Figure S1

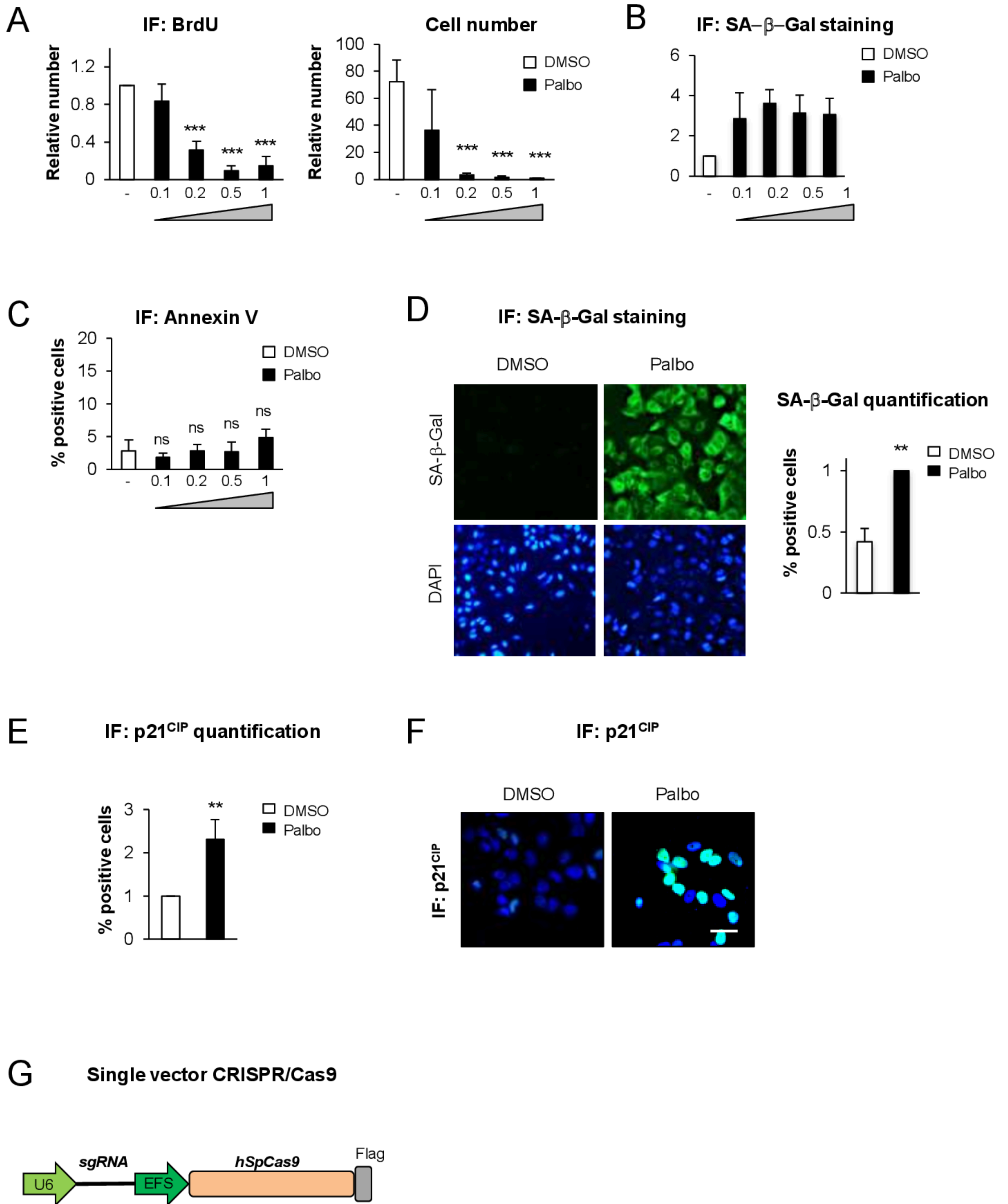


Figure S1. Palbociclib induces a senescent-like phenotype in MCF7 breast cancer cell line. (A) Quantification of BrdU incorporation and relative cell number in MFC7 treated with different concentrations (0.1, 0.2, 0.5 and 1 μM) of Palbociclib (Palbo) for 14 days. **(B)** SA-β-galactosidase (SA-β-Gal) staining in MCF7 cells treated with different concentrations of Palbo for 7 days. **(C)** The graph represents the percentage of Annexin V positive cells after 14 days Palbo treatment with different concentrations. **(D)** Representative images (left panel) and quantification (right panel) for SA-β-Gal staining in MCF7 cells treated with 200nM Palbo for 14 days. Graph shows the mean ± SEM of 4 independent experiments. Two-tailed Student´s t-test was used to calculate statistical significance. **(E)** Quantification and **(F)** representative pictures of the percentage of $p21^{CIP}$ positive cells upon 14 days 200nM Palbo treatment. Two-tailed Student´s t-test was used to calculate statistical significance. Scale bar: 50 μm. **(G)** Diagram for the lentiCRISPR*v2* one vector system used in the GeCKO library and to clone individual sgRNAs. The plasmid contains an expression cassette for human Cas9 (*hSpCas9*) and the sgRNA in the same vector. Related to **Figure 1**.

