<i>ADAM10</i>	214895_s_at
<i>AKT2</i>	203809_s_at
<i>ANXA11</i>	206200_s_at
<i>ANXA4</i>	201301_s_at
<i>APOL2</i>	221653_x_at
<i>ARHGEF5</i>	204765_at
<i>ATP11A</i>	216488_s_at
<i>ATP6AP1</i>	207809_s_at
<i>ATP6V0E1</i>	200096_s_at
<i>ATP7B</i>	204624_at
<i>AVPI1</i>	218631_at
<i>CAPZA2</i>	201237_at
<i>CARMIL1</i>	219573_at
<i>CCNG2</i>	211559_s_at
<i>CDH1</i>	201130_s_at
<i>CDKN2AIP</i>	218929_at
<i>CELSR1</i>	41660_at
<i>CPD</i>	201942_s_at
<i>CRYAB</i>	209283_at
<i>CRYL1</i>	220753_s_at
<i>DDX52</i>	212834_at
<i>DEFB1</i>	210397_at
<i>DEPTOR</i>	218858_at
<i>DPP4</i>	203716_s_at
<i>DRAM1</i>	218627_at
<i>DYNC2LI1</i>	203762_s_at

<i>DYRK2</i>	202970_at
<i>EEF1E1</i>	204905_s_at
<i>EIF2AK3</i>	218696_at
<i>ENTPD7</i>	220153_at
<i>FNDC3B</i>	218618_s_at
<i>FUCA1</i>	202838_at
<i>FXVD2</i>	205674_x_at
<i>GABARAPL1</i>	208869_s_at
<i>GDF15</i>	221577_x_at
<i>GNPTAB</i>	212959_s_at
<i>GPRC5C</i>	219327_s_at
<i>GPX4</i>	201106_at
<i>GRAMD2B</i>	218706_s_at
<i>GSE1</i>	212056_at
<i>HGD</i>	205221_at
<i>HKDC1</i>	220585_at
<i>HNF1B</i>	205313_at
<i>HOOK1</i>	219976_at
<i>IBTK</i>	210970_s_at
<i>IL1R2</i>	205403_at
<i>IQCK</i>	215130_s_at
<i>ITM2B</i>	217731_s_at
<i>IVNS1ABP</i>	201362_at
<i>KIAA0513</i>	204546_at
<i>LAMA5</i>	210150_s_at
<i>LMAN2L</i>	221274_s_at
<i>LPIN2</i>	202460_s_at
<i>MAP7</i>	215471_s_at
<i>MFHAS1</i>	213457_at
<i>MINDY2</i>	214691_x_at
<i>MITF</i>	207233_s_at
<i>MLH3</i>	204838_s_at
<i>MOAP1</i>	212508_at

<i>MPC2</i>	202427_s_at
<i>MYOF</i>	201798_s_at
<i>NCOA3</i>	207700_s_at
<i>NF2</i>	217150_s_at
<i>NR2C1</i>	204791_at
<i>NT5C2</i>	209155_s_at
<i>P2RX4</i>	204088_at
<i>PAX8</i>	121_at
<i>PCBD1</i>	203557_s_at
<i>PCCA</i>	203860_at
<i>PDE9A</i>	205593_s_at
<i>PDZK1IP1</i>	219630_at
<i>PEX7</i>	205420_at
<i>PGBD5</i>	219225_at
<i>PLA2G12A</i>	221027_s_at
<i>PLLP</i>	204519_s_at
<i>PLXNB2</i>	208890_s_at
<i>PPM1B</i>	213225_at
<i>PREPL</i>	212215_at
<i>PTPN3</i>	203997_at
<i>RAB11FIP1</i>	219681_s_at
<i>RAB9A</i>	221808_at
<i>RBKS</i>	57540_at
<i>RBM47</i>	218035_s_at
<i>RBPM5</i>	207836_s_at
<i>RNASET2</i>	217984_at
<i>RNF141</i>	219104_at
<i>SAV1</i>	218276_s_at
<i>SEMA4D</i>	203528_at
<i>SGK1</i>	201739_at
<i>SLC16A5</i>	206600_s_at
<i>SLPI</i>	203021_at
<i>SPTLC2</i>	203128_at

