

Supplementary Information

Autophagy-mediated ID1 turnover dictates chemo-resistant fate in Ovarian Cancer Stem Cells

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Supplementary Figure 1

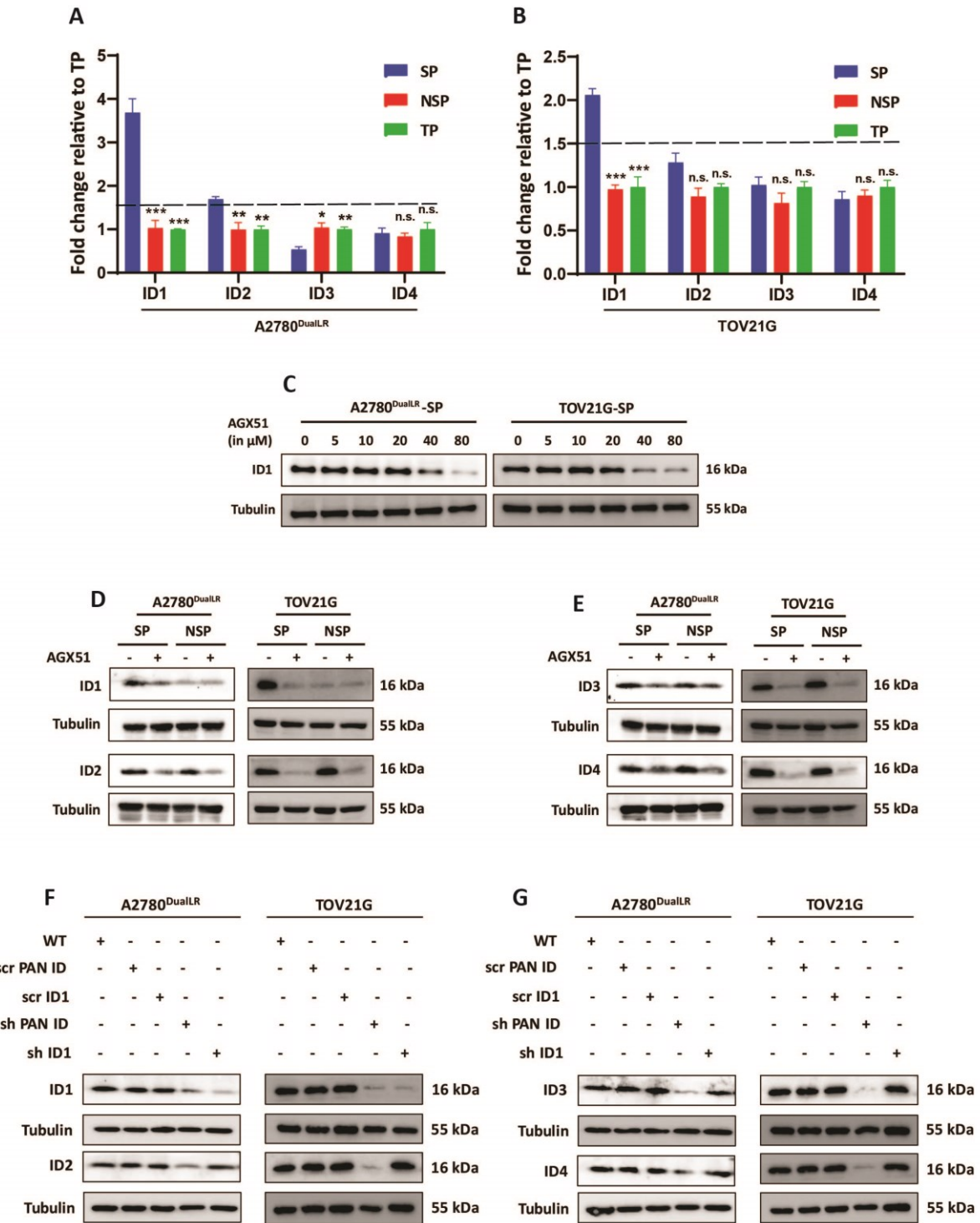


Figure S1: (A, B) RT-qPCR profiling for ID genes (ID1-ID4) revealed that ID1 had the highest upregulation (fold change above 1.5 threshold relative to TP cells) in SP cells (3.6-fold and 2-fold in A2780^{DualLR}-SP and TOV21G-SP respectively) compared to NSP and TP cells. ID2 was

1.7-fold upregulated in SP cells compared to NSP and TP of A2780^{DualLR} cells but non-significantly increased in SP cells compared to NSP and TP of TOV21G. There were no significant changes in the expression of ID3 and ID4 across SP, NSP and TP of A2780^{DualLR} and TOV21G cells. Data represented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-non-significant. **(C)** A2780^{DualLR} and TOV21G cells were treated with increasing concentrations of AGX51 (0, 5, 10, 20, 40, and 90 μ M) for 24 hours. Protein levels of ID1 were analysed by Western blot. 40 μ M AGX51 was identified as the optimal concentration for ID1 degradation in both cell lines. **(D, E)** SP and NSP cells were treated with 40 μ M AGX51 for 24 hours. Protein levels of ID1, ID2, ID3, and ID4 were analysed by Western blot. AGX51 treatment effectively reduced the expression of all four ID proteins in both SP and NSP cells of A2780^{DualLR} and TOV21G cells. **(F, G)** Western blot analysis was performed to assess the expression levels of ID1, ID2, ID3, and ID4 proteins in A2780^{DualLR} and TOV21G cells following shRNA-mediated knockdown. Cells were transduced with sh PAN-ID (targeting all ID proteins), sh ID1 (targeting only ID1), or their respective scrambled controls (scr PAN-ID and scrID1). Wild-type (WT) non-transduced cells served as a baseline control. sh PAN-ID effectively downregulated all four ID proteins, while sh ID1 specifically reduced ID1 levels. The scrambled controls showed no significant changes in ID protein expression compared to WT cells in both cell lines. SP: Side Population, NSP: Non-Side Population, TP: Total Population,

