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Supplemental Information

MicroRNA-Mediated Suppression of the TGF-β Pathway Confers Transmissible and Reversible CDK4/6 Inhibitor Resistance

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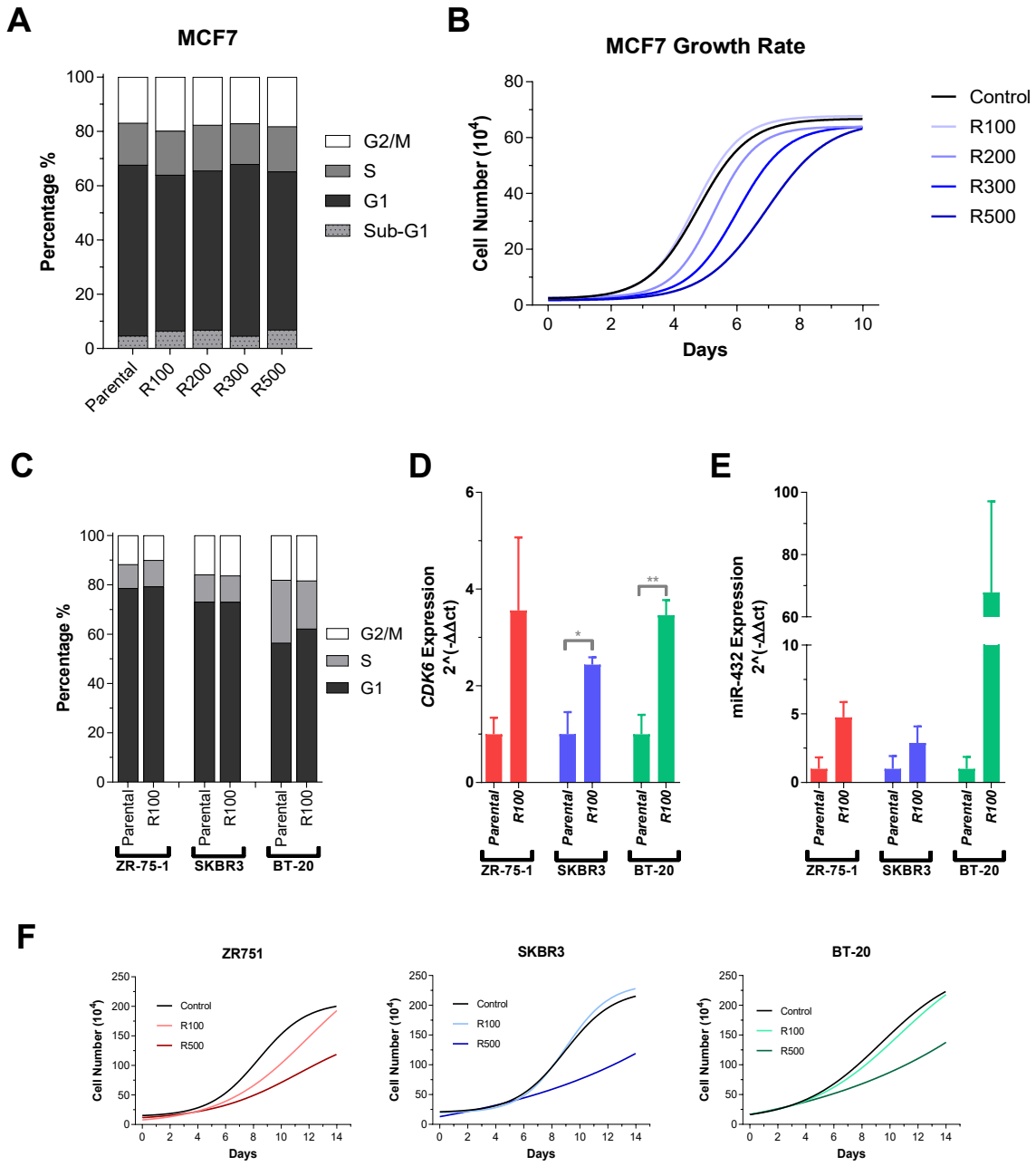

Figure S1. Generated CDK4/6 inhibitor resistant cell lines have dramatically increased CDK6 protein expression. Related to Figure 1.

