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Supplementary Materials for

SLC36A1-mTORC1 signaling drives acquired resistance to CDK4/6 inhibitors

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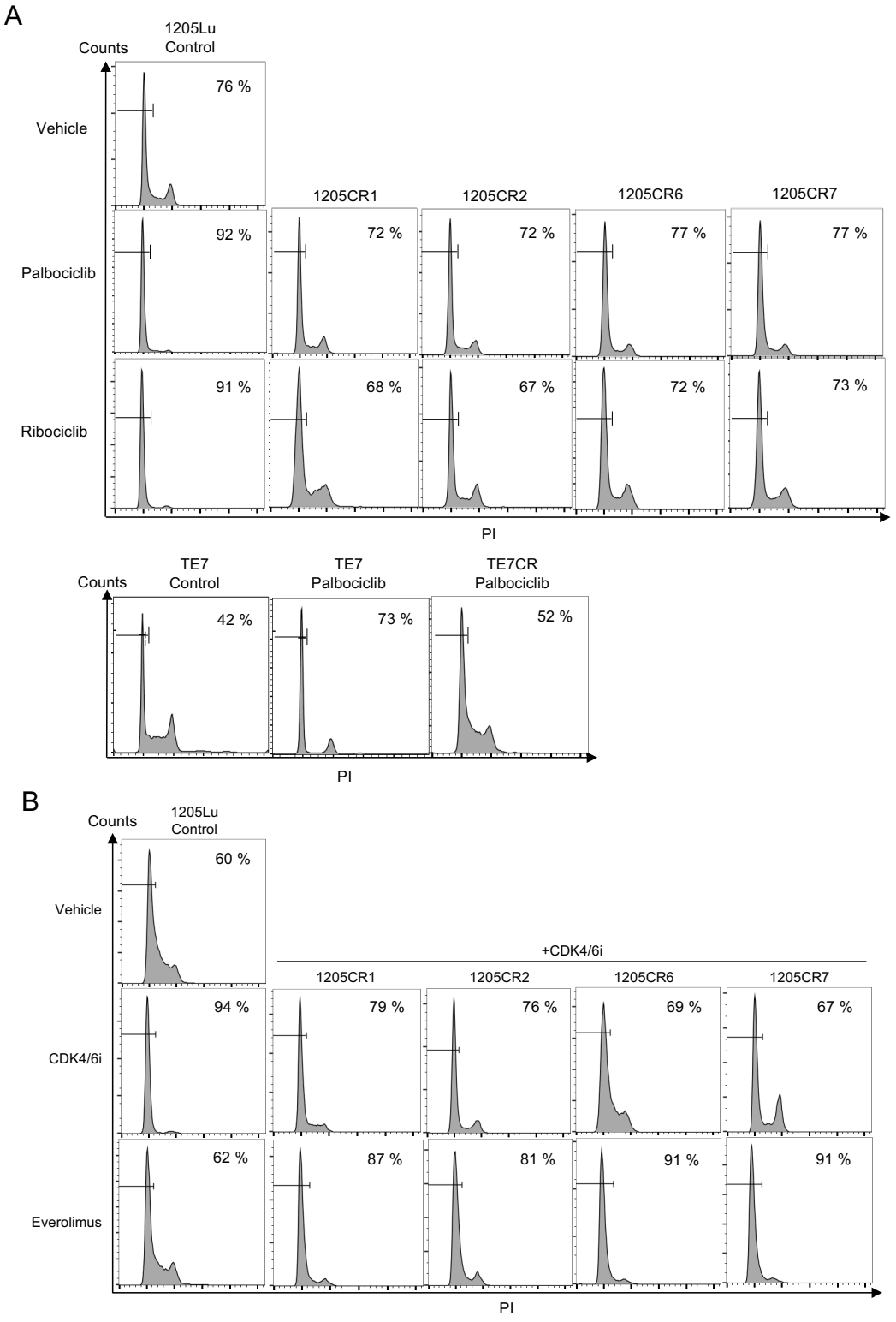
This PDF file includes:

Supplemental figures and figure legends

Supplemental Figure 1



Supplemental Figure 1 Continued

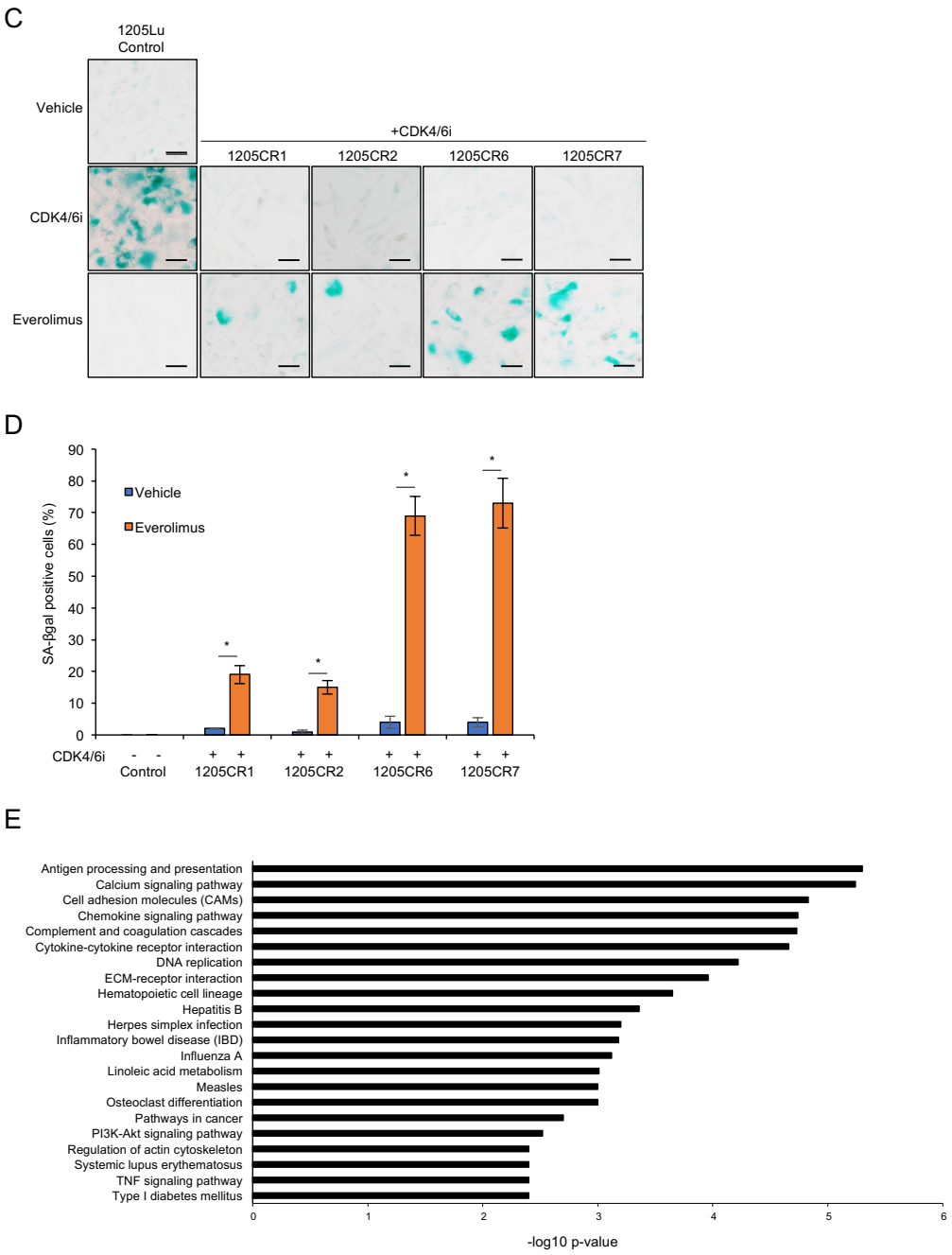


Fig. S1. Palbociclib-resistant melanoma cells retain broad resistance to CDK4/6i or CKD4/6i's. (**A**) FACS analysis of 1205Lu, 1205CR1, 2, 6 and 7 cells treated with or without ribociclib (1 μM) for 1 day (top panel). FACS analysis of TE7 and TE7CR cells

treated with or without palbociclib (1 μM) for 1 day (bottom panel) (**B**-**C**) 1205Lu cells were treated with or without palbociclib (1 μM) or everolimus (50 nM), 1205CR1, 2, 6 and 7 cells proliferating with palbociclib (1 μM) with or without everolimus (50 nM) for 1 day for FACS analysis (**A**) or 8 days for SA-βgal assay (**C**). (**D**) Quantification of SA-βgal positive cells from (**C**); data represent mean ± SD, * $P<0.01$ (two-tailed Student's t-test, n=3). (E) TOP biological pathways (Senescence vs Resistance) from RNA sequence.

Supplemental Figure 2

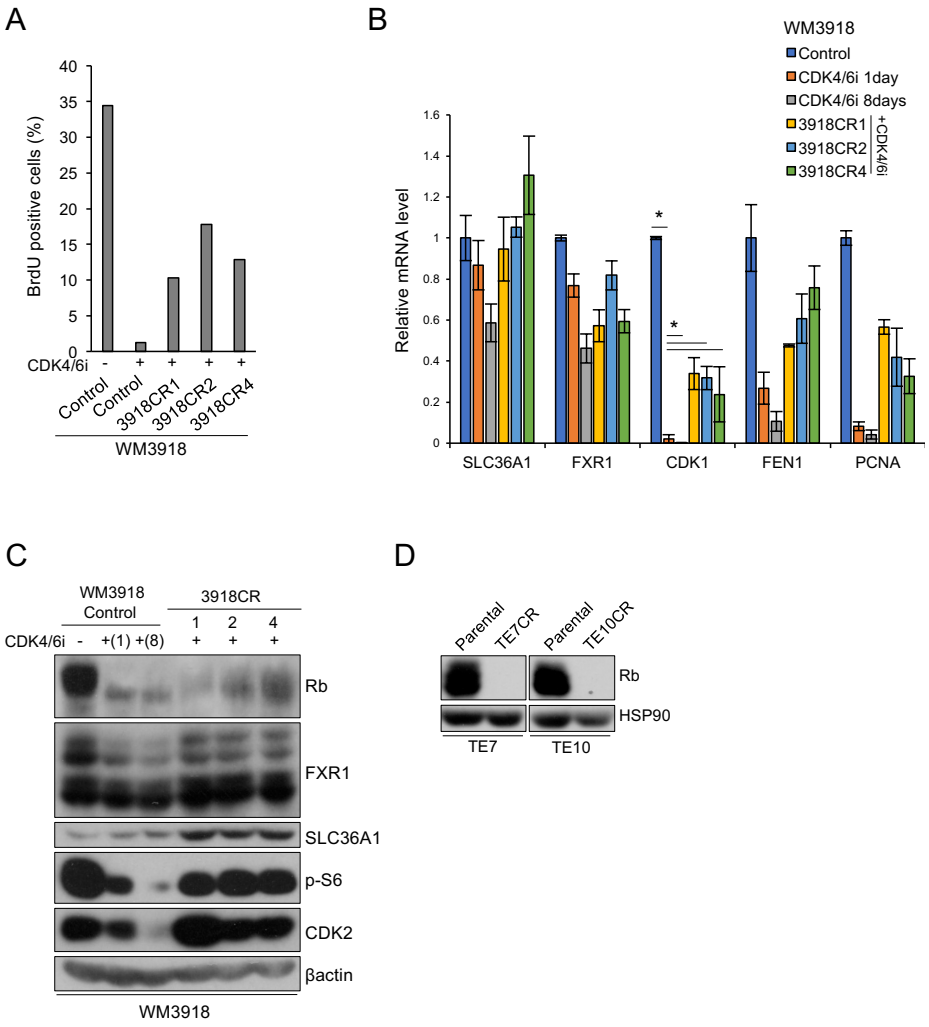


Fig. S2. Analyses of WM3918, TE7, and TE10 cells with CDK4/6i resistance. (**A**) WM3918 cells treated with or without palbociclib (1 μM) for 1 day and CDK4/6i-