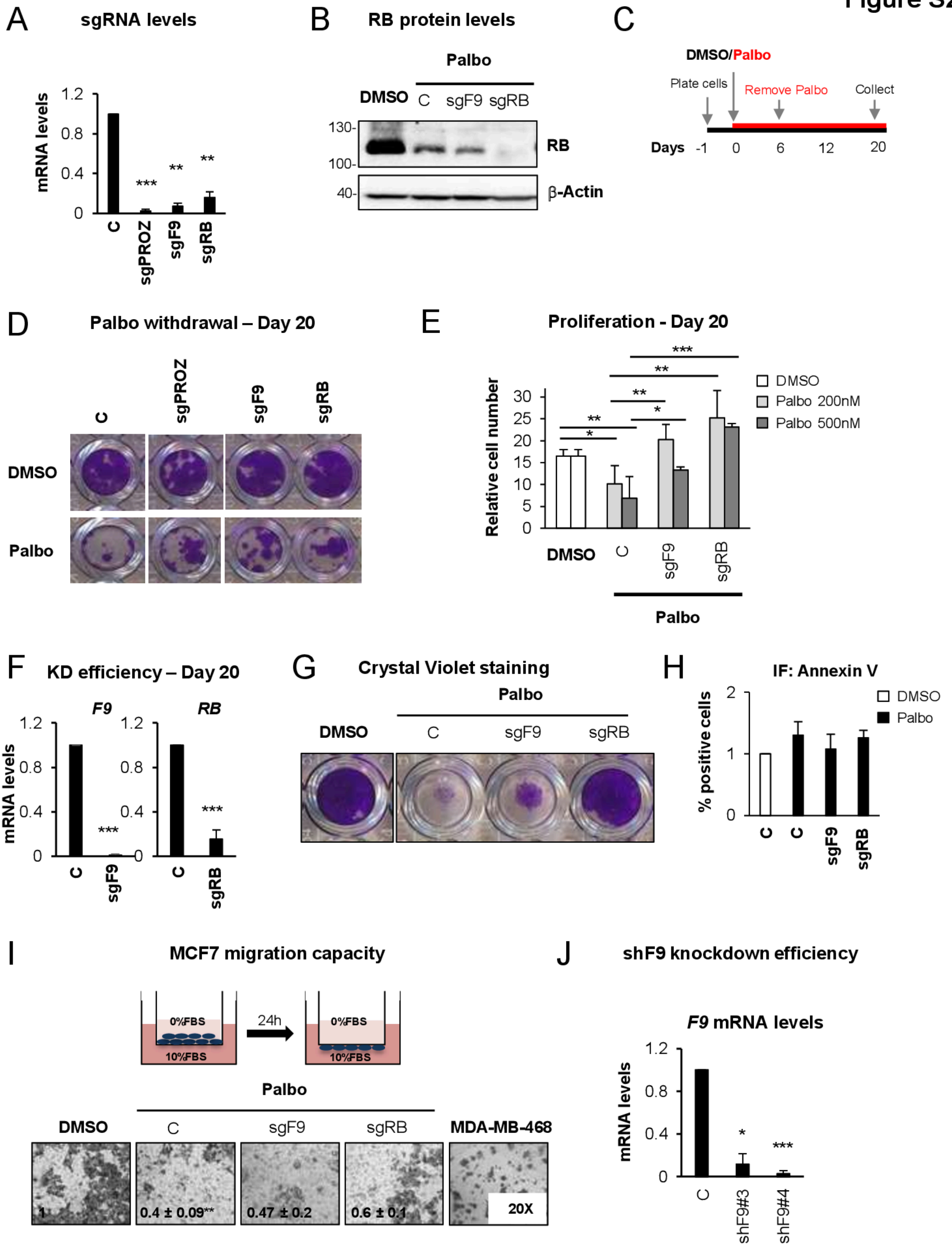


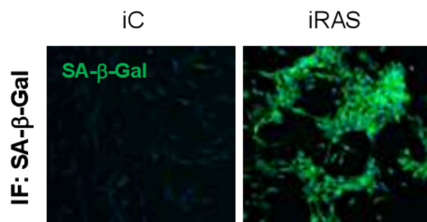
Figure S2
A
sgRNA levels
mRNA levels
1.2
0.8
0.4
0
***
**
**
C
sgPROZ
sgF9
sgRB
B
RB protein levels
Palbo
DMSO
C
sgF9
sgRB
130-
100-
40-
RB
β-Actin
C
DMSO/Palbo
Plate cells
Remove Palbo
Collect
Days
-1
0
6
12
20
D
Palbo withdrawal – Day 20
C
sgPROZ
sgF9
sgRB
DMSO
Palbo
E
Proliferation - Day 20
Relative cell number
DMSO
Palbo 200nM
Palbo 500nM
DMSO
C
sgF9
sgRB
Palbo
F
KD efficiency – Day 20
F9
RB
mRNA levels
C
sgF9
C
sgRB
G
Crystal Violet staining
Palbo
DMSO
C
sgF9
sgRB
H
IF: Annexin V
% positive cells
DMSO
Palbo
C
C
sgF9
sgRB
I
MCF7 migration capacity
0%FBS
10%FBS
24h
Palbo
DMSO
C
sgF9
sgRB
MDA-MB-468
1
0.4 ± 0.09**
0.47 ± 0.2
0.6 ± 0.1
20X
J
shF9 knockdown efficiency
F9 mRNA levels
mRNA levels
C
shF9#3
shF9#4

Figure S2. Validation of F9 loss-of-function efficiency and proliferative advantage in MCF7 cells. (A) Relative mRNA expression levels of *PROZ*, *F9* and *RB* in MCF7 after their respective sgRNA infection and selection. Graph shows the mean ± SEM of 2 independent experiments. Two-tailed Student´s t-test was used to calculate statistical significance compared to the Control sample. **(B)** Representative western blot showing RB knockout upon sgRB expression in MCF7 cells. β-actin was used as a loading control. Representative blot for 4 independent experiments. **(C)** Timeline and strategy followed to confirm that the senescence proliferative arrest is maintained after Palbo is removed and washed out. MCF7 cells were treated with DMSO or 500nM Palbo for 6 days, after which the drug was removed and the cells were grown and collected at day 