<i>STAT3</i>	208991_at
<i>SUCLA2</i>	202930_s_at
<i>SYNE2</i>	202761_s_at
<i>TBC1D8</i>	204526_s_at
<i>TPD52L1</i>	210372_s_at
<i>TRAPPC11</i>	218179_s_at
<i>TRIM2</i>	215945_s_at
<i>TSPAN12</i>	219274_at
<i>TUT7</i>	220933_s_at
<i>UGT2B28</i>	211682_x_at
<i>UXS1</i>	219675_s_at
<i>VAV3</i>	218807_at
<i>VLDLR</i>	209822_s_at
<i>VPS13C</i>	218396_at
<i>WSB1</i>	201294_s_at
<i>ZNF702P</i>	206557_at

17 DEG, differentially expressed genes; OCCC, ovarian clear cell carcinoma

19 **Supplementary Table 5**

20 Clinical characteristics of 117 patients with ovarian cancer and results of 8-OHdG and MDA
21 analyses.

	CCC	HGSC	P-value
N	72	45	
Age (years; median)			
<60	50	25	.1658
≥60	22	20	
FIGO stage (FIGO 2014 classification)			
I	49	5	(stage I, II vs. III, IV) <.0001*
II	4	4	
III	10	30	
IV	9	6	
8-OHdG IHC score			
0	3	35	<.0001*
1	10	28	
2	59	9	
MDA IHC score			
0	19	31	.0968
1	9	11	
2	17	3	

22 CCC, clear cell carcinoma; FIGO: International Federation of Gynecology and Obstetrics; HGSC,
23 high-grade serous carcinoma; IHC, immunohistochemistry; MDA, malondialdehyde; 8-OHdG, 8-
24 hydroxy-deoxyguanosine

25 *Statistical significance.

26 P-values were calculated using Fisher's exact test for age and FIGO stage and the chi-square test for
27 IHC scores.

28

29 **Supplementary Table 6**

30 Results of 8-OHdG and MDA analyses in 117 patients with ovarian cancer.

	CCC	HGSC	<i>P</i> -value
8-OHdG IHC score (early stage)			
0	1	3	.0010*
1	7	2	
2	45	4	
MDA IHC score (early stage)			
0	23	5	.4861
1	23	4	
2	7	0	
8-OHdG IHC score (advanced stage)			
0	2	16	.0180*
1	3	7	
2	14	13	
MDA IHC score (advanced stage)			
0	12	26	.7855
1	5	7	
2	2	3	

31 CCC, clear cell carcinoma; HGSC, high-grade serous carcinoma; IHC, immunohistochemistry;

32 MDA, malondialdehyde; 8-OHdG, 8-hydroxy-deoxyguanosine

33 *Statistical significance.

34 *P*-values were calculated using the chi-square test.

35

36 **Supplementary Table 7**

37 IC₅₀ values of 12 ovarian cancer cell lines after treatment with a ferroptosis inducer.

Cell line	Subtype	IC₅₀_erastin (μM)
RMG-I	OCCC	11.53
RMG-V	OCCC	8.485
KOC-5C	OCCC	4.272
KOC-7C	OCCC	14.28
JHOC-7	OCCC	2.191
JHOC-8	OCCC	2.448
OVCA420	HGSOC	1.713
OVCA433	HGSOC	0.8634
Hey	HGSOC	1.275
SK-OV-3	HGSOC	4.867
A2780	HGSOC	0.1379
TYK-nu	HGSOC	0.06632