resistant cells (3918CR1, 2 and 4) proliferating with palbociclib (1 μM) were subjected to BrdU incorporation for 45 minutes. BrdU positive cells were determined by FACS analysis. (**B**) QPCR analysis of samples from (**A**) using sets of primers for SLC36A1, FXR1, CDK1, FEN1 and PCNA. Data were normalized by GAPDH and represent mean ± SD, * $P<0.01$ (two-tailed Student's t-test, n=3). (**C**) Western blot analysis of lysates from (**A**) using antibodies to Rb, FXR1, SLC36A1, P-S6, CDK2 and βactin. (**D**) Western blot analysis of lysates from TE7, TE10, and CDK4/6i resistant cells (TE7CR and TE10 CR) using antibodies to Rb and HSP90.

Supplemental Figure 3

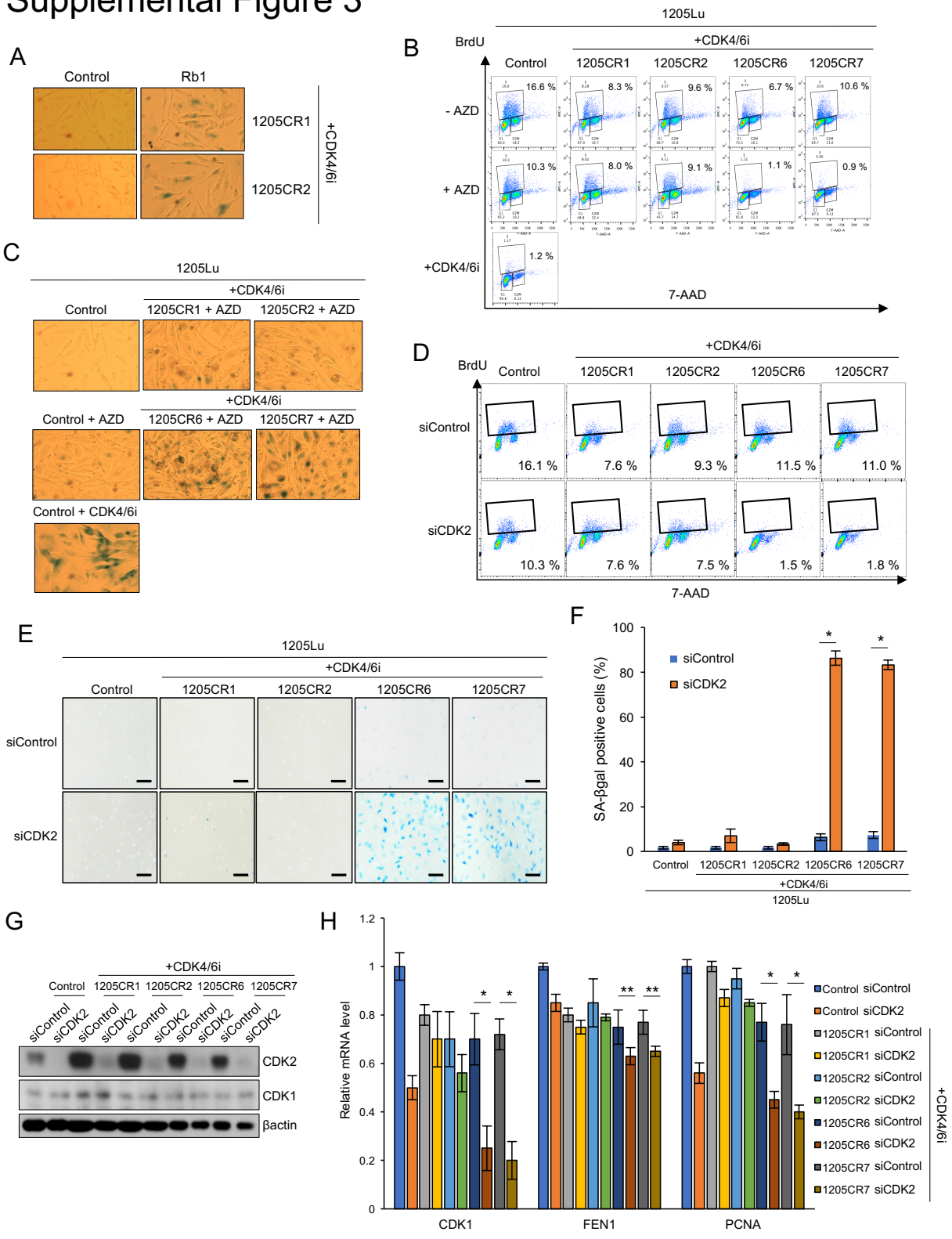


Fig. S3. Different acquired resistance mechanisms between 1205CR1-2 and 1205CR6-7 cells. (**A**) Representative images of SA-βgal staining in 1205CR1-2 cells infected with vehicle (Control) or Rb1. (**B**) 1205Lu, 1205CR1, 2, 6 and 7 cells were