20. **(D)** Crystal violet staining showing the proliferation rate of MCF7 cells expressing sgRNAs (sgPROZ, sgF9 and sgRB) at day 20. Palbo was removed after day 6 to confirm the induction of a stable cell cycle arrest. A representative experiment of 3 independent experiments is shown. **(E)** Relative cell number quantification in MCF7 control or expressing sgRNAs (sgF9, sgRB) using 200nM (light grey) or 500nM (dark grey) Palbo treatment for 20 days. Data represent the mean ± SEM of 3 independent experiments. **(F)** mRNA levels for *F9* and *RB* by qPCR in MCF7 cells 20 days after setting the experiment. Data represent the mean ± SD of 3 independent experiments. Two-tailed Student´s t-test was used to calculate statistical significance. **(G)** Crystal violet staining for MCF7 expressing sgF9 and treated with 500nM Palbo. A representative experiment is shown. sgRB is used as positive control. **(H)** Quantification for AnnexinV staining in MCF7 cells expressing sgF9 and sgRB upon Palbo treatment. Data represent the mean ± SEM of 3 independent experiments. **(I)** 24h MCF7 migration assays upon 500nM Palbo treatment for 20 days expressing sgF9 and sgRB. MDA-MB-468 cells were used as a positive control. Representative pictures of 3 independent experiments is shown. Data show the mean ± SEM of 3 independent experiments. One-Way ANOVA with Dunnett's multiple comparisons to Palbo was performed. **(J)** qPCR to confirm the efficacy of two independent shRNA targeting F9

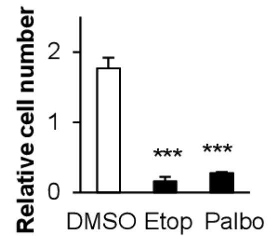
(shF9#3 and shF9#4). Data represent the mean ± SEM. Two tailed students t-test analysis was performed. Related to **Figure 2**.