A, Flow cytometry analysis of the cell cycle profile of palbociclib resistant MCF7 cells. Cells were initially made resistant to 100 nM (R100) which was then escalated culminating in cells resistant to 500 nM (R500). **B,** Growth rate of resistant cells compared to parental. **C,** Flow cytometry analysis of the cell cycle profile of parental and resistant ZR-75-1, SKBR3 and BT-20 cells. **D,** Quantitative real-time qPCR analysis of *CDK6* and **E,** miR-432 expression in parental and resistant ZR-75-1, SKBR3 and BT-20 cells. Data are reported as the mean ± SEM of three independent experiments. * $p < 0.01$, ** $p < 0.01$. **F,** Growth rate of ZR-75-1, SKBR3 and BT-20 cells cultured to palbociclib resistance.

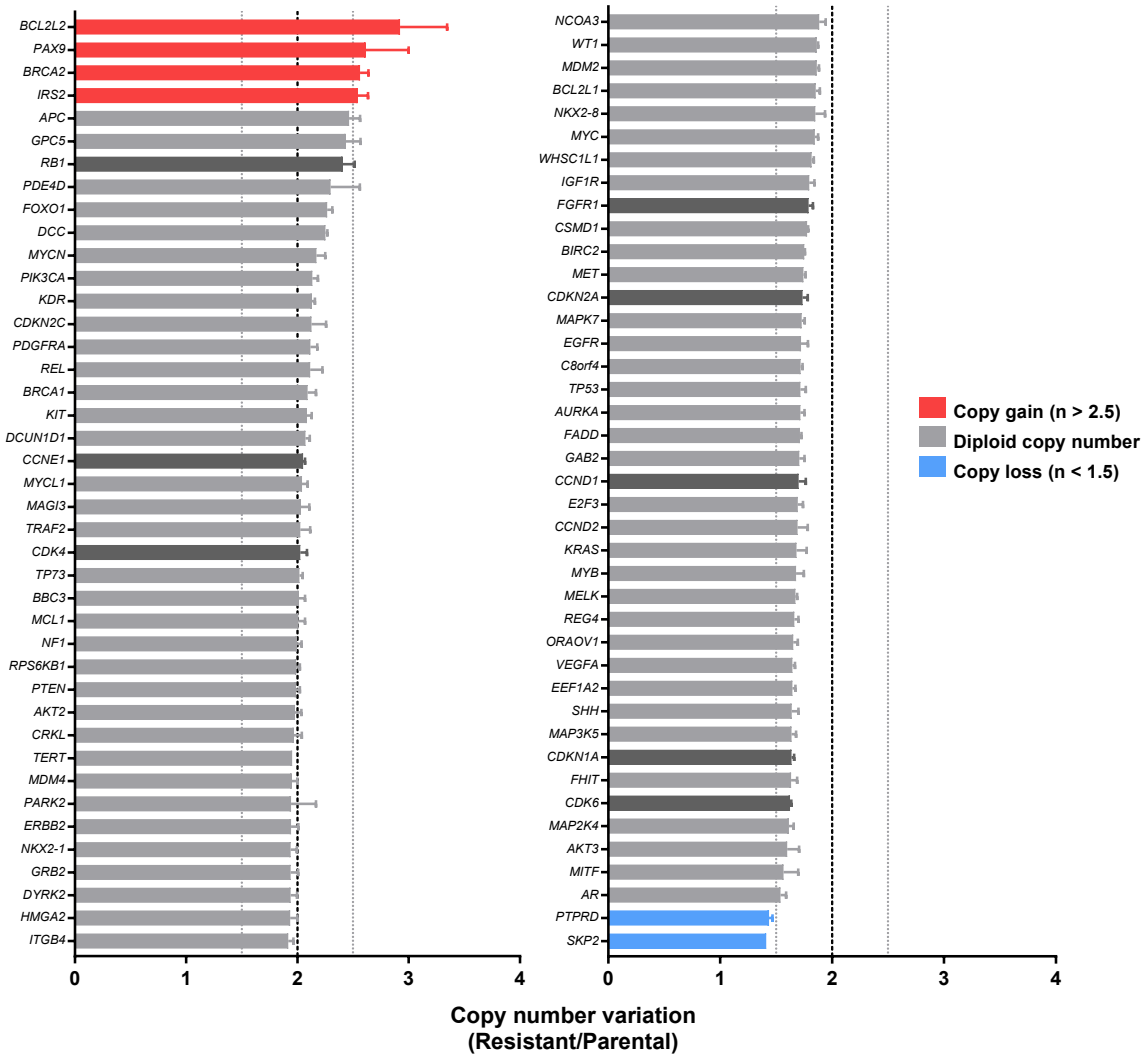

Figure S2. Copy Number variation analysis in parental and palbociclib resistant T47D cells. Related to Figure 1.