Supplementary Figure 2

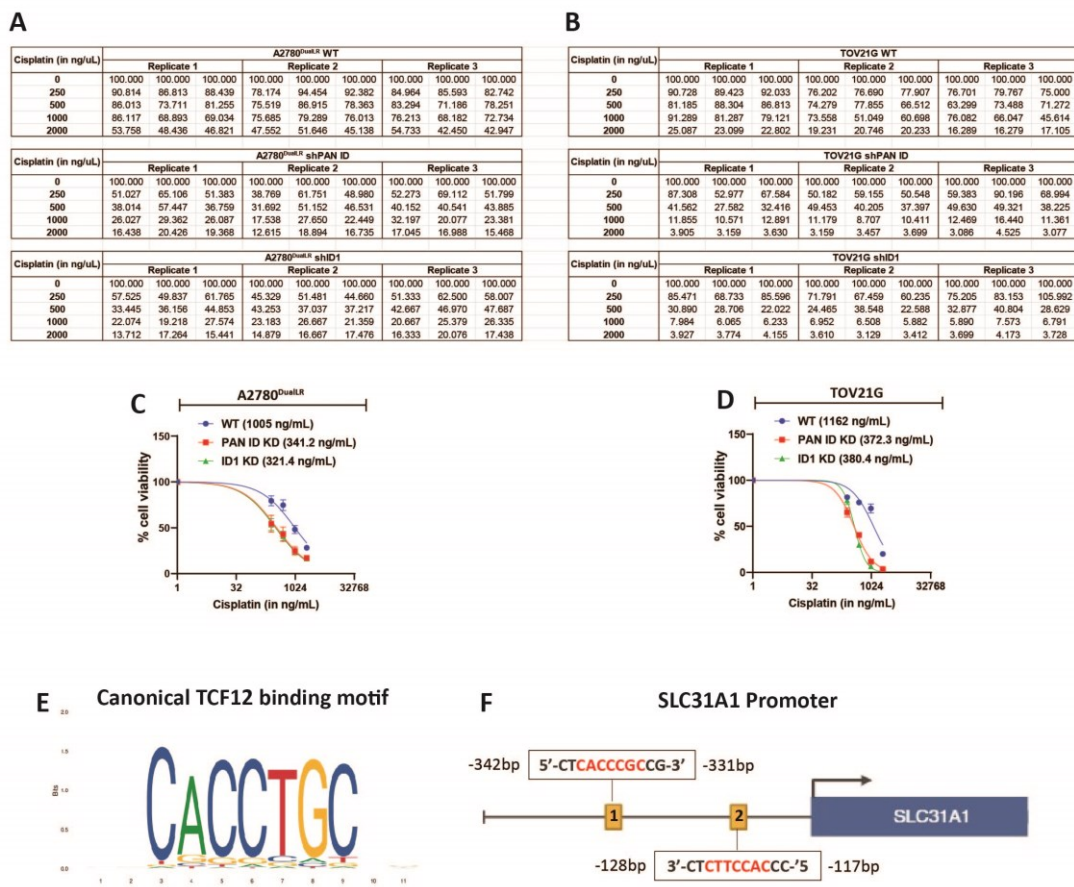


Figure S2: (A, B) Raw MTT assay data for A2780^{DualLR} and TOV21G cells treated with increasing concentrations of cisplatin. (C, D) Dose-response curves showing cell viability of wild-type (WT), shPAN-ID, and shID1 transduced A2780^{DualLR} and TOV21G cells in response to cisplatin. shRNA-mediated knockdown of PAN-ID or ID1 resulted in a marked decrease in cell viability and a 3-fold reduction in IC₅₀ values compared to WT cells. IC₅₀ values: A2780^{DualLR} (WT: 1005 ng/mL; shPAN-ID: 341.2 ng/mL; shID1: 321.4 ng/mL) and TOV21G (WT: 1162 ng/mL; shPAN-ID: 372.3 ng/mL; shID1: 380.4 ng/mL). Data represent mean $\pm$ SEM, n=3. (E) Schematic of human TCF12 canonical TCF12 binding motif obtained from JASPER Database. (F) Schematic of the SLC31A1 promoter showing two high-scoring putative TCF12 binding sites predicted by JASPAR.

