Supplemental Material (SM)

Materials and Methods:

Cell Culture: SKOV3 and SKOV3-derived cells were cultured in medium composed of 1:1 combination of MCDB 105 (Sigma Aldrich, Cat# M6395) and Medium 199 (Corning, Cat# 10-060-CV), supplemented with 10% FBS (Fisher Scientific, Cat# 35011CV) and 1% penicillin-streptomycin (Corning, Cat# 30-002-CI). Peo1 and Peo4 cells were cultured in RPMI-1640 with L-glutamine (Corning, Cat#10-040-CV) plus 10% FBS, 1% GlutaMAX (Gibco, Cat# 35050-061), 2mM Sodium Pyruvate (Gibco, Cat# 11360-070), and 1% penicillin-streptomycin. OVCAR5 and OVCAR5-derived cells were maintained in RPMI-1640 with L-glutamine (Corning, Cat# 10-040-CV) plus 10% FBS, 1% GlutaMAX, and 1% penicillin-streptomycin. OVCAR3 and its derived sublines were culture in ATCC-modified RPMI-1640 medium (ATCC, Cat# 30-2001) supplemented with 20% FBS, 1% penicillin-streptomycin, and 0.01 mg/mL recombinant human insulin (Gibco, Cat#12585-014). COV362 cells and its derived sublines were cultured in DMEM with L-glutamine, 4.5g/L glucose, without sodium pyruvate (Corning, Cat#10-017-CV) plus 10% FBS, 1% GlutaMAX, and 1% penicillin-streptomycin. Dissociated cells from OC tumors and xenografts were cultured in ultra-low attachment plates (Corning, Tewksbury, MA, USA) using MammoCult base stem cell medium (Stemcell Technologies, Cat#05620, Cambridge, MA, USA) supplemented with 10% MammoCult proliferation supplements (Stemcell technologies, Cat#05620), 4µg/ml heparin (Stemcell technologies, Cat#07980), 0.48µg/ml hydrocortisone (Stemcell Technologies, Cat#07904), and 1% penicillin-streptomycin.

Spheroid formation assay. A serial dilution (500, 1,000, 5,000, 10,000, 50,000 cells) of OVCAR5 cells transfected with shctrl and shFZD7 cells were cultured under non-attachment conditions in 96-well ultra-low attachment plates (Fisher Scientific, Cat#3474) in 100µl

MammoCult base stem cell medium. In other experiments, SKOV3, OVCAR5, COV362 or OVCAR3 cells, including wild type, cisplatin resistant, cells with FZD7 knockdown and FZD7 overexpressing cells, FACS sorted FZD7(+) and FZD7(-) cells derived from OC cell lines, or cells isolated from OVCAR5_shctrl and OVCAR5_shFZD7 xenografts were seeded into 96-well ultra-low attachment plates (1000-20,000 cells/well). Cells were cultured for 7 to 14 days and fresh medium was added every 2 to 3 days. Total numbers of spheroids were counted with an inverted microscope and numbers of viable cells were quantified with a Cell Counting Kit 8 (CCK8) or by measuring intracellular ATP levels with the CellTiter-Glo 3D cell viability assay (Promega, Cat# G9681) following the manufacturer's protocol. Briefly, a volume of CellTiter-Glo 3D Reagent equal to the amount of medium was added into each well, mixed for 30 minutes to induce cell lysis, and incubated for 35 minutes at room temperature to stabilize the luminescent signal. Luminescence was measured by using a microplate reader (SpectraMax GeminiXS, Molecular Devices, San Jose, CA, USA).

Clonogenic assay. OC cells (800 to 2,000 cells/well) were seeded in 6 well plates and allowed to attach overnight. Cells received experimental treatments and were cultured for 7–14 days to form colonies. Cell colonies were washed with PBS, fixed with 4% paraformaldehyde and stained with 0.05% crystal violet for counting.