Figure S3

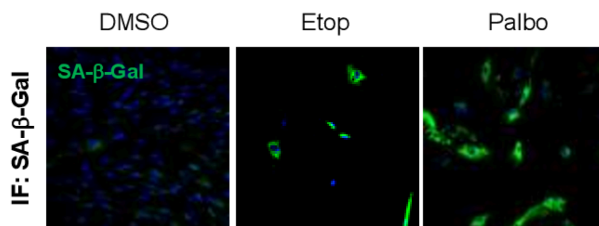
A SA- β- Gal activity - HFFF2



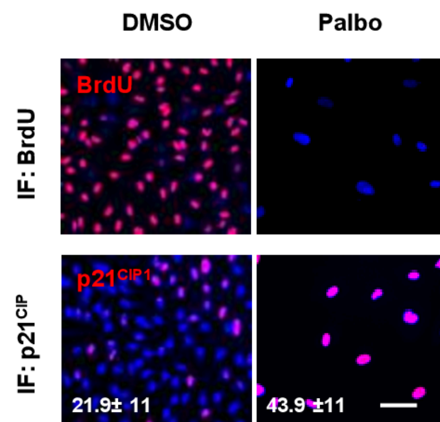
B Proliferation - HFFF2



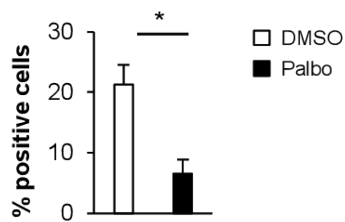
C SA- β- Gal activity - HFFF2



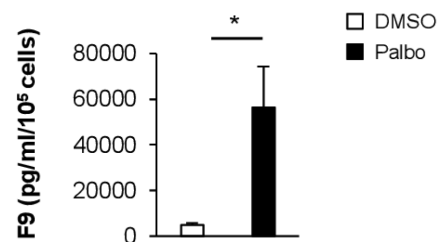
D Activation of senescence - HUVEC



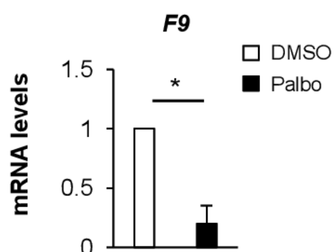
E IF: BrdU - HUVEC



F ELISA F9 - HUVEC



G SKMEL28



H F9 ectopic expression does not induces senescence in SKMEL28

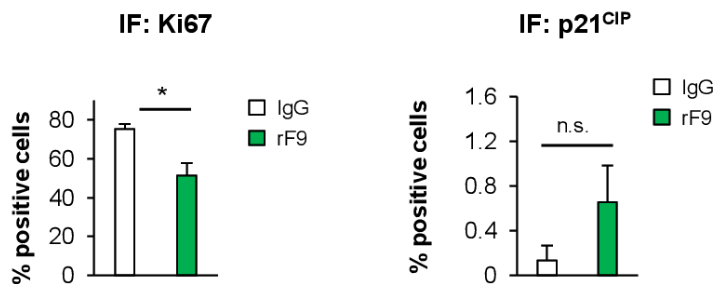
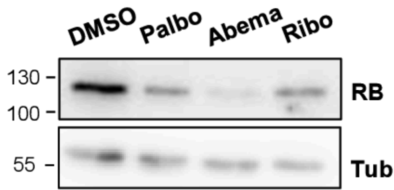