38 IC₅₀, half-maximal inhibitory concentration; OCCC, ovarian clear cell carcinoma

39

40 **Supplementary Table 8**

41 List of 27 KEGG pathways enriched in 111 commonly upregulated DEGs.

Description	LogP	Enrichment	Z-score
FoxO signaling pathway	−3.8978402	10.3782408	6.53554684
Lipid and atherosclerosis	−2.9146596	6.32348628	4.75935502
Measles	−2.7538774	7.82474561	4.89978006
Adipocytokine signaling pathway	−2.6724225	11.82217	5.46784571
Melanoma	−2.6196311	11.3295796	5.33148121
Pathways in cancer	−2.4514334	3.58449975	3.65055125
HIF-1 signaling pathway	−2.1156759	7.48375899	4.1200843
Toxoplasmosis	−2.0834179	7.28330116	4.0474784
Signaling pathways regulating pluripotency of stem cells	−1.7979681	5.7044037	3.42597535
Hepatitis C	−1.6914072	5.19573076	3.20236107
Hepatitis B	−1.6559896	5.0353687	3.12888608
Chemokine signaling pathway	−1.4673794	4.24859234	2.74354422
Focal adhesion	−1.4175573	4.05835686	2.64312376
Proteoglycans in cancer	−1.3962704	3.97916941	2.60035363
Rap1 signaling pathway	−1.3703594	3.88442728	2.54838915
Chemical carcinogenesis-receptor activation	−1.3602044	3.84778174	2.52804981
Carbohydrate digestion and absorption	−3.1563278	17.3559517	6.81778838
Thyroid hormone signaling pathway	−1.9921524	6.74156801	3.8448186
Autophagy—animal	−2.7312245	7.71375631	4.85481074
Mitophagy—animal	−2.6196311	11.3295796	5.33148121
Epithelial cell signaling in <i>Helicobacter pylori</i> infection	−2.6545543	11.6532819	5.4214693
Human papillomavirus infection	−1.4668991	3.28592036	2.54068028
mTOR signaling pathway	−2.5720418	6.97204897	4.54350789
Carbon metabolism	−2.0521103	7.093302	3.9775069
Lysosome	−1.8904622	6.17977068	3.62351136
Hippo signaling pathway	−1.6914072	5.19573076	3.20236107
Transcriptional misregulation in cancer	−1.4617063	4.22657891	2.73208571

42 DEG, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes

43

44 **Supplementary Table 9**

45 Clinical characteristics of 72 patients with OCCC in YAP1 IHC analysis.

	YAP1-low	YAP1-high	P-value
Characteristics	Low (<1)	High (≥1)	
Number	23	49	
Age (years)			
<60	16	34	.8832
≥60	7	15	
FIGO stage			
I, II	15	38	.3898
III, IV	8	11	
Residual tumor			
No	17	39	.7618
Yes	6	10	
Relapse			
No	11	36	*.0389
Yes	12	13	

46 FIGO, International Federation of Gynecology and Obstetrics; IHC, immunohistochemistry; OCCC,

47 ovarian clear cell carcinoma; YAP, Yes-associated protein 1

48 *Statistical significance.

49 P-values were calculated using Fisher's exact test.

50

51 **Supplementary Table 10**

52 Multivariate analysis of prognostic factors associated with PFS and OS in patients with OCCC in
53 validation datasets.

PFS	Univariate analysis			Multivariate analysis		
Parameter	HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
Age (years) (<60 vs. ≥ 60)	0.8192	0.613– 1.095	.1775	0.8679	0.6235– 1.208	.4013
FIGO stage (I, II vs. III, IV)	2.495	1.806– 3.447	$<.0001^{**}$	2.094	1.350–3.248	.0010 **
Residual tumor (no vs. yes)	2.259	1.559– 3.275	$<.0001^{**}$	1.570	0.997–2.473	.0515
*Signature score (high vs. low)	1.443	1.071– 1.918	.0154 **	1.577	1.127–2.208	.0079 **
OS	Univariate analysis			Multivariate analysis		
Parameter	HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
Age (years) (<60 vs. ≥ 60)	0.5236	0.3391– 0.8084	.0035	0.5944	0.3638– 0.9711	.0378 **
FIGO stage (I, II vs. III, IV)	6.336	4.038–9.944	$<.0001^{**}$	4.436	2.480–7.936	$<.0001^{**}$
Residual tumor (no vs. yes)	3.848	2.401–6.167	$<.0001^{**}$	1.808	1.034–3.159	.0376 **
*Signature score (high vs. low)	1.503	0.9792– 2.036	.06234	1.690	1.032–2.769	.0371 **

54 CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard
55 ratio; OCCC, ovarian clear cell carcinoma; OS, overall survival; PFS, progression-free survival

56 *GOBP_REGULATION_OF_HIPPO_SIGNALING.

57 ** Statistical significance.