subjected to a 45 minutes BrdU pulse following exposure to AZD5438 (AZD) treatment (0.5 μM). BrdU positive cells were determined by FACS analysis. (**C**) Representative images of SA-βgal staining in 1205Lu, 1205CR1, 2, 6 and 7 cells treated with AZD5438 (AZD) (0.5 μM) or palbociclib (1 μM) for 8 days. (**D**) 1205Lu, 1205CR1, 2, 6 and 7 cells transfected with siControl or siCDK2 were subjected to a 45 minutes BrdU pulse. BrdU positive cells were determined by FACS analysis. (**E**) Representative images of SA-βgal staining in 1205Lu, 1205CR1, 2, 6 and 7 cells after 8 days post transfection with siControl or siCDK2. Scale bars represent 100 μm. (**F**) Quantification of SA-βgal positive cells from (**E**); data represent mean ± SD, * $P<0.01$ (two-tailed Student's t-test, n=3). (**G**) Western analysis of lysates from 1205Lu, 1205CR1, 2, 6 and 7 cells transfected with siControl or siCDK2 using antibodies against CDK2, CDK1 and βactin. (**H**) QPCR analysis of samples from (**G**) using sets of primers for CDK1, FEN1 and PCNA. Data were normalized by GAPDH and represent mean ± SD, *$P<0.01$ (two-tailed Student's t-test, n=3), **$P<0.05$ (two-tailed Student's t-test, n=3).

Supplemental Figure 4

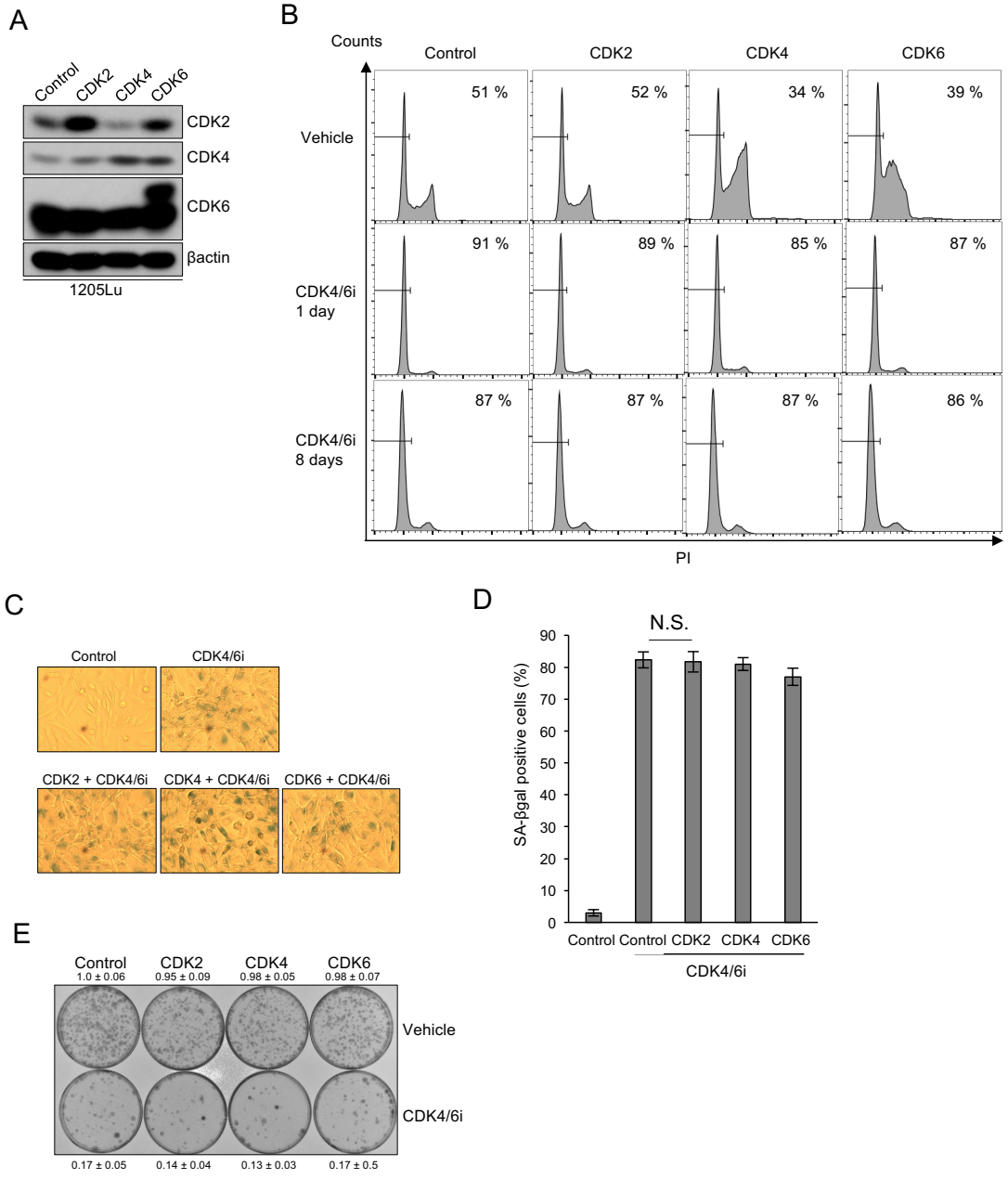


Fig. S4. Overexpression of CDKs does not override CDK4/6i-induced senescence in melanoma. (**A**) Western analysis of lysates from 1205Lu cells infected with either CDK2, CDK4 or CDK6 using antibodies to CDK2, CDK4, CDK6 and βactin. (**B**) FACS

analysis of cells from (**A**) treated with palbociclib (1 μM) for 1 day (middle panel) or 8 days (bottom panel) without palbociclib (top panel). (**C**) Representative images of SA-βgal staining in cells from (**A**) treated with or without palbociclib (1 μM). (**D**) Quantification of SA-βgal positive cells from (**C**); data represent mean ± SD, N.S., Not Significant (two-tailed Student's t-test, n=3). (**E**) Clonogenic colony formation assay of cells from (a) treated with or without palbociclib (1 μM) for 8 days. The numbers indicate quantification of colonies from three independent experiments.