In Vivo Experiments: To develop platinum resistant OC cells *in vivo,* female (6-8 weeks old) athymic nude mice (*Foxn1*^{nu}, Envigo) were injected subcutaneously (s.c.) with 2 million SKOV3 or OVCAR3 cells, or intraperitoneally (i.p.) with 2 million OVCAR5 cells to induce tumors. Mice were treated i.p. with PBS (control) or 25 mg/kg carboplatin (n = 3-5), once-a-week for 3 weeks starting when xenografts were > 10 mm^3 (SKOV3 and OVCAR3) or 2 weeks after i.p. inoculation (OVCAR5). Length (l), width (w) and height (h) of sc xenografts were measured

weekly using digital calipers and tumor volume (v) was calculated as v = ½ × l × w × h. Tumors were collected 1 week after last treatment and were used for isolation of cancer cells, RNA and protein extraction, or fixed with 10% formalin neutralizing buffer (Formal-fixx, Thermo Scientific, Ref# 9990244) for IHC. Two million OVCAR5 cells transduced with shRNAs targeting *FZD7* (OVCAR5_shFZD7) or scrambled shRNA control (OVCAR5_shctrl, n = 10 per group) were injected sc in 6-8 weeks female nude mice. Time to tumor initiation and tumor growth was monitored, as described above. Tumor initiation experiments used serial dilution (2,500, 5,000, and 10,000) of control and FZD7 targeting shRNA transduced cells injected sq in the flank of nude mice (n = 4 per group).

Patient Derived Xenografts: Pieces of HGSOC tumors obtained from consenting donors were subcutaneously implanted sc or intra-bursally in female NOD SCID gamma (NSG) mice (The Jackson Laboratory) and allowed to grow to 1-1.5cm diameter over the course of 3-4 months, as previously described (1). Collected PDX tumors were confirmed to be of high-grade serous carcinoma type by histological examination by a board certified pathologist. Pieces of the second generation PDX tumors were subcutaneously re-implanted into NSG mice. Treatment with carboplatin (once-a-week, i.p., 15mg/kg, n=5) or PBS (control, n=5) started when tumors reached 5-7 mm in length, and continued for 6 weeks. Body weights and tumor sizes were measured twice-a-week. Tumors were collected and processed as described.

Lipid peroxidation assay: Intracellular lipid peroxidation was determined by a Lipid Peroxidation Assay (Sigma-Aldrich, Cat# MAK085) following the manufacturer's protocol. Briefly, pellets of 1 or 3 million cells were homogenized on ice in 300 µl of the malondialdehyde (MDA) lysis buffer containing 3µl of butylated hydroxytoluene (BHT). After removal of insoluble materials, 600 µl of thiobarbituric acid (TBA) solution was added into each sample and incubated

at 95℃ for 60 minutes. Fluorescence was measure at 532/553 nm (excitation/emission) by using a microplate reader (SpectraMax GeminiXS, Molecular Devices).

Half maximal inhibitory concentration (IC_{50}): FZD7(+) and FZD7 (-) cells derived from SKOV3, OVCAR5, and COV362 cells were sorted by FACS and directly seeded at 10,000 cells/well into 96-well plates. Twenty-four hours after plating, cells were treated with cisplatin (0, 1, 2, 4, 6, 8, 12μM) for 24 hours. Cisplatin was removed (washed off), cells were cultured for 48 or 72 hours and cell viability was measured with a CCK8 kit. In other experiments, 1-5,000 OC cells were seeded in 96-well plates and treated with different concentrations of cisplatin (0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 250, 500 μM) or carboplatin (0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 250, 500 μg/ml) and proceeded as described above. To determine the IC_{50} of the glutathione peroxidase inhibitor ML-210, FZD7(+) and FZD7 (-) cells were treated daily with ML-210 (0, 0.25, 0.5, 1, 2μM) or DMSO (0 dose) for 96 hours, and cell viability was determined with a CCK8 kit. In other experiments, 1-5,000 OC cells were seeded in 96-well plates and treated with different concentrations of ML-210 (0, 2.5, 5, 10, 25, 50, 100, 250, 500, 750, 1000, 2000 nM) or RSL-3 (0, 2.5, 5, 10, 25, 50, 100, 250, 500, 750, 1000, 2000 nM) and proceeded as described above.

