Supplementary Information to

"Cdk4/6-inhibitor as a novel therapeutic approach for advanced Bladder Cancer independently of RB1 status"

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Supplementary Figures 1-13

Supplementary Tables 1, 2, 3, 7 and 8

Supplementary References

Supplementary tables 4-6 are separately submitted as spreadsheets

Supplementary Methods and Materials

Immunoblot and Immunohistochemistry

For Western Blot analysis, cell pellets or tissues were disrupted by freeze-thawing cycles in lysis buffer (20 mM HEPES pH 7.5, 1% Triton-100, 40 mM β-Glicerophosphate, 100 mM NaCl, 20 mM $MgCl_2$, 10 mM EGTA) supplemented with protease and phosphatase inhibitor cocktails (Roche), and centrifuged to obtain supernatant containing total protein. Thirty micrograms of protein per sample were resolved in 4-12% NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes (Amersham). Primary and secondary antibodies (Supplementary Table 1) were diluted in blocking solution. Secondary antibodies were purchased from Jackson ImmunoResearch.

Plasmid and lentiviral production

Transfection experiments to upregulate FOXM1 expression were performed using FuGENE®6 Transfection Reagent (Promega). The plasmid coding for FOXM1, (in pLVX backbone) was generously provided by Dr. A. Gandarillas (IDIVAL, Santander, Spain). For the knockdown of FOXM1, the same cells lines were transfected with 2 independent lentivirus-based shRNAs (MISSION® shRNA, Sigma Aldrich) targeting human FOXM1 mRNA (TRCN000015544, denoted as sh1, and TRCN000015546, denoted as sh2). The selection of the infected cells was performed for at least 15 days in puromycin-containing medium (0.5-1 µg/ml; Sigma Aldrich) and pooled clones were used. For knockdown of CDK4 (pLVTHM-shCDK4), CDK2 (pLVTHM-shCDK2), RB1 (pLVTHM-RB1) and empty vector (pLVTHM) were used for lentiviral production. The short hairpin sequences (5' to 3') used are:

CDK4: GCGTGTCCCTTTCCCTACCTTTATTTCAAGAGAATAAAGGTAGGGAAAGGGACTTTTTTGGAAAT
RB1: CGCGTGTTTCCTCTTCCAAAGTAATTCAAGAGATTACTTTGGAAGAGGAAACTTTTTTGGAAAT
CDK2: CGCGTGCAGAATCTGCTTATTAACATTCAAGAGATGTTAATAAGCAGATTCTGCTTTTTTGGAAAT

For lentiviral production, the second generation lentiviral vectors pLVTHM-shCDK4, pLVTHM-shCDK2, pLVTHM-RB1and pLVTHM were cotransfected with viral packaging plasmid (pMDLg/pRRE) and viral envelope plasmid (pMDG2). After incubation with polyethylenimine (PEI) (0.13µM), the solution was added drop-wise to the medium of a subconfluent p100 cell culture dish containing Human Embryonic Kidney (HEK) 293T cells. Cell culture medium was refreshed 6 hours after transfection of the 293T cells. For CDK4 knock-down in RT112 and 5637 cells, viral supernatants obtained 24hrs and 48hrs after transfection were filtered (0.22 µm) and added to these target cells for transduction. After 48hrs of incubation, infected cells were selected based on GFP fluorescence by BD InfluxTM Cell Sorter (BD Biosciences) using laser filter 530/30 (excitation laser line 488nm). Selected cells were amplified for further analysis by Western Blot and RT-qPCR.

Stock Solutions

Palbociclib (kindly provided by Pfizer SLU Spain) was dissolved in water at a stock concentration of 2 mg/ml (3.5 mM). Cisplatin (Selleckchem) was dissolved in water at 1 mg/ml

(3.33 mM) stock solution. PBS was used as a vehicle control. Working concentrations were prepared fresh for immediate use**.**

Cell viability and Cell Cycle assays

Cells were seeded at a density of 5000 cells/well in a 96-well plate. After 24 hours, cells were treated with a range of concentrations of palbociclib or CDDP for 24 hours Combined viability assays were performed according the Chou-Talalay method for 2 drugs (1). Cell viability was evaluated by XTT Cell proliferation kit II (Roche), measured with GENios pro microplate reader (Tecan). Background absorbance (medium only) was subtracted, and the data (average of six replicates of each drug concentration) were normalized as percent of vehicle control (PBS). Each experiment was performed five to ten times. Cell cycle analysis was performed by flow cytometry in a Becton Dickinson LSR Fortessa cell analyzer. Cells were cultured in 12-well plates and Palbociclib was added at IC50. After 24 hours of treatment, cells were harvested, washed with PBS and fixed in 70% ice-cold ethanol overnight at 4ºC. The samples were then centrifuged and suspended in PBS containing 2 µg/ml DAPI and 0.05% NP40 for at least 2 hours. Data analysis was performed with FlowJo 7.6.5 software using the cell cycle analysis tool. Each experiment was performed at least twice, with 3 replicates of each concentration per experiment.