Supplementary Figure 3

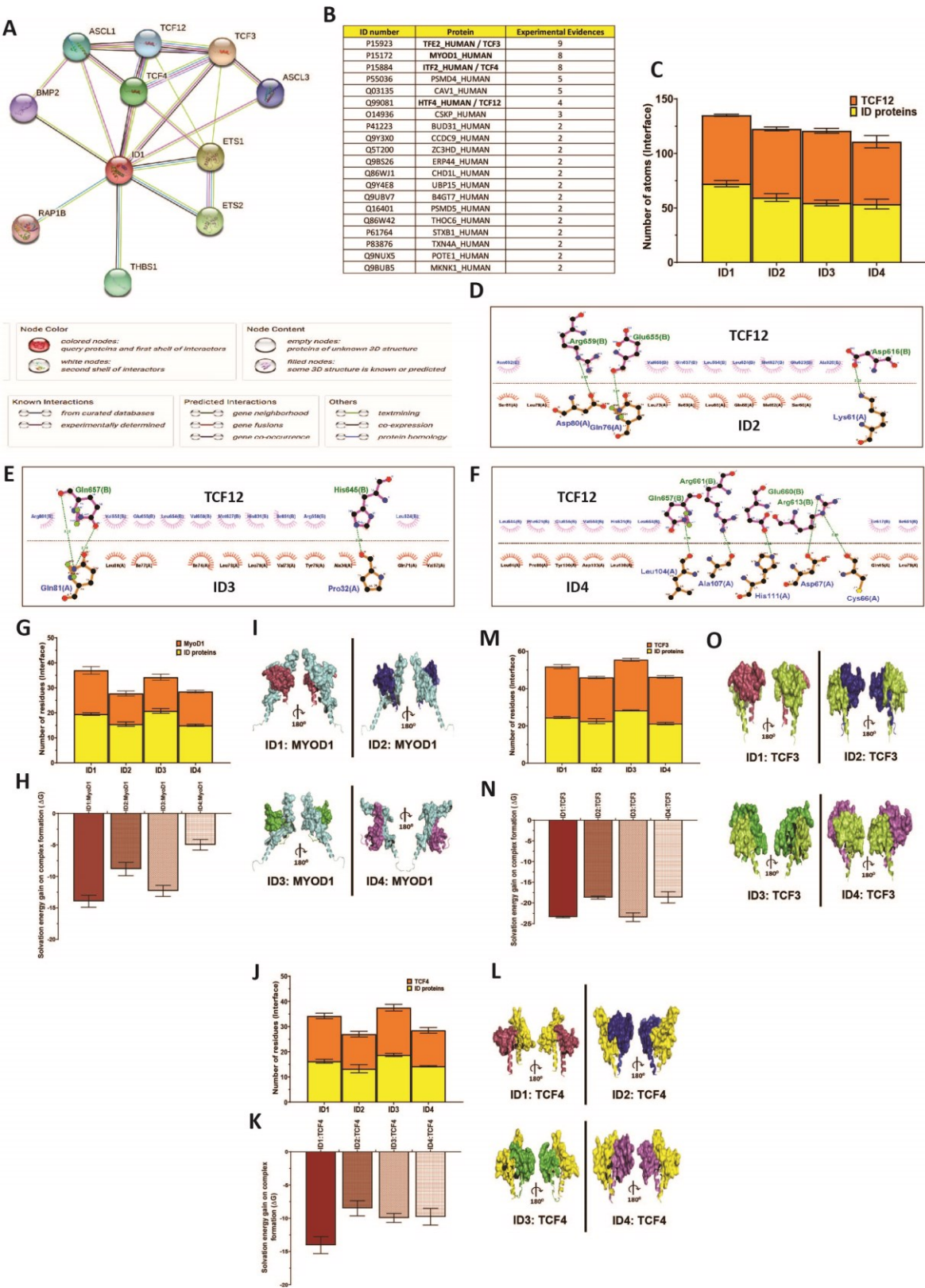
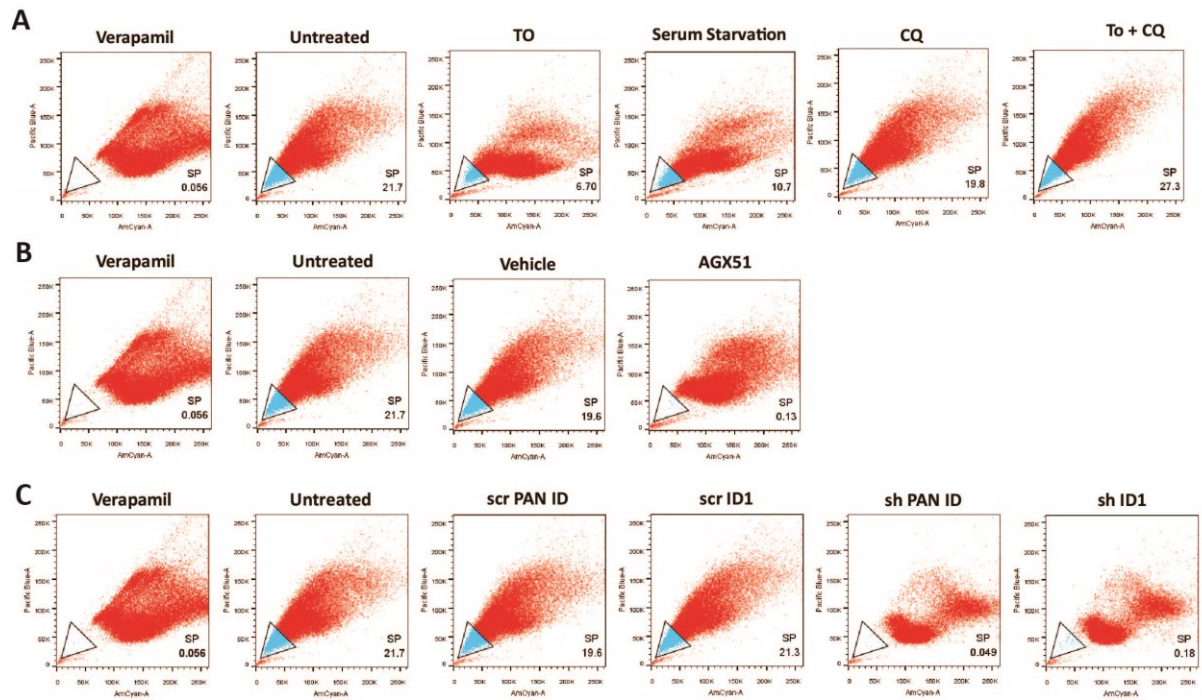