Cloning strategy: The FZD7 coding sequence plus a C-terminal Myc-DDK tag was PCR-amplified from pCMV6-Entry mammalian vector (Origene, cat # RC204167) using forward primer 5'-TATGGATCCATGCGGGACCCCGGCG-3' and reverse primer 5'-TTAGAATTCTTACTTATCGTCGTCATCCTTGTAATCCAGG-3' to introduce BamHI and EcoRI restriction sites. The PCR products were resolved by agarose gel electrophoresis, purified using a QIAquick gel extraction kit (Qiagen, #28704), and digested using BamHI (Thermo Scientific, #FD0054) and EcoRI (Thermo Scientific, #FD0274) endonucleases. Similarly, pcDNA 3.1 empty vector was digested with BamHI and EcoRI to create insertion sites. The

DNA insert encoding the FZD7 sequence was then ligated into the pcDNA 3.1 vector. To verify correct insertion, Sanger sequencing was performed using universal T7 and BGH primers flanking the inserted sequence. The sequence of the newly constructed plasmid is given in Supplementary Figure S10.

RNA extraction and quantitative RT-PCR analysis. RNA was isolated by using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and quantified with a NanoDrop spectrophotometer (Thermo Scientific). For *mRNA* expression studies, 0.2 to 1 μg of total RNA was reverse-transcribed into cDNA with an iScript cDNA synthesis kit (Bio-Rad, Berkeley, California) according to the manufacturer's instructions. Quantitative real-time PCR analysis was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Berkeley, California) and a 7900HT real-time PCR instrument (Applied Biosystems, Foster City, CA). The RT-PCR reaction used the following parameters: 94℃ for 10 min, 40 cycles of amplification at 94 ℃ for 15 s and 60 ℃ for 1 min, and an extension step of 7 min at 72℃. Data were normalized using expression of the 18S gene. Relative expression of target genes was calculated using the $2^{-\Delta(\Delta C_T)}$ method where $\Delta C_T = C_{T,\ target} - C_{T,\ 18S}$ and $\Delta(\Delta C_T) = \Delta C_{T,\ stimulated} - \Delta C_{T,\ control}$. Primer sequences (Integrated DNA Technologies, USA) are in Supplemental Table S4. RNA from less than 500,000 FACS sorted cells was isolated with a RNeasy Micro Kit and corresponding protocol (Qiagen, Cat#74004). RNA quantity and quality were determined using a NanoDrop spectrophotometer and a Qubit® 2.0 Fluorometer (Invitrogen). RNA samples were used for real-time RT-PCR as described above.

Western Blotting: Protein lysates were prepared using radio immunoprecipitation assay (RIPA) buffer, and protein concentrations were quantified with the Bradford assay (Biorad Protein Assay Reagent, BioRad, Berkeley, CA). Proteins (20μg) were resolved by SDS-PAGE, and electroblotted onto PVDF membranes. The antibodies against P63 (rabbit monoclonal, Cat# ab124762, used at 1:1000), GPX4 (rabbit monoclonal, Cat# ab125066, used at 1:1000) and FZD7 (rabbit polyclonal, Cat# ab64636, used at 1:500) were purchased from Abcam (Cambridge, MA). Mouse monoclonal GAPDH antibody was from Meridian Life Science (Memphis, Tennessee, Cat# H86504M, used at 1:10000). Mouse monoclonal β-catenin antibody was from ECM Biosciences (Versailles, KY, Cat# CM181, used at 1:1000). HRP-conjugated donkey-anti-rabbit polyclonal antibody (Cat#NA9340, used at 1:2000) was purchased from GE Healthcare (Pittsburgh, PA) and HRP-conjugated goat-anti-mouse antibody (Cat#haf007, used at 1:2000) was from R&D System (Minneapolis, MN). Membranes were blocked in TBST containing 5% BSA for an hour, and then incubated with primary antibody at 4℃ overnight and with HRP-conjugated secondary antibody for 1 hour at room temperature. Signal was generated using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Cat# 34577) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Cat# 34095) enhanced chemiluminescent HRP system. Images of protein bands were captured by a luminescent image analyzer with a CCD camera (LAS 3000, Fuji Film) and band intensities were quantified by densitometric analysis using Gel-Pro Analyzer 3.1 software. The antibodies used are included in the main manuscript. Densitometric analysis of each band was measured using ImageJ (https://imagej.nih.gov/ij) for quantification.