58

59 **Supplementary Table 11**

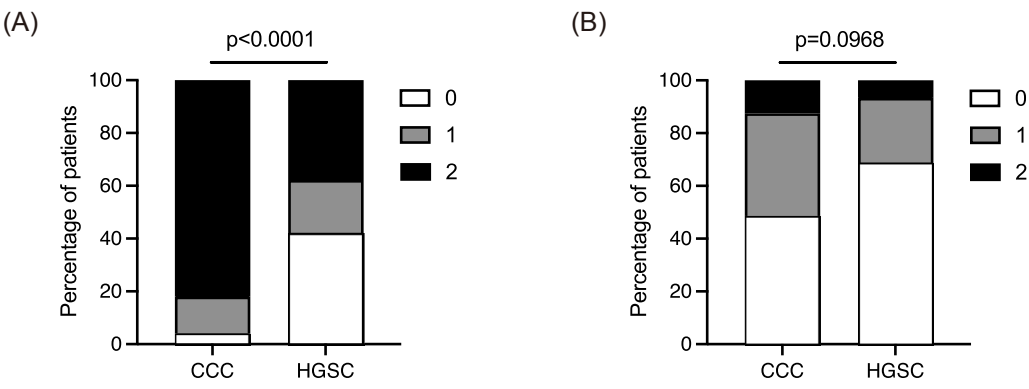
60 The contingency table between expressions of YAP1 and ZDHHC7

	Low expression of nuclear YAP1	High expression of nuclear YAP1	Total
Low expression of ZDHHC7	9	21	30
High expression of ZDHHC7	14	28	42
Total	23	49	72

61

62

Supplementary Figure 1



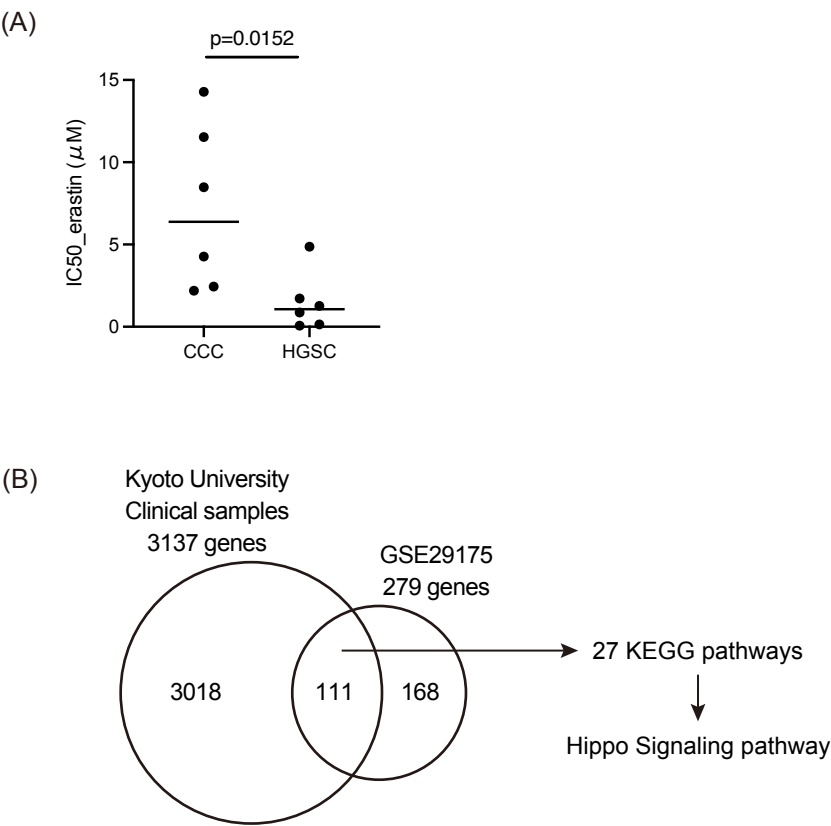
Supplementary Figure 1. 8-OHdG and MDA immnostaining in clinical ovarian clear cell carcinoma (CCC) and high-grade serous carcinoma (HGSC) patients.

(A) Distribution of 8-OHdG IHC scoring of patients with CCC and HGSC in Kyoto University Hospital.