Figure S3: (A, B) STRING and AGILE analysis showing the potential interactions and functional relevance of ID1 (C) Graph showing the number of atoms involved in stabilizing the ID1-4 interaction with TCF12. (D-F) Lig-plot analysis of (D) ID2:TCF12, (E) ID3:TCF12 and (F) ID4:TCF12 dimer revealing dramatic decrease in H-bonds and increase in the van-der waal interaction. (G, J, N) Graph showing the total number of residues involved in stabilizing the (G) ID1-4: MyoD1 dimer, (J) ID1-4: TCF4 dimer and (N) ID1-4: TCF3 dimer. Each bar denotes the mean $\pm$ S.D. of top four HADDOCK structures. (H, K, N) Graph showing the thermodynamic feasibility of (H) ID1-4 interaction with MyoD1, (K) ID1-4 interaction with TCF4 and (N) ID1-4 interaction with TCF3. (I, L, O) Representative 3-D structures of ID1-4 interactions with (I) MyoD1 (L) TCF4 and (O) TCF3.

Supplementary Figure 4

A2780^{DualLR}



TOV21G

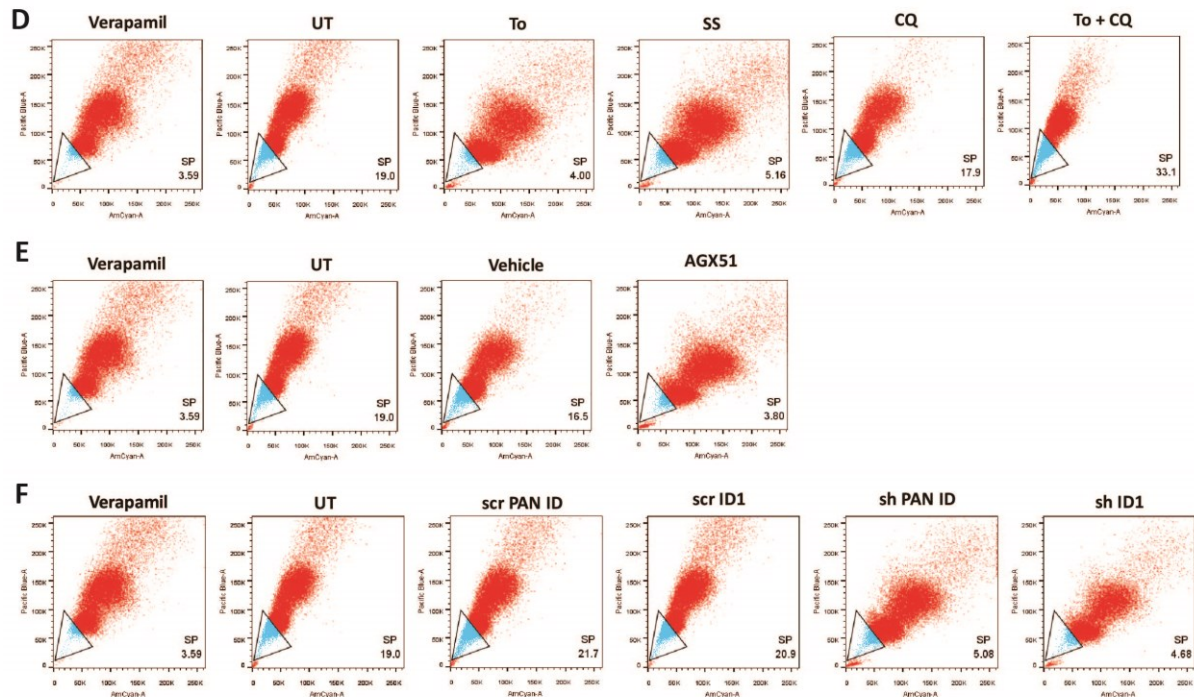


Figure S4: (A, D) FACS plots showing the percentage of SP cells after autophagy modulation in A2780^{DualLR} (A) and TOV21G (D) cells. Treatments include autophagy induction (Torin 1 and serum starvation), blockade (Chloroquine, CQ), and dual manipulation (Torin 1 + CQ). **(B, E)** FACS plots displaying the percentage of SP cells following AGX51 treatment (pharmacological