Copy number variation was analyzed using nanoString nCounter v2 Cancer CNV Assay. Copy number estimations are expressed as resistant cell/parental cell. Copy number gains were taken as a ratio of > 2.5, copy number loss was taken as a ration of < 1.5, as per manufactures instructions. Dark grey bars are highlighted as they have been previously implicated in CDK4/6 inhibitor resistance. Data are reported as the mean ± SEM of three technical repeats.

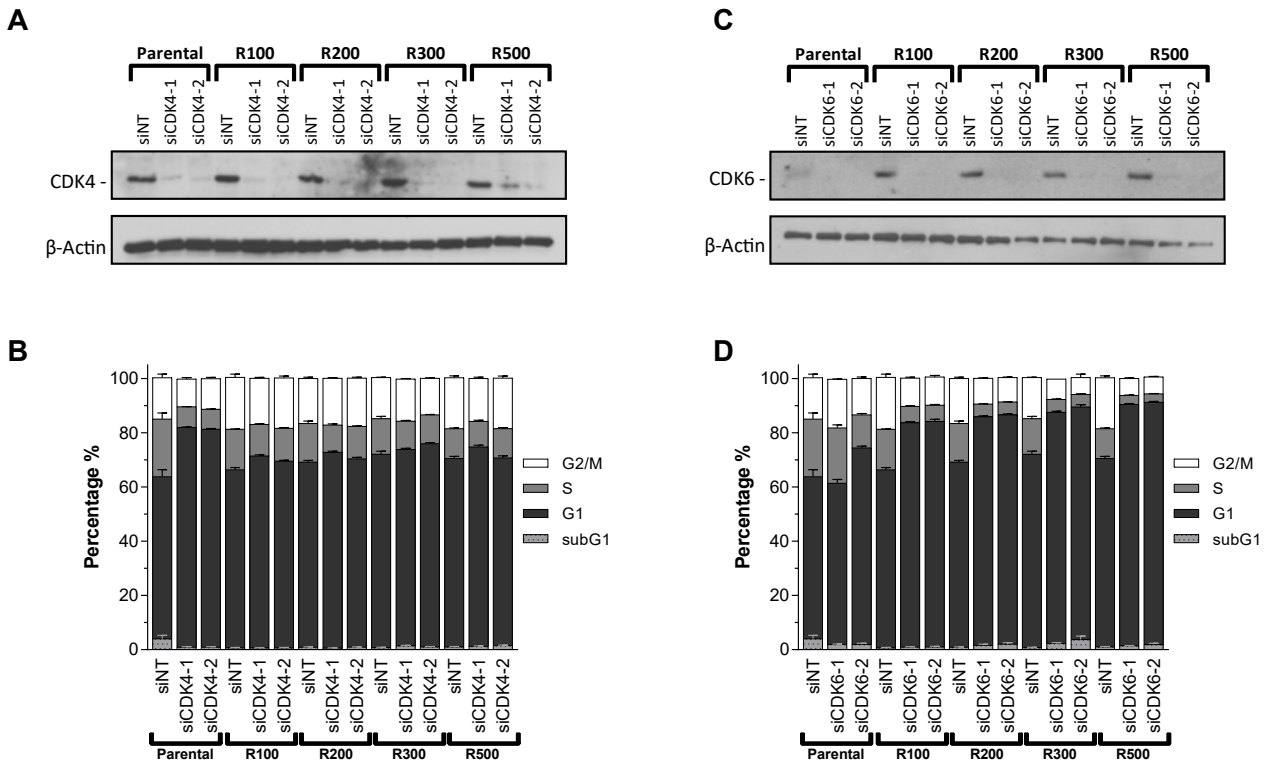

Figure S3. CDK6, but not CDK4, knockdown resensitizes palbociclib resistant T47D cells. Related to Figure 2.