(B) Distribution of MDA IHC scoring of patients with CCC and HGSC in Kyoto University Hospital.

P-values were calculated with Chi-square test.

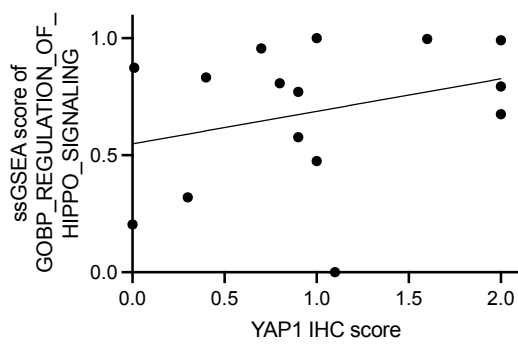
Supplementary Figure 2



Supplementary Figure 2.

(A) IC₅₀ values with erastin in six ovarian CCC and six HGSC cell lines. *P*-values were calculated with Mann-Whitney U test. (B) Enriched KEGG pathways in commonly upregulated 111 DEGs. DEGs were identified from CCC cell lines and clinical samples compared to HGSC cell lines or clinical samples. GSE29175, GSE39204/GSE55512 microarray datasets were analyzed. FDR-*q* values less than 0.05 are considered DEGs. KEGG pathways with *P*-value less than 0.05 are considered as enriched pathways.