Immunohistochemistry (IHC): Sections (5 μm) of paraffin-embedded tissues, or ovarian cancer tissue microarrays were heated at 56℃ for 20 mins and deparaffinized with xylene, followed by

re-hydration through decreasing concentration of ethanol (100%, 90%, 70%, 50%, 0%). Antigen retrieval was performed with citrate buffer (10 mM, pH 6.0) for 30 minutes at 95°C as previously described (2). Peroxidase activity was eliminated with 10% hydrogen peroxide (Fisher Scientific, Cat# H324500) for 10 mins, and then tissues were incubated with 0.5% normal goat serum (DAKO, Hamburg, Germany, Cat# K0672) in PBS for 1 hour. Anti-FZD7 (Abcam, Cat# 64636, 1:50), anti-GPX4 (Abcam, Cat# ab125066, 1:250), or rabbit IgG (Santa Cruz, Cat# sc-2027,1:500) were added to tissue sections and incubated overnight at 4°C. This was followed by treatment with avidin-biotin peroxidase reagents of a DAKO Detection Kit (DAKO, Hamburg, Germany, Cat# K0672) and with liquid DAB substrate chromogen (DAKO, Cat# K3467). Sections were counterstained with hematoxylin (Agilent Technologies, Cat# CS700) and cover-slipped. Protein staining was measured as a H-score, which is the product between staining intensity (0 to 3+) and percentage of stained cells (0-100%).

Cell transfection. OC cells were seeded, grown to 70% confluence, and then transduced with lentiviral particles containing shRNA in the presence of polybrene (8 μg/ml) for 48 hours. Lentiviral transduction particles containing three shRNAs targeting FZD7 were used (shFZD-1, Cat#TRCN0000008345; shFZD7-2, Cat#TRCN0000008343, and shFZD7-3, Cat#TRCN0000357012). Cells transduced with scrambled shRNA (Mission Lentiviral Transduction Particles, Sigma-Aldrich, St Louis, MO, USA) were used as controls. Stable knockdown of p63 used a pool of lentiviral particles containing shRNA targeting p63 (sc-36161-v, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). Control cells were transduced with scrambled shRNA (sc-108080, Santa Cruz Biotechnology Inc.). Transduced cells were selected with puromycin (2 μg/ml for OVCAR5 and SKOV3, and 0.5μg/ml for OVCAR3 cells). FZD7 was cloned into pcDNA3.1 expression vector (see SM). 1μg of the FZD7 expression vector or

empty vector was transfected into SKOV3 and OVCAR5 cells using lipofectamine 2000 (Invitrogen, Cat# 11668-019), according to the manufacturer's protocol. Stably transfected cells were selected with 200μg/ml G418 (Geneticin, Thermo Fisher, Cat# 10131027) by using single colony selection strategy. Transiently transfected cells were collected 48 and 72 hours post-transfection.

Clonogenic survival assay. OC cells (800 to 2,000 cells/well) were seeded in 6 well plates and allowed to attach overnight. Cells received experimental treatments and were cultured for 7–14 days to form colonies. Cell colonies were washed with PBS, fixed with 4% paraformaldehyde and stained with 0.05% crystal violet for counting.

RNA Sequencing: The RNA-seq libraries (n=3 per experimental group) were prepared using the NEBNext Ultra II RNA library prep kit from Illumina (New England Biolabs Inc., Ipswich, MA). *mRNA* was isolated from 1 mg of total RNA and used for first-strand cDNA synthesis. This was followed by second-strand cDNA synthesis, end repair of the cDNA library, dA-tailing of the cDNA library, adaptor ligation, and PCR enrichment. The RNA-seq libraries were checked by using a BioAnalyzer, and then sequenced on an Illumina NextSeq500 system with single-end, 75-bp read length settings. For quality control, raw fastq files were pre-processed using TrimGalore (0.4.4) and cutadapt (1.14) with single-end trimming mode, Phred score cutoff of 20 and minimum sequence length cutoff of 20 bp (3). Trimmed reads were aligned to the ENSEMBL human genome version GRCh38 using STAR (2.5.2)(4) and SAMtools (5). Mapped reads were then counted using HTSeq (6).