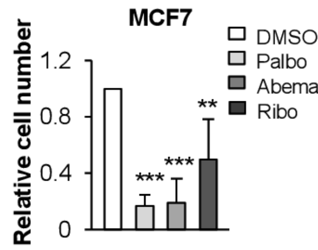
Figure S3. Evaluation of senescence in different human primary cultures and additional cancer cell lines. (A) Representative images of SA-β-galactosidase activity (SA-β-Gal). Human primary fibroblasts (HFFF2) expressing an empty vector ER:EV (iC) or ER:H-RAS^{G12V} (iRAS) were treated with 200nM 4OHT for 6 days to induce senescence. SA-β-Gal is shown by the incorporation of the fluorescent compound C_{12}FDG (green). **(B)** HFFF2 relative cell number after 2 days treatment with 50 μM of Etoposide followed by 5 days with fresh media or 7 days with 1μM Palbociclib. Data show the mean ± SEM of 3 independent experiments. Two-tailed students t-test was performed compared to Control sample. **(C)** HFFF2 treated with 50μM of Etoposide for 2 days followed by 5 days with fresh media and 1μM Palbo for 7 days were incubated for 8h with C_{12}FDG compound. SA-β-gal activity (green) was determined by fluorescent signal and representative images are shown. **(D)** Representative IF images for p21^{CIP1} and BrdU in HUVEC (human umbilical vein endothelial cells) control or treated with 500nM Palbo for 7 days. Representative images are shown from 3 independent experiments for BrdU and 4 for p21^{CIP}. Scale bar: 50 μm. **(E)** The graph represents the quantification for the percentage of HUVEC cells staining positive for BrdU. The data represent the mean ± SEM of 3 independent experiments. Two-tailed Student´s t-test was used as test. **(F)** F9 quantitative ELISA measured in the conditioned media of HUVEC treated or not with Palbo for 7 days. Data represent the mean ± SEM of 4 independent experiments. Two-tailed Student´s t-test was used. **(G)** *F9* mRNA levels analysed by qPCR in in SKMEL28 treated with 500nM Palbo for 20 days. Please note data presented here are the same as SKMEL28 in **Figure 5D**. Student t-test statistical analysis performed. **(H)** SKMEL28 melanoma cells were treated with 10μg/mL of recombinant F9 (rF9) twice for 6 days. Ki67 and p21^{CIP} levels were determined by IF. Data show the mean ± SEM of 3 independent experiments. Two-tailed Student´s t-test was used as statistical analysis. Related to **Figure 3**.