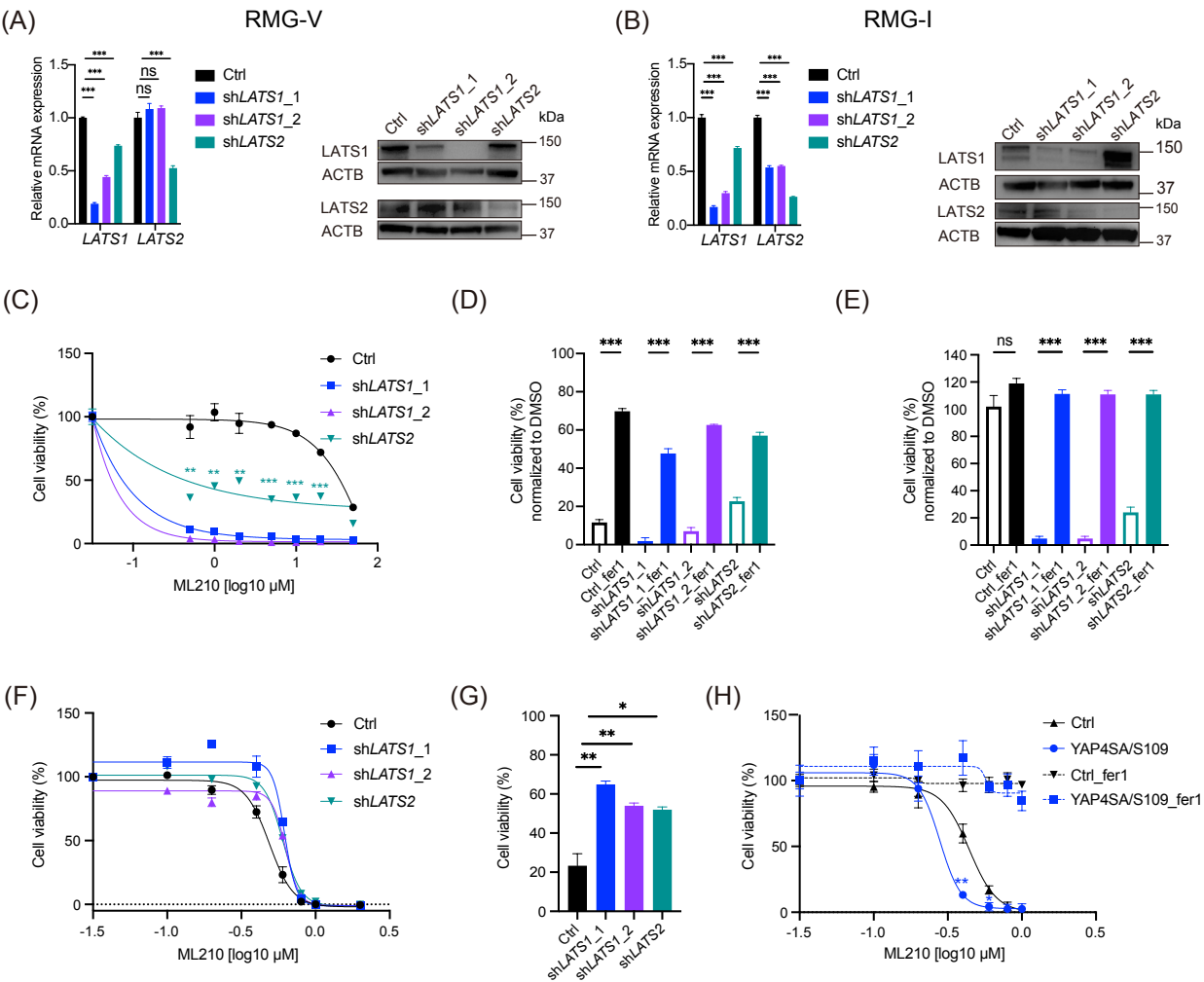
Supplementary Figure 3



Supplementary Figure 3.

Correlation between YAP1 IHC score and normalized ssGSEA score of GOBP_REGULATION_OF_HIPPO_SIGNALING in Kyoto University clinical samples from 15 patients with clear cell carcinoma (CCC) (GSE39204 and GSE55512). *P*-value and *R*-value were calculated using the Spearman correlation coefficients.

Supplementary Figure 4

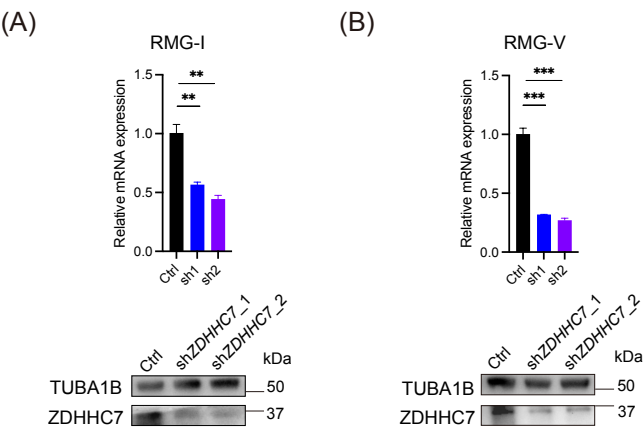


Supplementary Figure 4. YAP activation induces ferroptosis in ovarian clear cell carcinoma.

(A)(B) RT-PCR and immunoblotting of RMG-V-sh*LATS1*/*LATS2* (A), RMG-I-sh*LATS1*/*LATS2* (B) cells; n=3. (C)(F) Growth inhibition curves of RMG-V-sh*LATS1*/*LATS2* (C) and RMG-I-sh*LATS1*/*LATS2* (F) treated with erastin for 72 h, and (G) Cell viability of RMG-I-sh*LATS1*/*LATS2* cells treated with 0.6 μ M ML210; n=3. (D)(E) RMG-V Cell viability treated with 10 μ M erastin (D) or 1 μ M ML210 (E) with or without 1 μ M ferrostatin-1 for 72 h; n=3. (H) Growth inhibition curves of RMG-I-YAP4SA/S109 treated with or without 1 μ M ferrostatin-1 for 72 h; n =3.

Cell viabilities were normalized to DMSO. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (unpaired *t*-test). Averaged data are presented as the mean with SEM.