A, Western blot analysis of CDK4 expression and **B,** cell cycle analysis by flow cytometry 72 hours after CDK4 siRNA transfection. Data are reported as the mean ± SEM of three independent experiments. **C,** Western blot analysis of CDK6 expression and **D,** cell cycle analysis by flow cytometry 72 hours after CDK6 siRNA transfection. Data are reported as the mean ± SEM of three independent experiments.

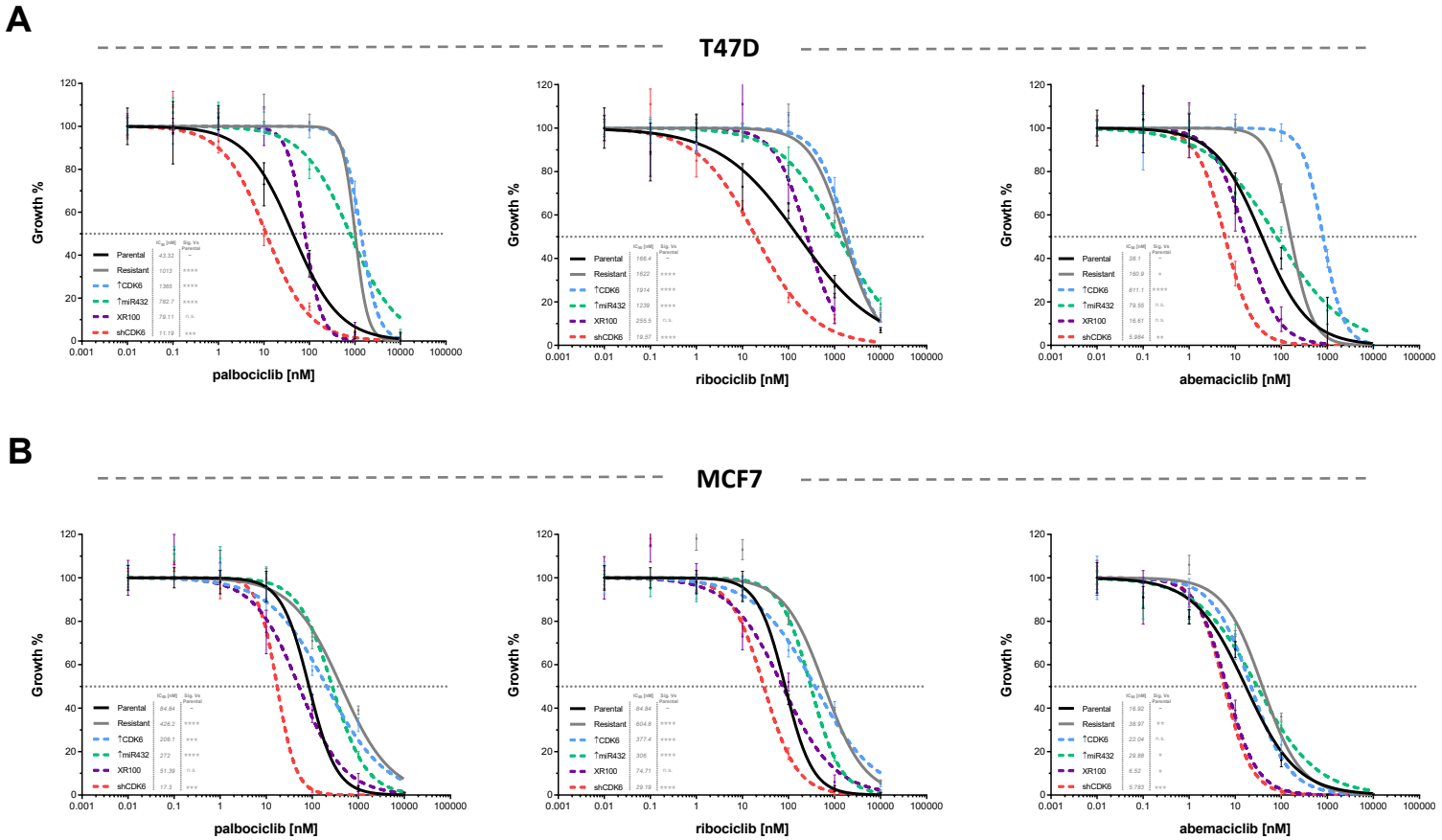

Figure S4. Palbociclib resistant cells are significantly more resistant to ribociclib, and moderately more resistant to abemaciclib. Related to Figures 1, 2, 3, 5 and 7. **A,** T47D and **B,** MCF7 Cells were treated with either DMSO, palbociclib, ribociclib or abemaciclib for 5 days. Subsequently cell growth was quantified and normalized to DMSO treated control. Cells were either parental, palbociclib resistant (100nM), CDK6 overexpressing, miR-432-5p overexpressing, ex-resistant or CDK6 knockdown. Data are mean ± SEM (n>3), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