depletion of ID1) in A2780^{DualLR} (B) and TOV21G (E) cells. Vehicle control showed no significant change compared to untreated cells. **(C, F)** FACS plots illustrating the percentage of SP cells after genetic knockdown (shPAN-ID and shID1) in A2780^{DualLR} (C) and TOV21G (F) cells. Scrambled controls (scrPAN-ID and scrID1) showed no significant changes compared to non-transduced wild-type cells. SP cells were identified based on DCV dye efflux. Data represent n=3 independent experiments. The experiments were conducted together as single set of data: (Set A-C) and (Set D-F). For each set a single Verapamil-treated control and a single untreated has been control utilized for comparative analysis. SP: Side Population, NSP: Non-Side Population, TP: Total Population, TO: Torin 1, CQ: Chloroquine, AGX51: PAN ID Inhibitor, SS: Serum Starvation, scr PAN-ID: Scrambled PAN-ID, scr ID1: scrambled ID1, sh PAN ID: PAN ID Knockdown, sh ID1: ID1 knockdown

Supplementary Figure 5

A2780^{DualLR} model

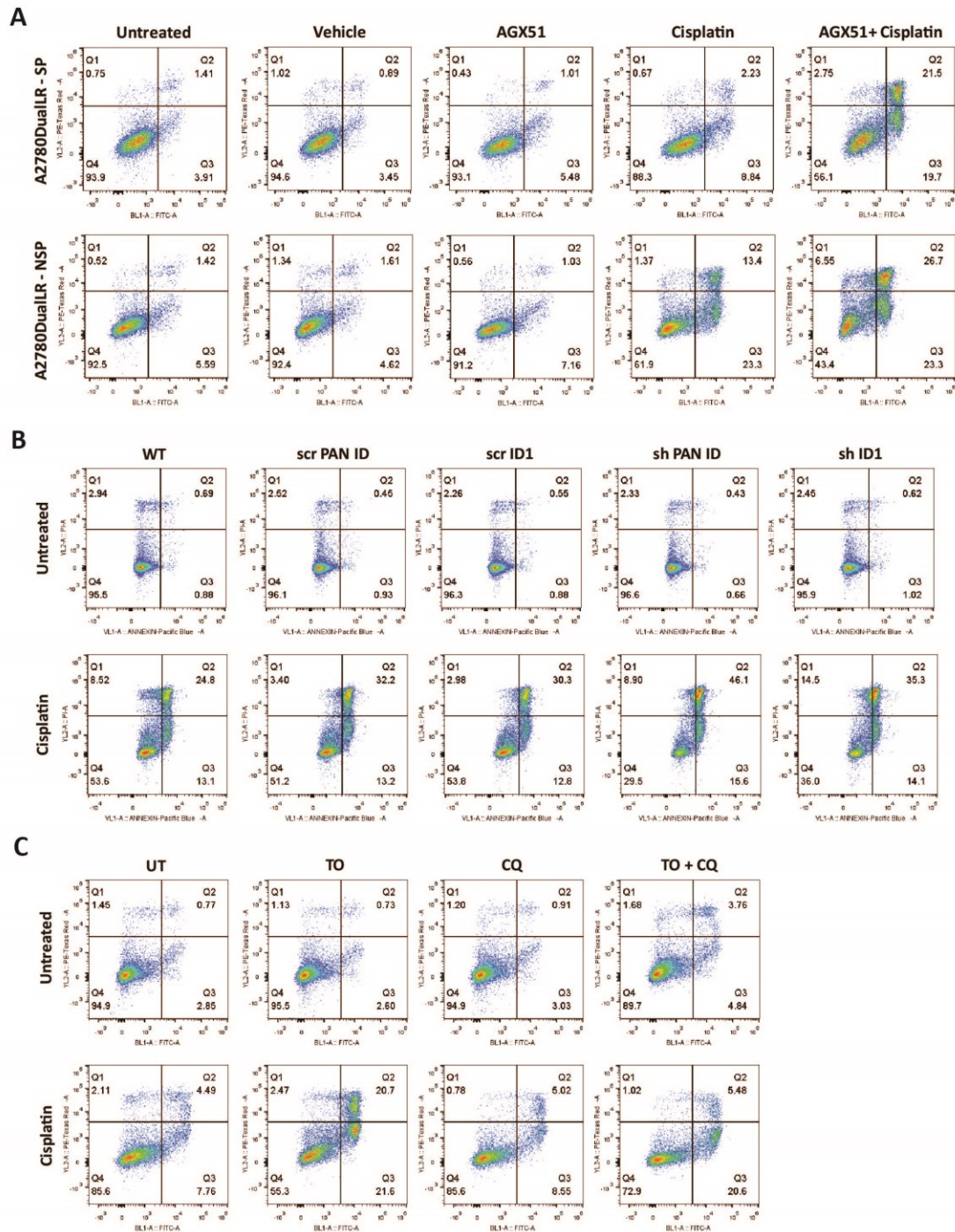


Figure S5: Annexin V-PI FACS plots showing the percentage of cell death (necrosis + early and late apoptosis) after cisplatin treatment in A2780^{DualLR} (SP, NSP and TP cells) under various conditions: **(A)** Pharmacological ID1 depletion using AGX51 in SP and NSP cells: The