Supplemental Figure 5

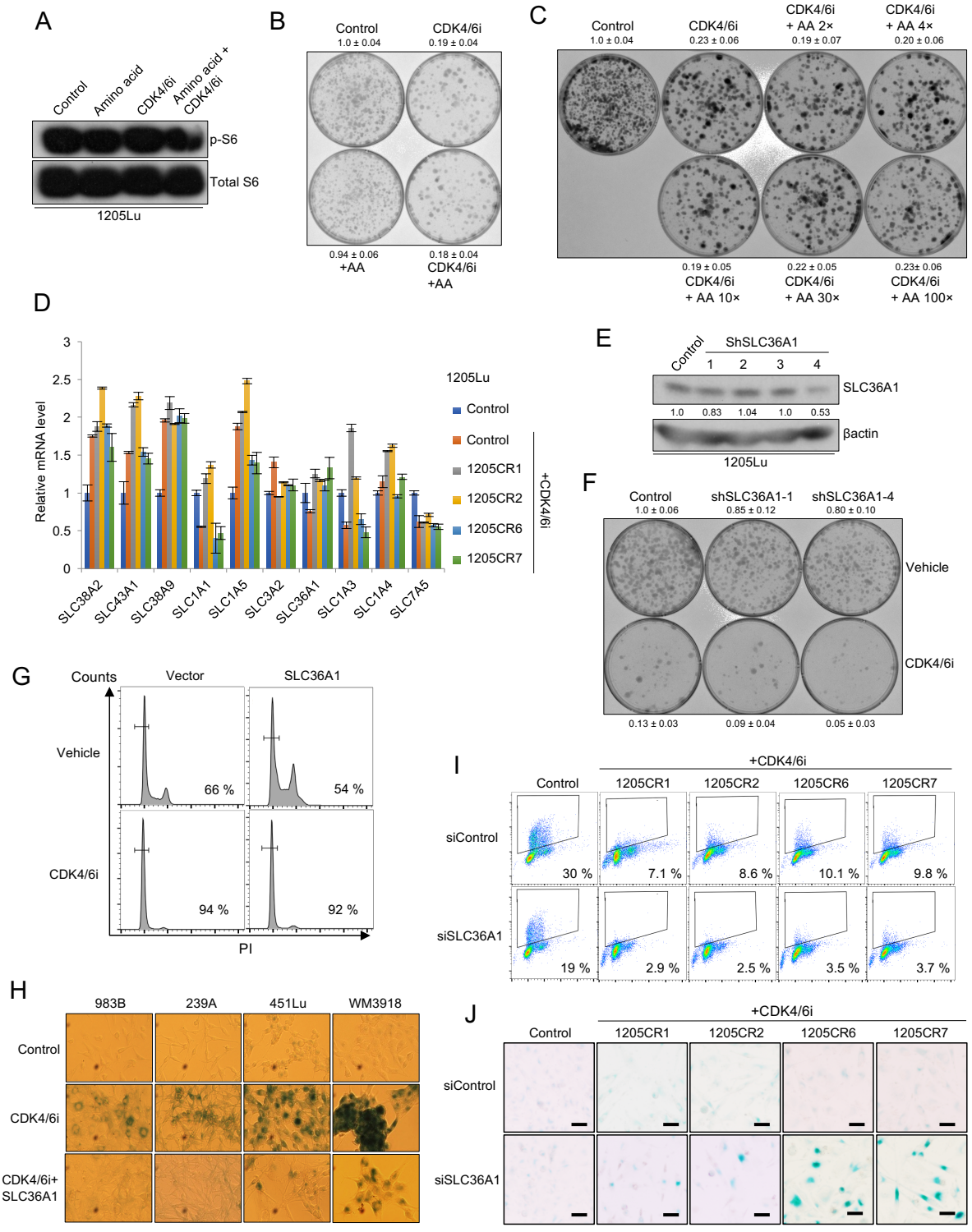
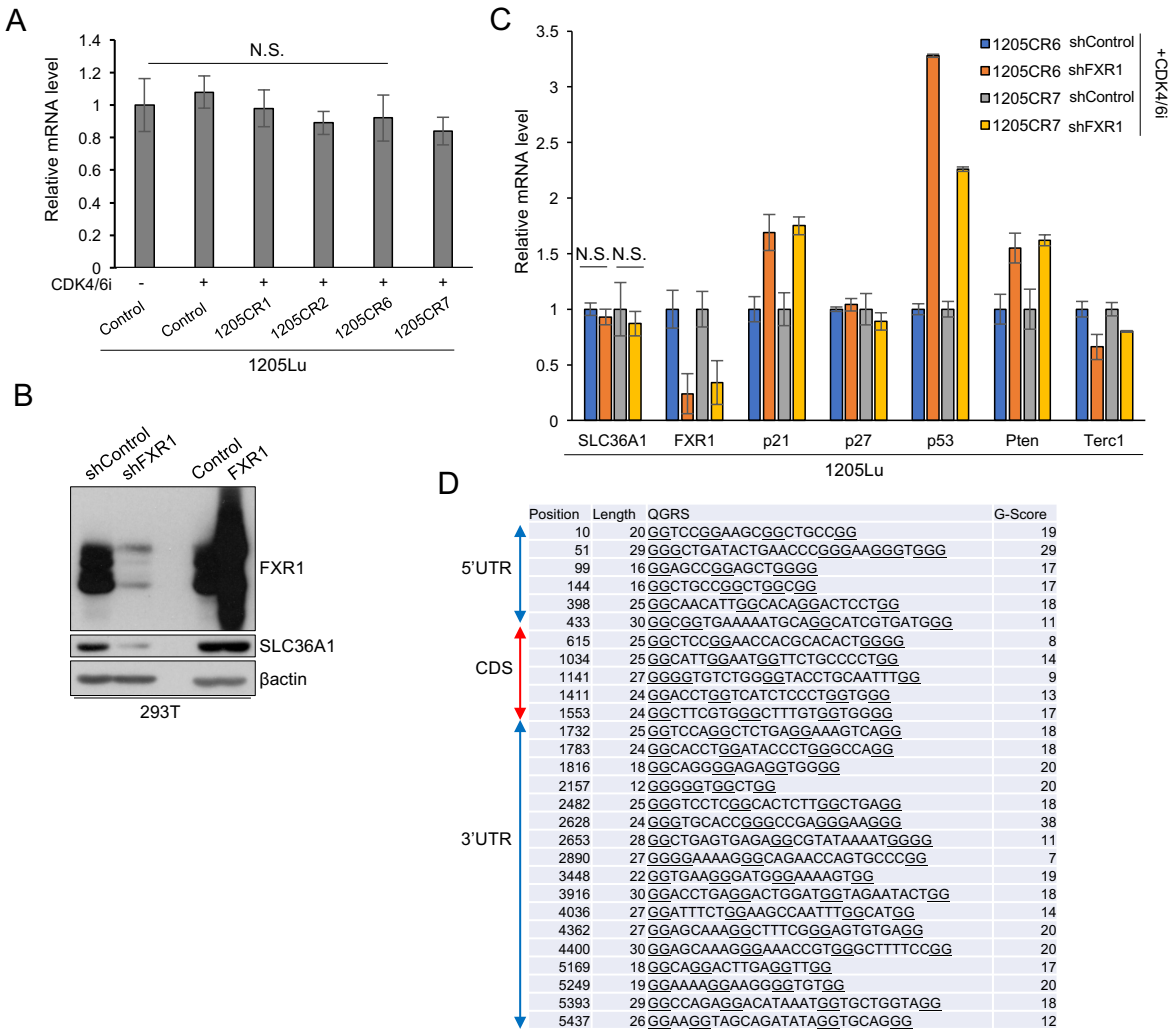