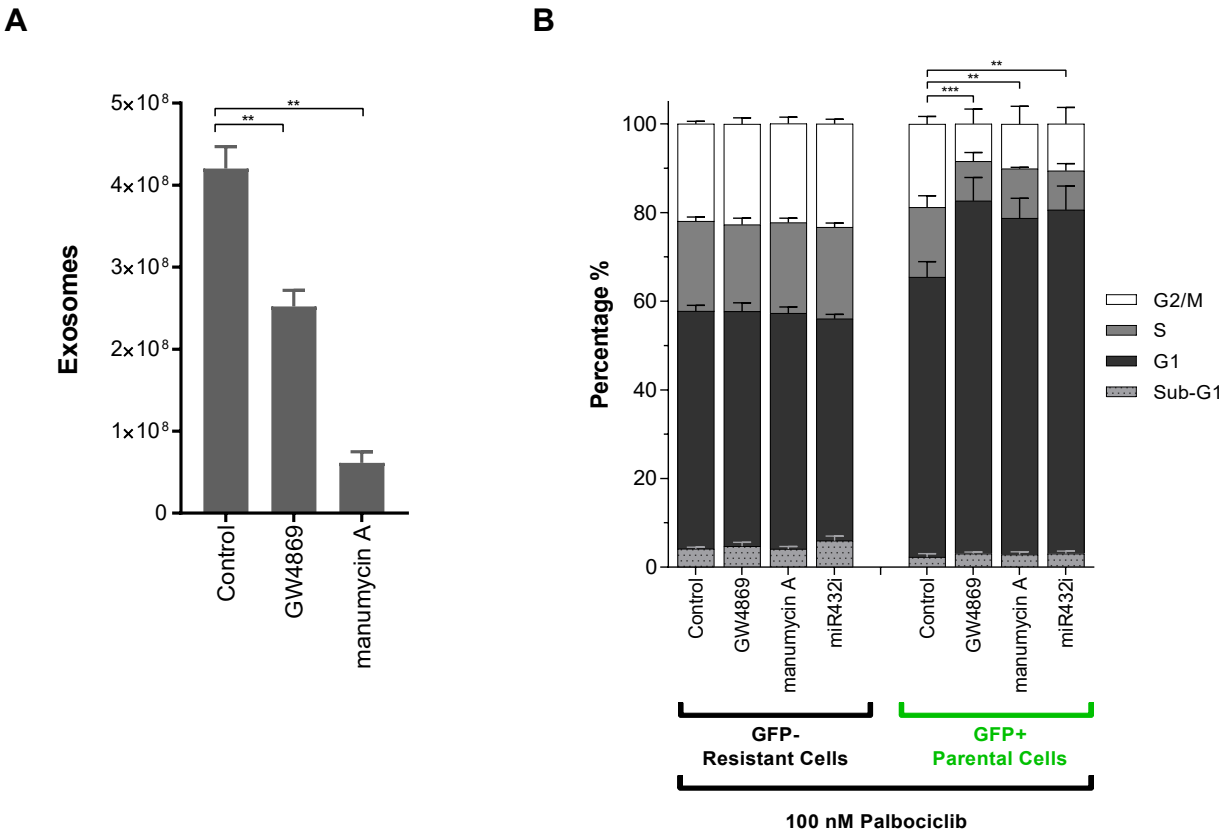

Figure S5. Inhibition of exosome production reduces efficacy of resistance transmission from resistance to parental cells. Related to Figure 4.

A, Cells were treated with either DMSO, 10 μM GW4869 or 10 μM manumycin A for 48 hours prior to exosome harvest and quantification. Data are reported as the mean ± SEM of three independent experiments. ** $p < 0.01$. **B,** Palbociclib resistant GFP- cells were cocultured with GFP parental cells in the presence of either DMSO, GW4869, manymycin A or miR-432-5p inhibitor for 72 hours. Subsequently, cells were harvested and analyzed by flow cytometry for GFP expression and cell cycle profile. Data are reported as the mean ± SEM of three independent experiments. Statistical comparisons illustrated the difference in G1 populations. ** $p < 0.01$, *** $p < 0.001$.