Intracellular reactive oxygen species (ROS). Intracellular ROS levels were measured by monitoring the oxidation of cell permeable 2′,7′-dichlorofluorescein diacetate (DCFHDA, Sigma-Aldrich) to fluoros-pectrophotomete at excitation and emission wavelengths of 480 and 535 nm,

respectively, measuring intracellular hydroxyl, peroxyl and other ROS activity. 150,000 cells cultured in 35 mm glass bottom dish were treated with 1 or 2μM ML210 alone or with 800 nM DFOA for 24 hours. Cell cultures were then treated with 10 μM DCFDA (Abcam, Cat#ab113851) for 15 minutes to detect ROS level through confocal fluorescence microscopy. ROS level was measured as integral fluorescence intensity normalized by the cellular area in a frame (n=15 frames) using ImageJ (https://imagej.nih.gov/ij).

Cell cycle analysis. FZD7(+) and FZD7(-) cells FACS sorted from OVCAR5 and COV362 and shRNA control or targeting FZD7 stably transduced SKOV3 and OVCAR5 cells were trypsinized washed with PBS. Cell pellets were suspended in 1ml cold 1X PBS buffer and fixed by adding the cell suspension dropwise to an equal volume of cold absolute ethanol. Fixed cells were stored at -20 ºC for 12 hours and stained with propidium iodide (PI)/RNase staining buffer (Cat # 550825, BD Pharmingen PI/RNase staining buffer) according to the manufacturer's protocol. The percentage of cells in each cell cycle phase were analyzed by using FlowJo as described in our previous studies (7).

***In vivo* limited dilution assay.** Serial limited number (2,500, 5,000, and 10,000) of OVCAR5_shctrl and shFZD7 cell were subcutaneously injected into nude mice (n=4 per group). Time to tumor initiation and tumor growth were monitored. Stem cell frequencies were calculated by using the Extreme Limiting Dilution Analysis (http://bioinf.wehi.edu.au/software/elda/), as described in our previous studies (8).

Wnt3a and IWR-1 treatment. 1.5 million of SKOV3 and OVCAR5 OC cells transfected with shctrl, shFZD7, FZD7-pcDNA3.1 and empty vector were treated with 1% BSA, WNT3a (150ng/ul) (Cat# 5036WN010CF, R&D Systems), and a beta-catenin pathway inhibitor, IWR-1-

endo (1-5 μM) (sc-295215A, Santa Cruz) alone or in combination for 24 hours. Cells were lysed in RIPA buffer for western blotting analysis.

HGSOC ferroptosis signature: The ferroptosis signature of Pt-R HGSOC cells was derived by comparing platinum-resistant to platinum sensitive HGSOC cells, grouped based on arbitrary cutoff dose of 5μM, using the reported IC_{50} of those cells to cisplatin (9, 10). RNA-seq data of platinum resistant HGSOC cells (OVCAR4, OVCAR8, COV362 and SNU119, $IC_{50} >$5μM) and platinum sensitive HGSOC cells (TYKNU, IGROV1, OVCAR3, $IC_{50} <$5μM) in the form of gene counts were downloaded from the Cancer Cell Line Encyclopedia (CCLE). The raw counts were normalized using variance stabilizing transformations inside the DESeq2 package [Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2]. A heatmap of the ferroptosis pathway was generated from the normalized reads using pheatmap package.

Analysis of data from The Cancer Genome Atlas (TCGA). Processed Transcript-Per-Million (TPM) data were downloaded from TCGA-TARGET-GTEx Toil RNA-Seq Recompute Compendium (11) on UCSC Xena browser (12). 419 ovarian cancer samples from TCGA and 88 healthy ovarian control samples from GTEx were obtained. For isoform-level analysis, lowly expressed transcript isoforms were filtered out if they were not expressed in ≥ 90% of all samples. All genes and isoforms were annotated using Ensembl 82 (GRCh38.p3). **Correlation analysis between gene pairs:** Pearson correlation coefficient was calculated using log-transformed fold-change values of two genes of comparison. Outliers and influential points, with leverage exceeding twice of average of diagonal elements of hat matrix and Cook's distance exceeding 10% confidence ellipsoid, were removed. The test statistic follows a t-distribution with n-2 degrees of freedom and the statistical significance of the correlation was determined by corresponding p-value. **Survival analysis:** Kaplan-Meier survival curves were plotted using R package 'survival'

(R version 3.6.0). The high or low expression groups were defined based on statistically determined cutoff point that maximizes absolute value of the standardized two-sample linear rank statistic (13). The statistical significance of survival difference between groups with high/low level of expression was determined using log-rank test.

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