Fig. S5. The impacts of adding amino acids and SLC36A1 in the context of CDK4/6i-induced senescence. (**A**) Western analysis of lysates from 1205Lu cells cultured in medium supplemented with (proline, alanine and glycine) for 1 day using

antibodies to phospho-S6 and total S6. (**B**) Clonogenic colony formation assay of 1205Lu cells treated with palboiclib (1 μM), or cultured in medium supplemented with (proline, alanine and glycine) or both for 8 days or 1205Lu cells without treatment (Control). The numbers indicate quantification of colonies from three independent experiments. (**C**) Clonogenic colony formation assay of 1205Lu cells treated with palbociclib (1 μM), cultured with varying concentrations of amino acids as indicated for 8 days and 1205Lu cells without treatment (Control). The numbers indicate quantification of colonies from three independent experiments. (**D**) QPCR analysis of samples from 1205Lu cells treated with vehicle (Control), palbociclib (1 μM) for 8 days or in CDK4/6i-resistant clones grown in presence of palbociclib (1 μM) (1205CR1, 2, 6 and 7) using sets of primers for indicated on the bottom of figure. Data were normalized by GAPDH (n=3). (E) Western analysis of lysates from 1205Lu cells infected with different hairpin-mediated knockdown of SLC36A1 (ShSLC36A1-1, ShSLC36A1-2, ShSLC36A1-3 and ShSLC36A1-4) using antibodies to SLC36A1 and βactin. The numbers indicate quantification of SLC36A1 determined by SLC36A1/βactin ratio. (F) Clonogenic colony formation assay of cells infected with ShControl (Control), ShSLC36A1-1 or ShSLC36A1-4 after treatment with or without palbociclib (1 μM) for 8 days. The numbers indicate quantification of colonies from three independent experiments. (G) FACS analysis of cells expressing empty vector or SLC36A1 treated with or without palbociclib (1 μM) for 1 day. (H) Representative images of SA-βgal staining in 983B, 239A, 451Lu and WM3918 cells introduced with vector control or SLC36A1 after treatment of DMSO or palbociclib (1 μM) for 8 days. (I) 1205Lu (Control), 1205CR1, 2, 6 and 7 cells were transfected with SiControl or SiSLC36A1 and then subjected to a 45

minutes BrdU pulse. BrdU positive cells were determined by FACS analysis. (J) Representative images of SA-βgal staining in 1205Lu (Control), 1205CR1, 2, 6 and 7 cells after 8 days post transfection with siControl or siSLC36A1. Scale bars represent 100 μm.