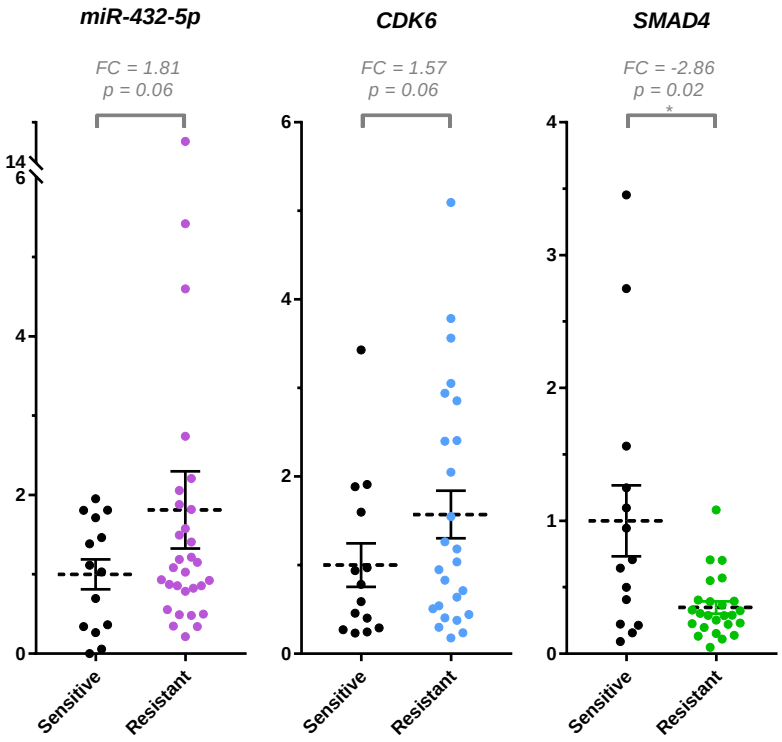

Figure S6 miR-432-5p, CDK6 and SMAD4 expression in CDK4/6 inhibitor treated patient biopsies. Related to Figure 6.

miR-432-5p, *CDK6* and *SMAD4* expression was analyzed in tumor biopsy samples from 44 patients who received CDK4/6 inhibitors. Samples were grouped based on the radiological response and normalized to the average expression in the sensitive group. miR-432-5p miRNAseq data is represented as normalized counts per million (CPM), *CDK6* and *SMAD4* real-time qPCR data is represented as normalized $2^{-\Delta\Delta CT}$. P-values were determined using a Welch's *t*-test.