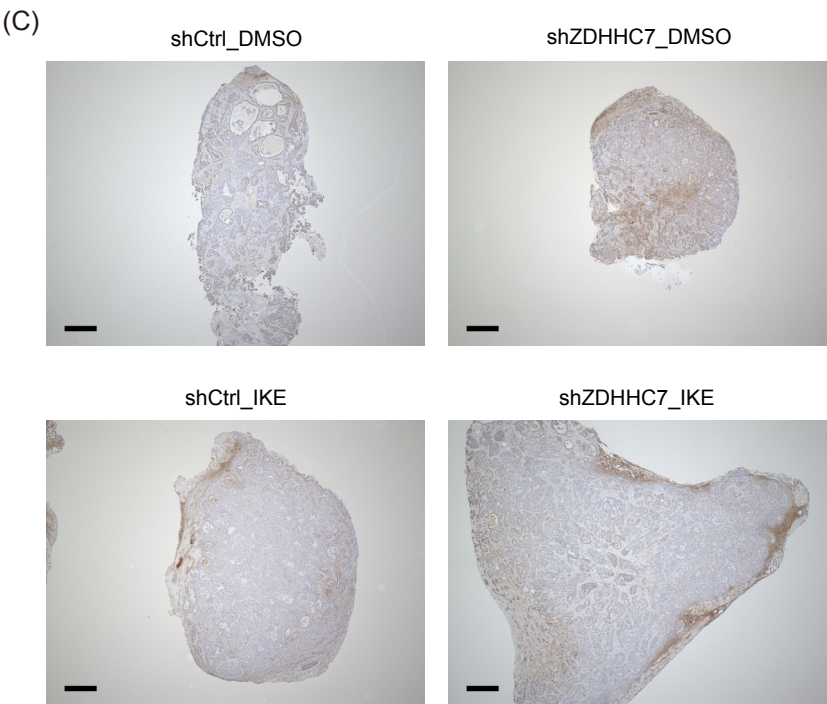
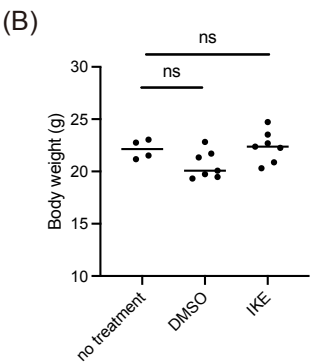
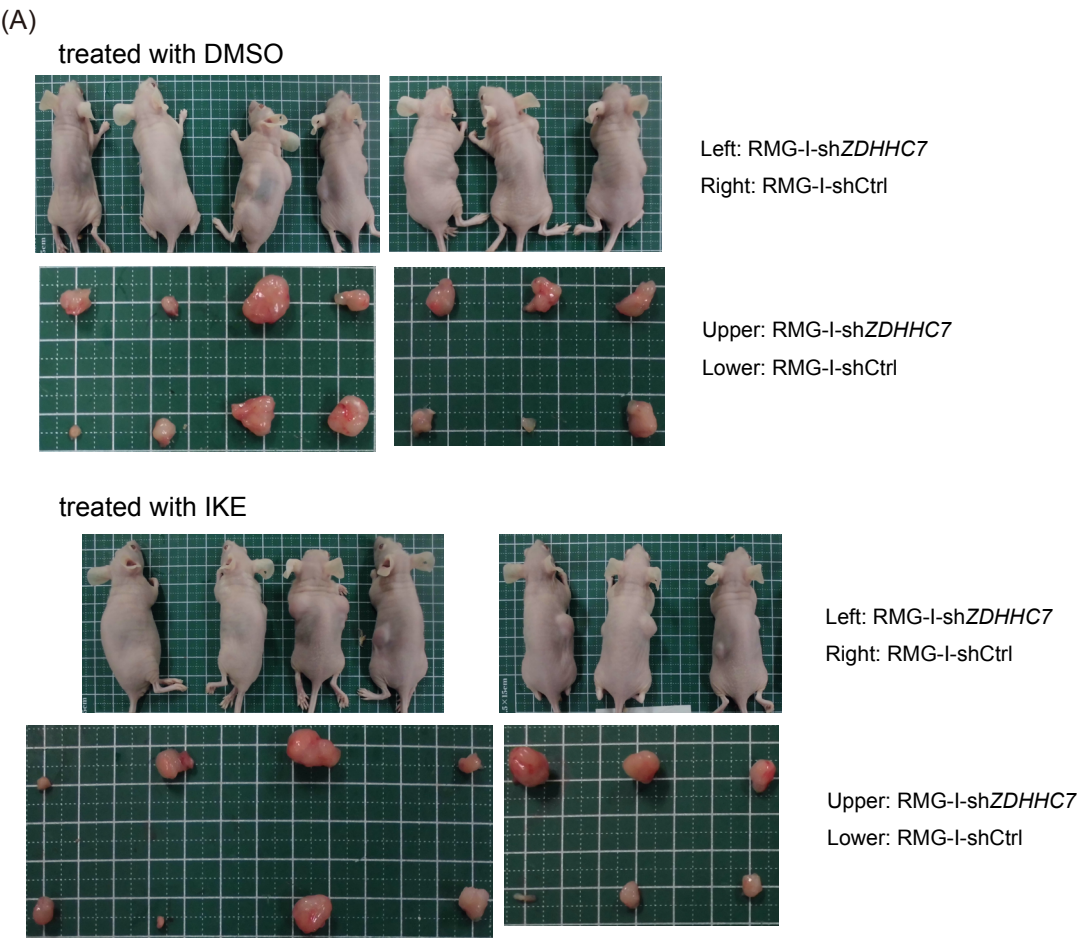
Supplementary Figure 5