Supplemental Figure 6



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144	16	GGCTGCCGGCTGGCGG	17
398	25	GGCAACATTGGCACAGGACTCCTGG	18
433	30	GGCGGTGAAAAATGCAGGCATCGTGATGGG	11
615	25	GGCTCCGGAACCACGCACACTGGGG	8
1034	25	GGCATTGGAATGGTTCTGCCCCTGG	14
1141	27	GGGGTGTCTGGGGTACCTGCAATTTGG	9
1411	24	GGACCTGGTCATCTCCCTGGTGGG	13
1553	24	GGCTTCGTGGGCTTTGTGGTGGGG	17
1732	25	GGTCCAGGCTCTGAGGAAAGTCAGG	18
1783	24	GGCACCTGGATACCCTGGGCCAGG	18
1816	18	GGCAGGGGAGAGGTGGGG	20
2157	12	GGGGGTGGCTGG	20
2482	25	GGGTCCTCGGCACTCTTGGCTGAGG	18
2628	24	GGGTGCACCGGGCCGAGGGAAGGG	38
2653	28	GGCTGAGTGAGAGGCGTATAAAATGGGG	11
2890	27	GGGGAAAAGGGCAGAACCAGTGCCCGG	7
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4036	27	GGATTTCTGGAAGCCAATTTGGCATGG	14
4362	27	GGAGCAAAGGCTTTCGGGAGTGTGAGG	20
4400	30	GGAGCAAAGGGAAACCGTGGGCTTTTCCGG	20
5169	18	GGCAGGACTTGAGGTTGG	17
5249	19	GGAAAAGGAAGGGGTGTGG	20
5393	29	GGCCAGAGGACATAAATGGTGCTGGTAGG	18
5437	26	GGAAGGTAGCAGATATAGGTGCAGGG	12

Fig. S6. FXR1 regulates SLC36A1 protein expression. (**A**) QPCR analysis of samples from 1205Lu cells treated with or without palbociclib (1 μM) for 8 days, and 1205CR1, 2, 6 and 7 cells for FXR1. Data were normalized by GAPDH and represent mean ± SD.

N.S., Not Significant (two-tailed Student's t-test, n=3). (**B**) Western analysis of lysates from 293T cells introduced with ShFXR1 or FXR1 using antibodies to FXR1, SLC36A1 and βactin. (**C**) QPCR analysis of samples from 1205CR6 and 1205CR7 cells with or without ShFXR1 for genes indicated on the bottom of the panels. Data were normalized by RPS18S and represent mean ± SD. N.S., Not Significant (two-tailed Student's t-test, n=3). (**D**) G-Score of SLC36A1 calculated by QGRS Mapper (http://bioinformatics.ramapo.edu/QGRS/index.php).

Supplemental Figure 7

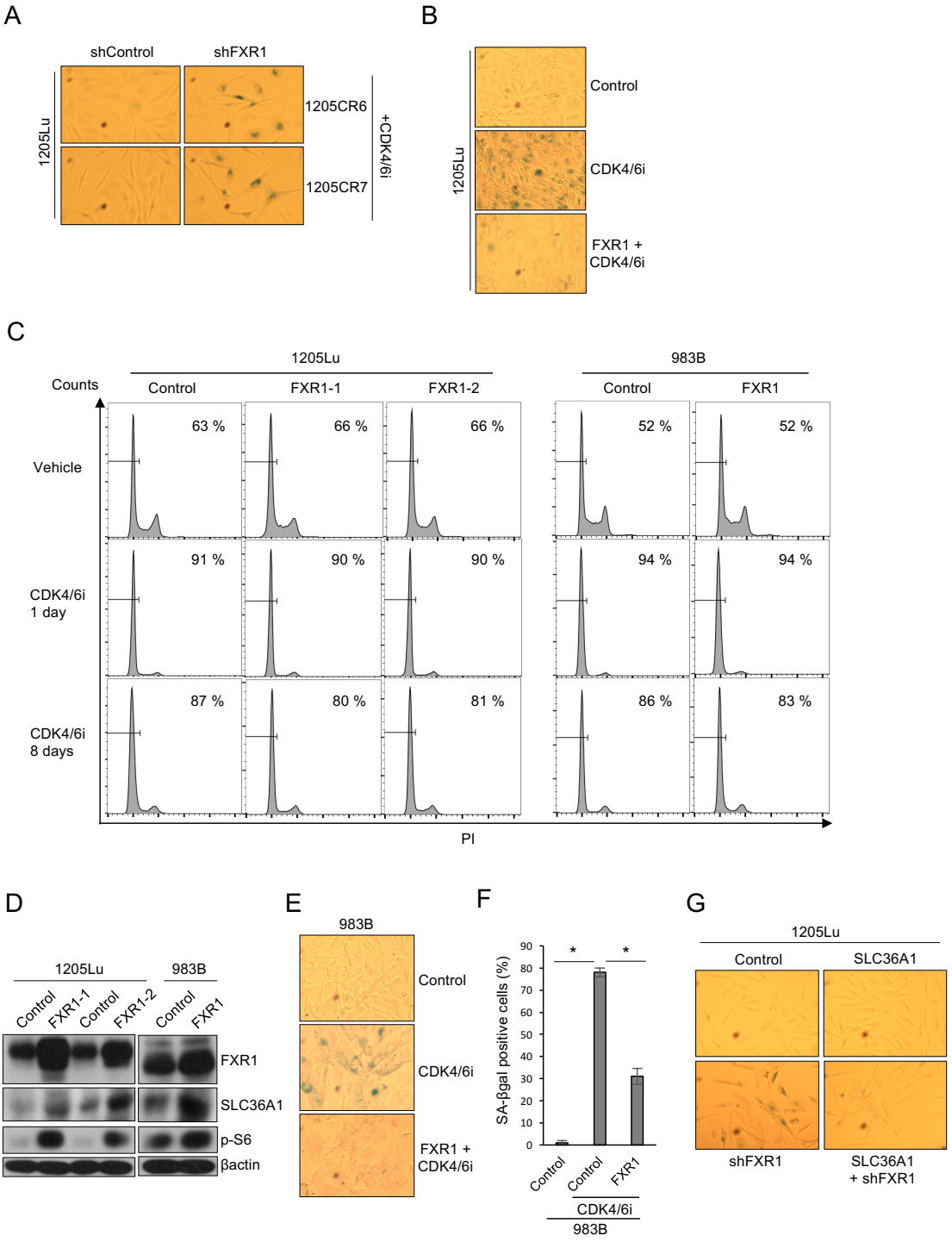

Fig. S7. FXR1 overrides CDK4/6i-induced senescence. (**A**) Representative images of SA-βgal staining in 1205CR6 and 1205CR7 cells introduced with vehicle (Control) or ShFXR1. (**B**) Representative images of SA-βgal staining in 1205Lu cells introduced with