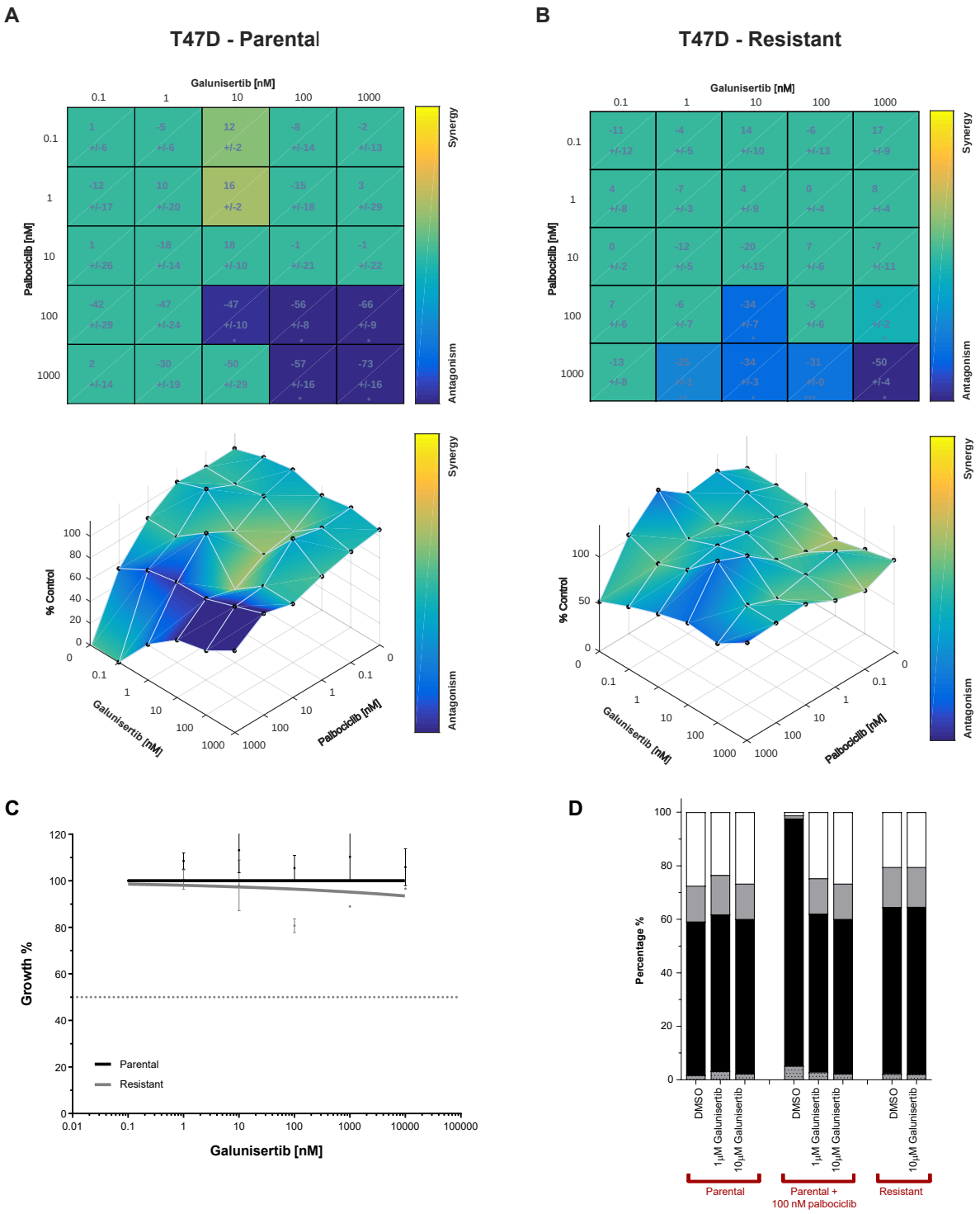

Figure S7. Combined galunisertib and palbociclib is antagonistic. Related to Figure 6.
A, T47D parental and **B,** resistant Cells were treated with either DMSO, palbociclib or galunisertib for 5 days. Subsequently cell growth was quantified and normalized to DMSO treated control. BLISS Synergy/ antagonism score was modelled using Combenefit software. **C,** T47D parental and resistant cells were treated with escalating dose of galunisertib for 5 days. Subsequently cell growth was quantified and normalized to DMSO treated control. **D,** Cell cycle analysis of T47D cells treated with galunisertib and/or palbociclib for 24 hours.