combination of AGX51 and cisplatin showed a synergistic effect in both cell populations. NSP cells showed more death compared to SP indicating their resistant nature. **(B)** Genetic knockdown of ID1. shPAN-ID and shID1 cells exhibited increased cell death compared to their respective scrambled controls (scr PAN-ID and scr ID1). Scrambled controls showed no significant difference in cell death compared to wild-type (WT) non-transduced cells. **(C)** ID1 depletion via autophagy modulation: Torin 1 treatment combined with cisplatin led to increased cell death. This effect was partially reversed in the Torin 1 + Chloroquine (TO+CQ) group treated with cisplatin, suggesting that the observed effects are mediated by autophagy induction. Cells were treated with 10X IC50 of cisplatin of A2780^{DualLR} for 24 hours. SP: Side Population, NSP: Non-Side Population, TP: Total Population, TO: Torin 1, CQ: Chloroquine, AGX51: PAN ID Inhibitor, SS: Serum Starvation, scr PAN-ID: Scrambled PAN-ID, scr ID1: scrambled ID1, sh PAN ID: PAN ID Knockdown, sh ID1: ID1 knockdown

Supplementary Figure 6

TOV21G model

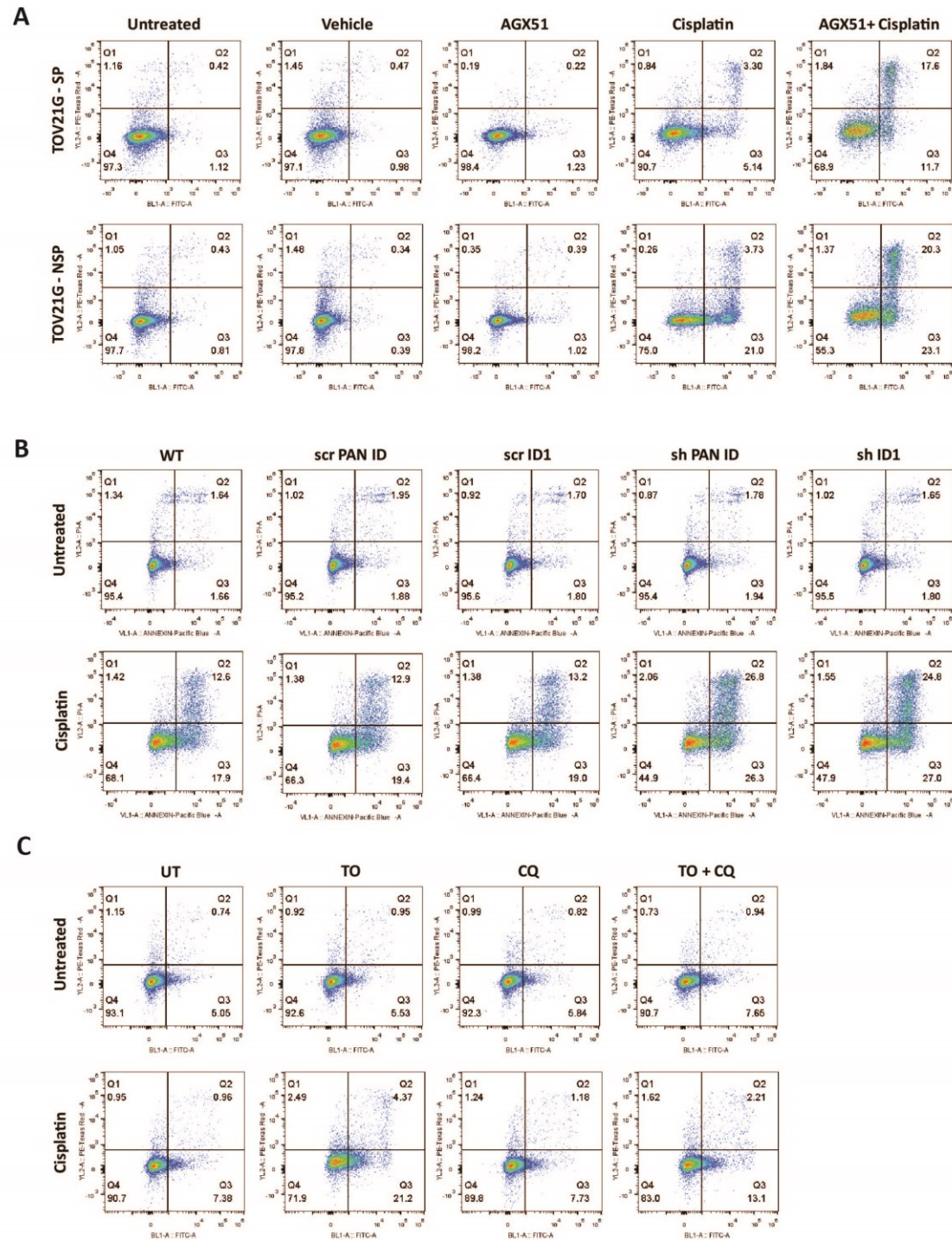


Figure S6: Annexin V-PI FACS plots showing the percentage of cell death (necrosis + early and late apoptosis) after cisplatin treatment in TOV21G (SP, NSP and TP cells) under various conditions: **(A)** Pharmacological ID1 depletion using AGX51 in SP and NSP cells: The combination of AGX51 and cisplatin showed a synergistic effect in both cell populations. NSP