vehicle (Control) or FXR1 with or without treatment of palbociclib (1 μM) for 8 days. (**C**) FACS analysis of 1205Lu and 983B cells overexpressing FXR1 (FXR1-1 or FXR1-2) treated with vehicle, palbociclib (1 μM) for 1 day (middle panel) or 8 days (bottom panel). (**D**) Western blot analysis of lysates from 1205Lu and 983B cells with vehicle (Control) or FXR1 using antibodies to FXR1 and βactin. (**E**) Representative images of SA-βgal staining in 983B cells introduced with vehicle (Control) or FXR1 with or without treatment of palbociclib (1 μM) for 8 days. (**F**) Quantification of SA-βgal positive cells from (**E**). Data represent mean ± SD, * $P<0.01$ (two-tailed Student's t-test, n=3). (**G**) Representative images of SA-βgal staining in 1205Lu cells introduced with vehicle (Control), ShFXR1, SLC36A1 or ShFXR1 + SLC36A1.

Supplemental Figure 8

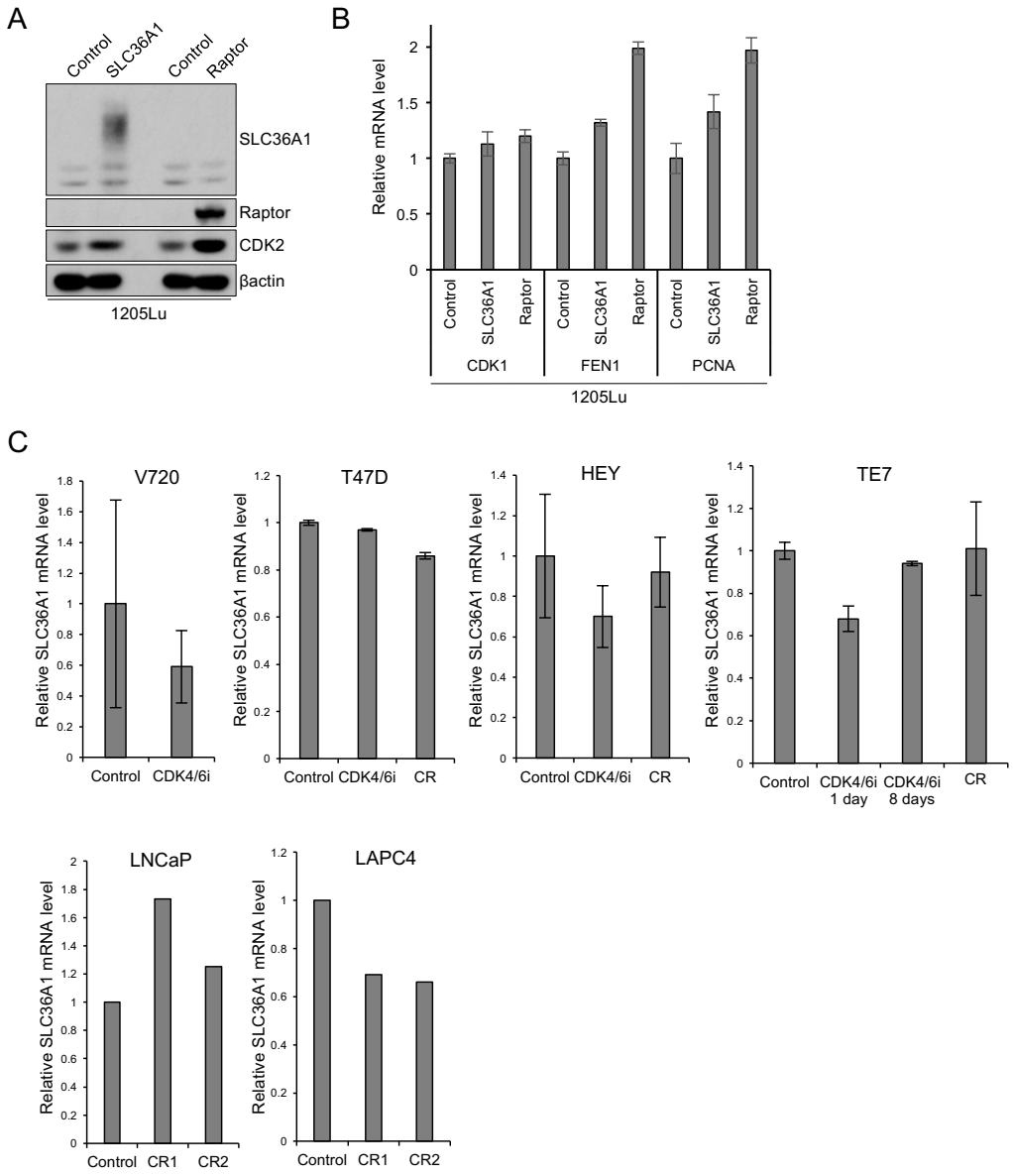

Fig. S8. RNA-seq analysis of additional cancers with CDK4/6i resistance. (**A**) Western blot analysis of lysates from 1205 cells overexpressed with either SLC36A1 or Raptor using antibodies to SLC36A1, Raptor, CDK2 and βactin. (**B**) QPCR analysis of

samples from (**A**) for CDK1, FEN1 and PCNA. Data were normalized by GAPDH and represent mean ± SD. (**C**) Analysis of RNA seq data in GEO database repository (V720; GSE40513, T47D; GSE117743, HEY; GSE63529, TE7; GSM3101838, LNCaP and LAPC4; GSE99675) for SLC36A1.