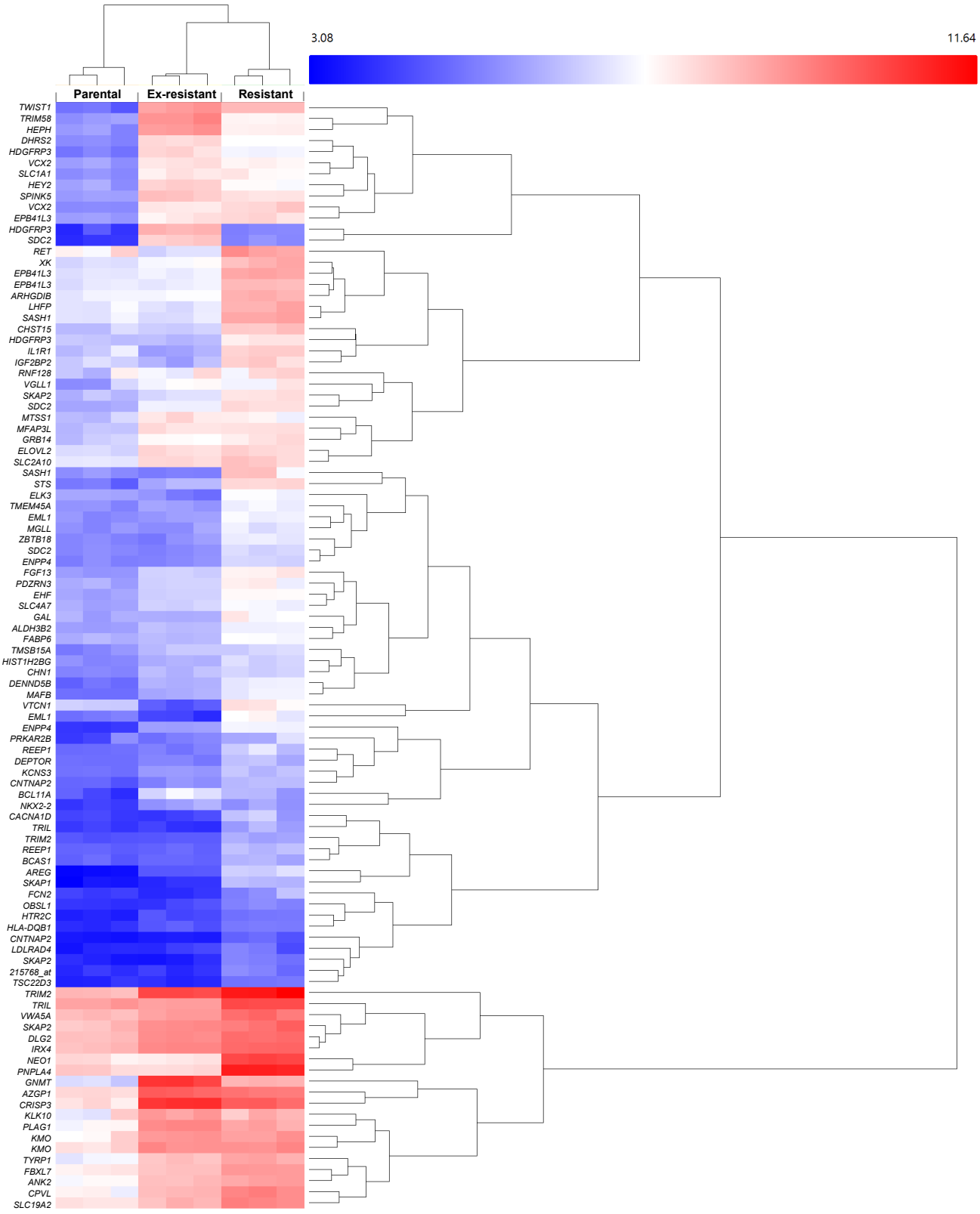

Figure S8. Gene expression in ex-resistant cells is more closely related to resistant than parental cells. Related to Figure 7.

Hierarchal clustering performed using 100 of the most significantly changed genes across parental, resistant and ex-resistant T47D cells, determined by gene expression analysis.

Gene	Primer	Sequence (5' - 3')
CDK6	Forward	TGCACAGTGTCACGAACAGA
CDK6	Reverse	ACCTCGGAGAAGCTGAAACA
CDK4	Forward	CCCGAAGTTCTTCTGCAGTC
CDK4	Reverse	CTGGTCGGCTTCAGAGTTTC
GAPDH	Forward	GAGTCAACGGATTTGGTCGT
GAPDH	Reverse	TTGATTTTGGAGGGATCTCG
CCND1	Forward	GATCAAGTGTGACCCGGACT
CCND1	Reverse	TCCTCCTCTTCCTCCTCCTC
CCND3	Forward	TGATTTCCTGGCCTTCATTC
CCND3	Reverse	AGCTTCGATCTGCTCCTGAC
TGFBR1	Forward	GAGCATGGATCCCTTTTTGA
TGFBR1	Reverse	ATGTGAAGATGGGCAAGACC
TGFBR2	Forward	GGGGAAACAATACTGGCTGA
TGFBR2	Reverse	GAGCTCTTGAGGTCCCTGTG
TGFBR3	Forward	CCAAGATGAATGGCACACAC
TGFBR3	Reverse	CCATCTGGCCAACCACTACT
SMAD4	Forward	CCCCAGAGCAATATTCCAGA
SMAD4	Reverse	GGCTCGCAGTAGGTAACTGG
SMAD3	Forward	GATACGTGGACCCTTCTGGA
SMAD3	Reverse	ACCTTTGCCTATGTGCAACC

Table S1. Primers for qPCR. Related to STAR Methods