Supplementary Figure 5.

(A)(B) RT-PCR and immunoblotting of ZDHHC7 from RMG-I (A) and RMG-V (B) cells. TUBA1B was used as control.

Supplementary Figure 6

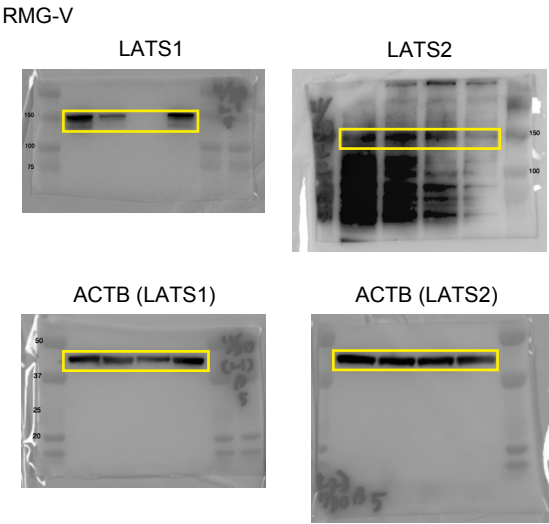


Supplementary Figure 6.

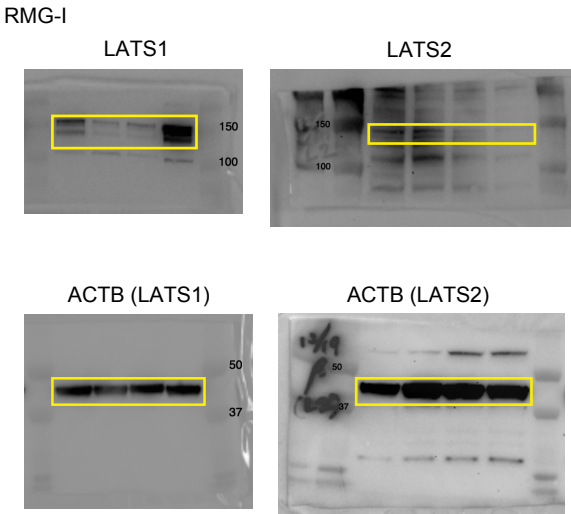
(A) All images of the mice and RMG-I tumors obtained from *in vivo* experiment. (B) Body weight of the mice at the end of the treatment. (C) Whole images of MDA staining using RMG-I xenograft tumors. Note that many of the cells experienced cell death in shZDHHHC7_IKE sample. Scale bar, 250mm.

Supplementary Figure 7

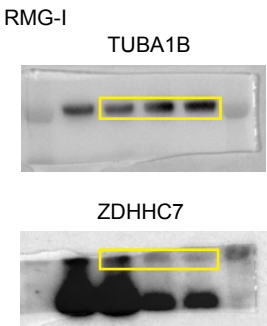
Supplementary Figure 4A



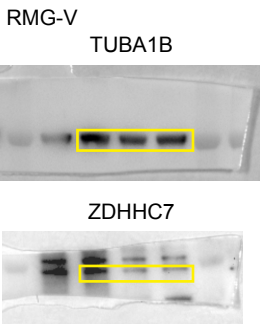
Supplementary Figure 4B



Supplementary Figure 5A



Supplementary Figure 5B



Supplementary Figure 7. Raw data of immnoblottng