Figure S4

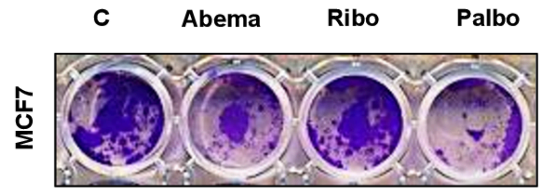
A RB protein levels - MCF7



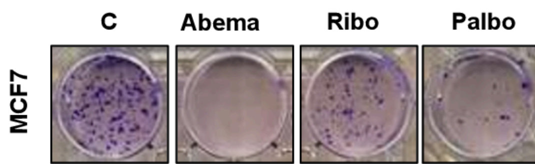
B Relative cell number



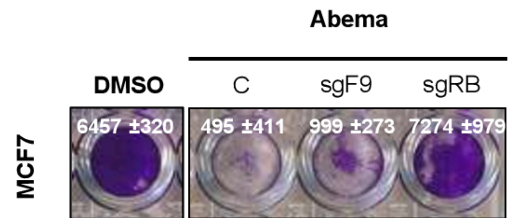
C CDK4/6 inhibitors treatment



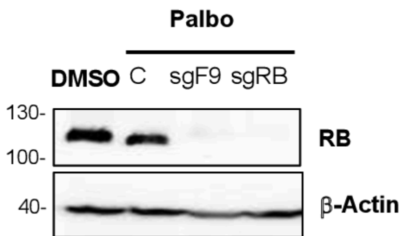
D Drug withdrawal



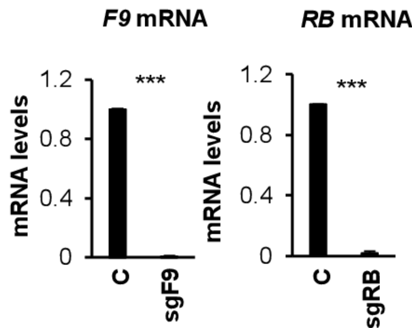
E *sgF9* prevents Abema-induced arrest



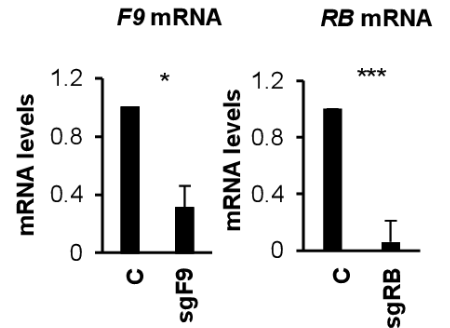
F RB protein levels - T47D



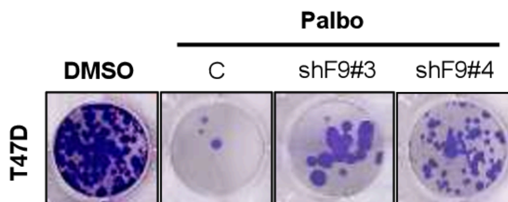
G Knockout efficiency in T47D



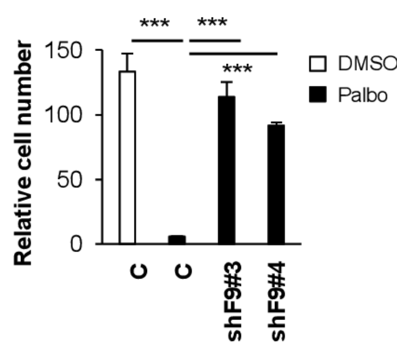
H Knockout efficiency in MDA-MB-468



I shF9 induces proliferation in T47D



J Proliferation – T47D



K Knockdown efficiency in T47D

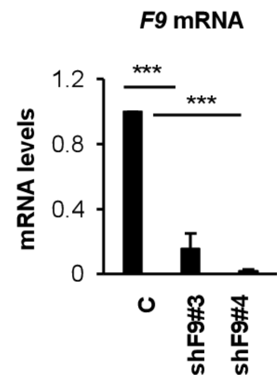


Figure S4. CDK4/6 inhibitors response in a variety of cancer cell lines. (A) Immunoblot for RB in MCF7 cells treated with Palbo, Abema and Ribo show a decrease in the levels of RB confirming a proliferative arrest. **(B)** Relative cell number quantified after MCF7 cells were treated with 1μM of different CDK4/6 inhibitors (Palbociclib, Abemaciclib, Ribociclib). Data show the mean ± SD of 5 independent experiments. Two-tailed student's t-test analysis was performed. **(C)** Clonogenicity assay shows the proliferation rate of MCF7 cells after 14 days treatment with 1μM Abema, 1μM Palbo and 1μM Ribo. Representative experiment of 2 biological replicates. **(D)** Crystal violet staining showing the effect on proliferation after 6 days treatment with Palbo, Abema and Ribo. After drug withdrawal, cells were washed and the experiment was stopped 20 days after the initial treatment. Representative experiment is shown. **(E)** Crystal violet staining for colony formation assay in MCF7 cells expressing sgF9 or sgRB treated with 1μM Abema for 20 days. Representative experiment of 3 independent experiments is shown. Quantifications shows mean ± SD for relative cell number. **(F)** Representative western blot showing RB knockout in T47D cells. β-actin was used as a loading control. Blot representative of 3 independent experiments. **(G)** *F9* and *RB* mRNA levels were determined by qPCR in T47D cells expressing sgF9 or sgRB. Data show the mean ± SEM of 3 independent experiments. Two-tailed student' t-test analysis was performed. **(H)** Knockout efficiency for sgF9 and sgRB in MDA-MB-468 cells. mRNA levels were determined by qPCR. Data show the mean ± SEM of 3 biological replicates. Two-tailed student's t-test analysis was performed. **(I)** Crystal violet staining showing the proliferative rate of T47D cells expressing two independent shRNA targeting F9 (shF9#3 and shF9#4) treated with 1μM Palbo for 20 days. Representative experiment of 3 biological replicates. **(J)** Proliferation rate of T47D expressing shF9#3 and shF9#4 treated with 1μM Palbo for 20 days. The data represent the mean ± SEM of 7 independent experiments. One Way ANOVA with Dunnett's multiple comparisons to Palbo C sample was performed. **(K)** qPCR analysis for the levels of *F9* mRNA in T47D cells expressing shF9#3 and shF9#4. Data show the mean ± SEM of 3

independent experiments. Two-tailed student' t-test analysis was performed. See also to **Figure 4**.

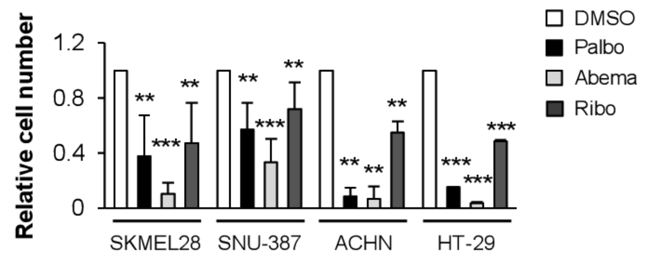
Figure S5

A Panel of 22 cell lines tested

Renal	ACHN
Melanoma	SKMEL28
Breast	MCF7
Brain	U87MG
Liver	SNU-387
Colon	HT-29
Melanoma	SKMEL2
Renal	A498
Pancreatic	Capan-2
Prostate	PC3
Lung	A549
Colon	HT-116
Melanoma	SKMEL5
Bladder	HT-1376
Breast	BT-549
Bladder	HT-1197
Ovarian	SKOV-3
Ovarian	OVCAR-3
Brain	U118MG
Lung	NHI-23
Pancreatic	PANC1
Prostate	DU-145