cells showed more death compared to SP indicating their resistant nature. **(B)** Genetic knockdown of ID1. shPAN-ID and shID1 cells exhibited increased cell death compared to their respective scrambled controls (scr PAN-ID and scr ID1). Scrambled controls showed no significant difference in cell death compared to wild-type (WT) non-transduced cells. **(C)** ID1 depletion via autophagy modulation: Torin 1 treatment combined with cisplatin led to increased cell death. This effect was partially reversed in the Torin 1 + Chloroquine (TO+CQ) group treated with cisplatin, suggesting that the observed effects are mediated by autophagy induction. Cells were treated with 10X IC50 of cisplatin of TOV21G for 24 hours. SP: Side Population, NSP: Non-Side Population, TP: Total Population, TO: Torin 1, CQ: Chloroquine, AGX51: PAN ID Inhibitor, SS: Serum Starvation, scr PAN-ID: Scrambled PAN-ID, scr ID1: scrambled ID1, sh PAN ID: PAN ID Knockdown, sh ID1: ID1 knockdown

Supplementary Figure 7

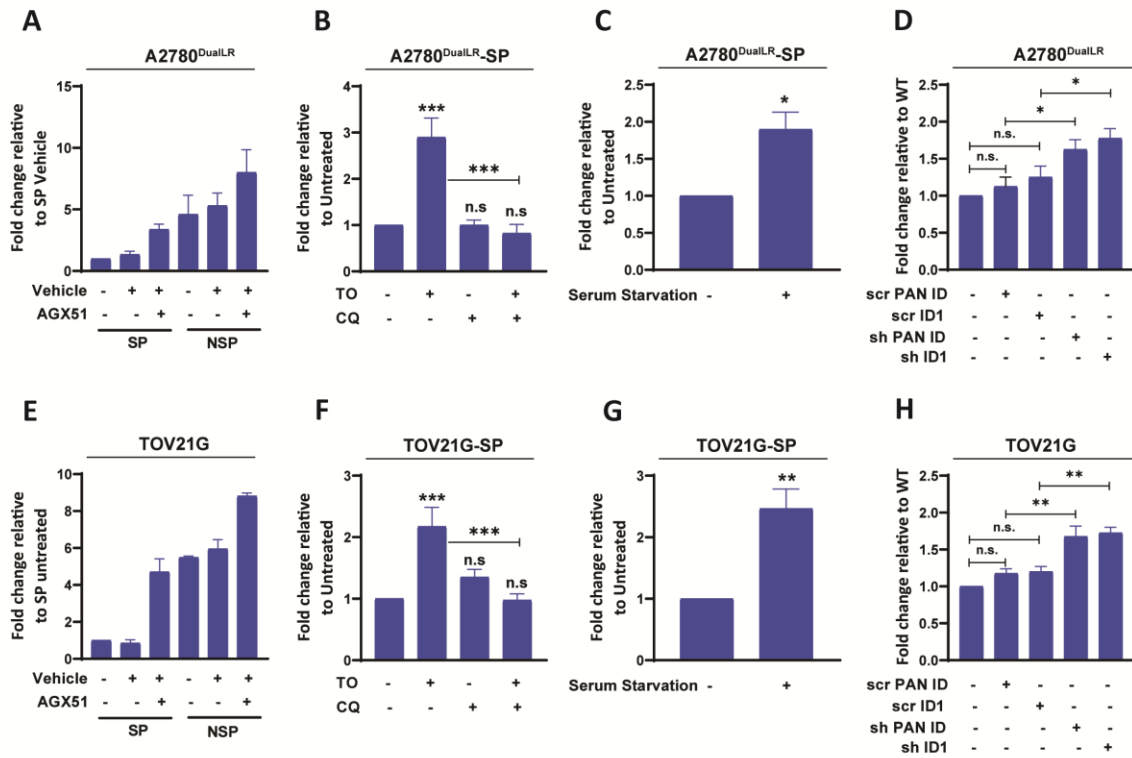


Figure S7: (A, E) Densitometric analysis of SLC31A1 protein levels in A2780^{DualLR} and TOV21G cells (n=2). SP cells showed lower SLC31A1 expression compared to NSP and TP cells. ID1 depletion by AGX51 treatment increased SLC31A1 levels in both SP and NSP cells, with the highest increase observed in NSP cells. Vehicle control (0.4% DMSO) showed no changes in protein levels. (B, F) Quantification of SLC31A1 protein levels following autophagy-mediated depletion of ID1 (n=3). Torin1 treatment led to significant upregulation of SLC31A1 protein. Simultaneous treatment with Torin1 and CQ resulted in decreased SLC31A1 protein levels compared to Torin1 alone in both A2780^{DualLR}-SP and TOV21G-SP cells, indicating autophagy induction-specific effects. (C, G) Densitometric analysis of SLC31A1 protein levels after serum starvation (n=3). Both A2780^{DualLR}-SP and TOV21G-SP cells showed significant increases in SLC31A1 protein levels following serum starvation. (D, H) Quantification of SLC31A1 protein levels following genetic depletion of ID1 (n=3). Both sh PAN-ID and sh ID1 depletion of ID1 led to increased levels of SLC31A1 protein, with the