Responding to ≥ 2 CDK4/6 inhibitors

B Cell number in cells treated with CDK4/6i



C Continuous drug treatment

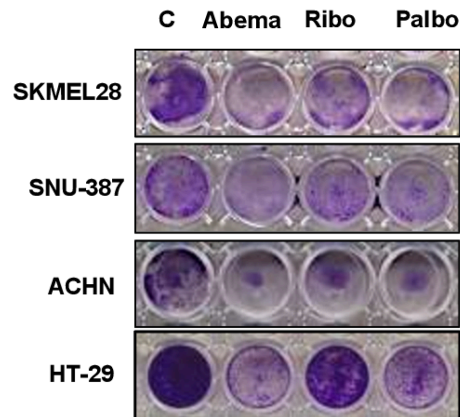
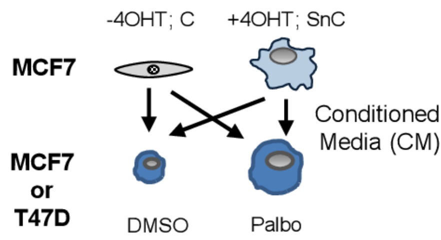


Figure S5. Response of other cancer cell lines to different CDK4/6 inhibitors

(A) Panel of cancer cell lines used in the Primary Screen to determine the efficacy of increasing concentrations of other CDK4/6 inhibitors on proliferation. The cell lines highlighted in orange are the ones that responded to two or more inhibitors. **(B)** Quantification of relative cell number from 8 different cancer cell lines selected for the Secondary Screen that responded to more than two CKD4/6 inhibitors. 1μM CDK4/6 inhibitor concentration was used. Data show the mean ± SD of 5 independent experiments for SKMEL28 and SNU-387 and 2 for HT-29 and ACHN. Student's t-test analysis was performed. **(C)** Crystal violet staining showing the effect on proliferation of Abema, Ribo and Palbo in the selected cancer cell lines after 14 days of continuous drug treatment. Related to **Figure 5**.