highest increase observed in shID1 cells of both A2780^{DualLR} and TOV21G cells. Scrambled controls (scr PAN ID and scr ID1) showed no significant changes in SLC31A1 protein levels compared to non-transduced wild-type cells. Data represents mean $\pm$ SEM. Statistical analysis was performed using Student's t-test for comparing untreated and drug-treated group. For comparisons involving multiple groups, one-way ANOVA was used, followed by Dunnett's post-hoc test when comparing multiple groups to a control, or Bonferroni post-hoc test when comparing all groups to each other, whichever is appropriate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.- non-significant. SP: Side Population, NSP: Non-Side Population, TP: Total Population, TO: Torin 1, CQ: Chloroquine, AGX51: PAN ID Inhibitor, SS: Serum Starvation, scr PAN-ID: Scrambled PAN-ID, scr ID1: scrambled ID1, sh PAN ID: PAN ID Knockdown, sh ID1: ID1 knockdown

Table S1. List of antibodies used in this study

Primary Antibodies			
Antibody	Source	Identifier	Company
ABCG2	Rabbit	A17908	ABclonal, MA, USA
ID1	Mouse	sc-133104	SCBT, TX, USA
ID2	Mouse	sc-398104	SCBT, TX, USA
ID3	Mouse	sc-56712	SCBT, TX, USA
ID4	Mouse	sc-365656	SCBT, TX, USA
LAMP1	Mouse	H4A3	DSHB, IA, USA
LC3B	Rabbit	2775S	CST, MA, USA
NANOG	Mouse	sc-293121	SCBT, TX, USA
OCT-3/4	Rat	MAB1759	Biotechne, MN, USA
p62/SQSTM1	Rabbit	ab155686	CST, MA, USA
SLC31A1	Rabbit	A0773	ABclonal, MA, USA
SLC31A1 (I.H.C. grade)	Rabbit	APREST72883	Sigma Aldrich, DA, DE
TCF12	Rabbit	A4146	ABclonal, MA, USA
TCF12 (ChIP grade)	Mouse	sc-28364-X	SCBT, TX, USA
TCF12	Rabbit	11825	CST, MA, USA
α -Tubulin	Mouse	T5168	Sigma Aldrich, DA, DE
Rabbit Gamma Globulin Control	Rabbit	31887	Invitrogen, USA
Mouse Gamma Globulin Control	Mouse	31878	Invitrogen, USA
Secondary antibodies			
Alexa Fluor™ 568	Rat	A11004	Thermofisher, MA USA
Anti-Mouse IgG HRP conjugate	Goat	4416	Thermofisher, MA USA

Anti-Rabbit IgG HRP conjugate	Goat	A6154	Thermofisher, MA USA
DyLight™ 488	Rabbit	35552	Thermofisher, MA USA
DyLight™ 633	Mouse	35512	Thermofisher, MA USA

Table S2. List of oligonucleotide sequences used in this study

Primer Name	Sequences	Purpose
shPAN-ID	5'-CTACGACATGAACGGCTGTTA-3'	PAN ID shRNA plasmid construction
shID1	5'-AGGTGAGCAAGGTGGAGATTC-3'	ID1 shRNA plasmid construction
scr PANID	5'- ACTTCGAGAACTGTCGGAAC-3'	scrambled PAN ID shRNA plasmid construction
scrID1	5'-GGAGTGTGCGTAGACGATAAG-3'	scrambled ID1 shRNA plasmid construction
SLC31A1 F	5'-AGGACTCAAGATAGCCCGAGAGA-3'	qRT-PCR
SLC31A1 R	5'-CCTGGGACAGGCATGGAA-3'	qRT-PCR
OCT4A F	5'- CCTGAAGCAGAAGAGGATCACC-3'	qRT-PCR
OCT4A R	5'-AAAGCGGCAGATGGTCGTTTGG-3'	qRT-PCR
NANOG F	5'-CTCCAACATCCTGAACCTCAGC-3'	qRT-PCR
NANOG R	5'-CGTCACACCATTGCTATTCTTCG-3'	qRT-PCR
GAPDH F	5'-TGCACCACCAACTGCTTAGC-3'	qRT-PCR
GAPDH R	5'-GGCATGGACTGTGGTCATGAG-3'	qRT-PCR
Site 1 F	5'- AACAATCCGCCGGTATCTCT-3'	CHIP-qRT-PCR

Site 1 R	5'- TGAAAGCCAACGCAATGTTCG-3'	CHIP-qRT-PCR
Site 2 F	5'- ACGGTCTCTGGACCGAAAGT-3'	CHIP-qRT-PCR
Site 2 R	5'-CGCGCGAGCCTCTCATTT-3'	CHIP-qRT-PCR
GAPDH F	5'-TACTAGCGGTTTTACGGGCG-3'	CHIP-qRT-PCR
GAPDH R	5'-TCGAACAGGAGGAGCAGAGAGCGA-3'	CHIP-qRT-PCR