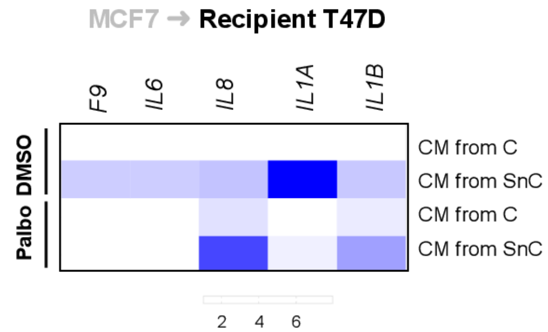
Figure S6

A Cross-talk between MCF7 and other cells

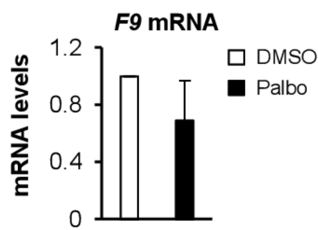


B Cross-talk MCF7 ➜ T47D or MCF7

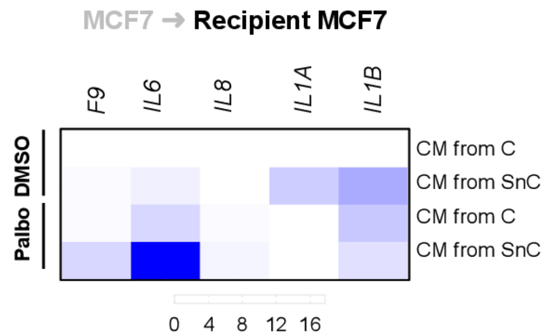
MCF7 ➜ Recipient T47D



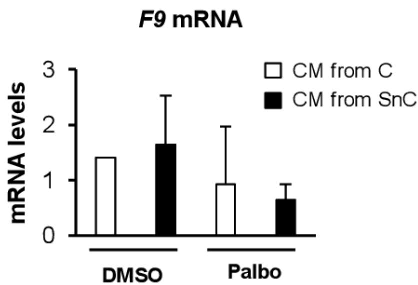
C MDA-MB-468



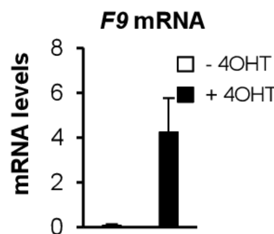
MCF7 ➜ Recipient MCF7



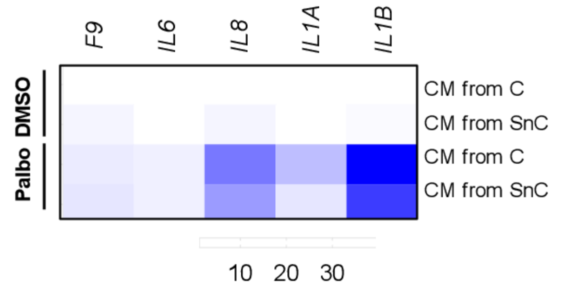
D Cross-talk iRAS ➜ MDA-MB-468



E F9 mRNA – HFFF2



F MCF7 ➜ Recipient SKMEL28



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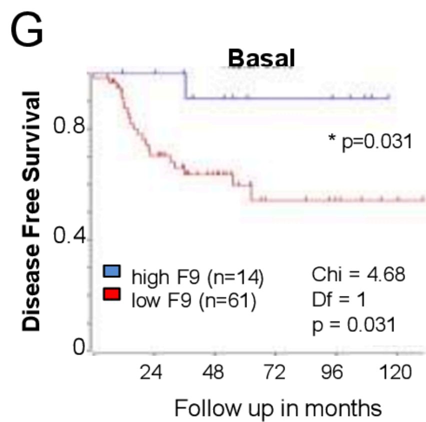

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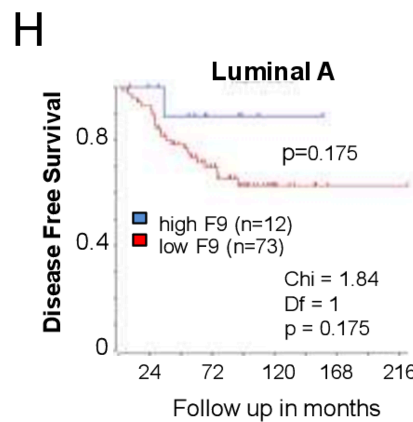

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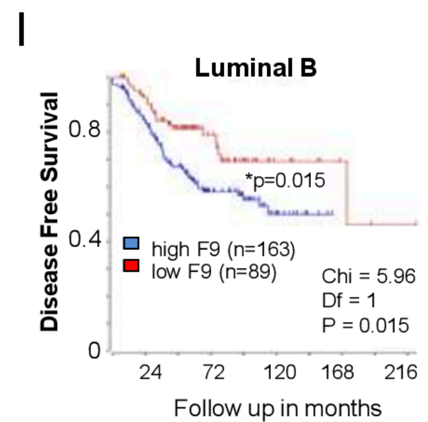

Figure S6. Influence of the intercellular cross talk between different cell cultures. (A) Schematic representation of the experimental design to identify the cross-talk between Palbo-induced MCF7 cells and other cancer cell lines. (**B**) Quantification of different SASP and *F9* mRNA levels in recipient cells (MCF7 and T47D) treated with the CM from MCF7 cells treated with Pablo or not. (**C**) mRNA levels of *F9* in MDA-MB-468 breast cancer cell line. Data represent the mean ± SEM of 3 independent experiments. (**D**) Cross-talk between iRAS human primary fibroblasts undergoing senescence or not and treatment of MDA-MB-468 with their CM. Data represent the mean ± SEM of 3 independent experiments. (**E**) *F9* mRNA levels for HFFF2 iRAS fibroblasts undergoing senescence upon +4OHT treatment. Data show the mean ± SEM of 3 independent experiments. (**F**) *F9* and other SASP mRNA levels in SKMEL28 human melanoma cancer cell lines exposed to the conditioned media of proliferative or senescence MCF7 cells. (**G, H, I**) Kaplan-Meier survival curves for different breast cancer subtypes: (**G**) Basal, (**H**) Luminal A and (**I**) Luminal B showing the expression levels of F9 high (blue) or low (red) and disease-free survival probability[30]. Dataset calculated with R2.