Tumor xenografts

For xenografts, RT112 or 5637 cells were trypsinized and resuspended in a mixture (1:1) of PBS with Matrigel (BD Biosciences). Five million cells in suspension were subcutaneously injected in each flank of 20 immunocompromised nude (nu/nu) mice (Janvier Saint-Berthevin, France). Only females at 8-10 weeks of age were used. When tumor volume reached between 150-250 mm^3, mice were randomized in 4 groups (n = 5) to receive the different treatments: vehicle (PBS, Sigma), Palbociclib, CDDP or combined Palbociclib +CDDP. Palbociclib was dissolved in vehicle and 150 mg/kg was administrated intraperitoneally 5 days a week. CDDP was injected intraperitoneally 1 day per week with a dose of 6 mg/kg. The treatment with Palbociclib and CDDP consisted of a combination of the above mentioned injections. Data in Supp Fig 9A' correspond to similar experiments using RT112 cells in which the treatment was extended for 30 days.

Patient series and clinical data University Hospital "12 de Octubre" cohort.

Tumor and their corresponding non tumoral paired bladder, with available medical records were analyzed from 87 patients (pathological and clinical data are shown in Supp Table 2) who had been consecutively evaluated at the Urology Department between January 2009 and October 2011. The tumor samples were collected by multiple cold-cup biopsies from the exophytic part and from the normal mucosa of the bladder of patients undergoing transurethral resection. All the samples were kept in RNAlater. The histopathological status was confirmed by the Pathology Department, following the latest World Health Organization and TNM Classification of Malignant Tumors guidelines. All the patients were followed up within a local program according to European Association of Urology guidelines. Informed consent was obtained from all the patients and the study was approved by the Ethical

Committee for Clinical Research of University Hospital 12 de Octubre. Samples and united data from patients included in this study were provided by the Biobanco i+12 in the Hospital 12 de Octubre integrated in the Spanish Hospital Biobanks Network (RetBioH; www.redbiobancos.es) following standard operation procedures with appropriate approval of the Ethical and Scientific Committees (ref. 10/050). The construction and analysis of tissue microarray containing all the human samples has been reported elsewhere (2,3).

EPICURO Study

Resources from the EPICURO Study including 832 newly diagnosed urothelial bladder cancer (UBC) cases aged 22–80 years with a median follow-up of 70.7 months (range 0.7–117.7 months) and available tumor tissue were used to replicate previous results on FOXM1 prognostic value. Patients were consecutively recruited between 1998 and 2001 in 18 hospitals of Spain (4). Diagnoses were uniformly reviewed by a panel of expert pathologists according to TNM and WHO-ISUP classifications. All tumor samples were collected prior to the administration of any intravesical or systemic therapy. Clinical information related to diagnostic procedures, tumor characteristics and treatment was collected from medical records. Follow-up information was annually updated from hospital records up to 10 years. In this series, NMIBC recurrence was defined as the appearance of a new NMIBC following a previous negative follow-up cystoscopy, and progression, as the development of a MIBC. In patients initially presenting with MIBCs, any tumor reappearance after treatment was considered progression Informed consent was obtained from study participants in accordance with the Institutional Review Board of the Ethics Committees of participating hospitals that approved the study (IRB Hospital del Mar, ref. 2008/3296/1). Tissue microarrays (TMA) containing tumor cores of 0.6-mm in diameter represented in duplicate and selected from the most representative regions of the tumor were used to assess Thr600 phosphorylated FOXM1 protein expression as described previously. Associations between phosphorylated FOXM1 expression and demographic and clinico-pathological parameters were assessed using X^2 test. Among NMIBCs, expression was also assessed in low-grade/risk (pTaG1/G2) and high-grade/risk (pTa/pT2G3). Kaplan-Meier method was applied to display recurrence-free, progression-free, and overall disease-specific. Univariable and multivariable Cox-proportional hazards analysis was used to calculate hazard ratios (HR) and 95% confidence intervals (CI).

Gene expression microarray analyses

Cell pool transcriptomes were obtained from 3 different p100 dishes. Total RNA was extracted using miRNeasy Mini Kit (Qiagen). cDNAs from 12 ng total RNA were generated, fragmented, biotinylated, and hybridized to the GeneChip Human Transcriptome Array (HTA 2.0) (Affymetrix).

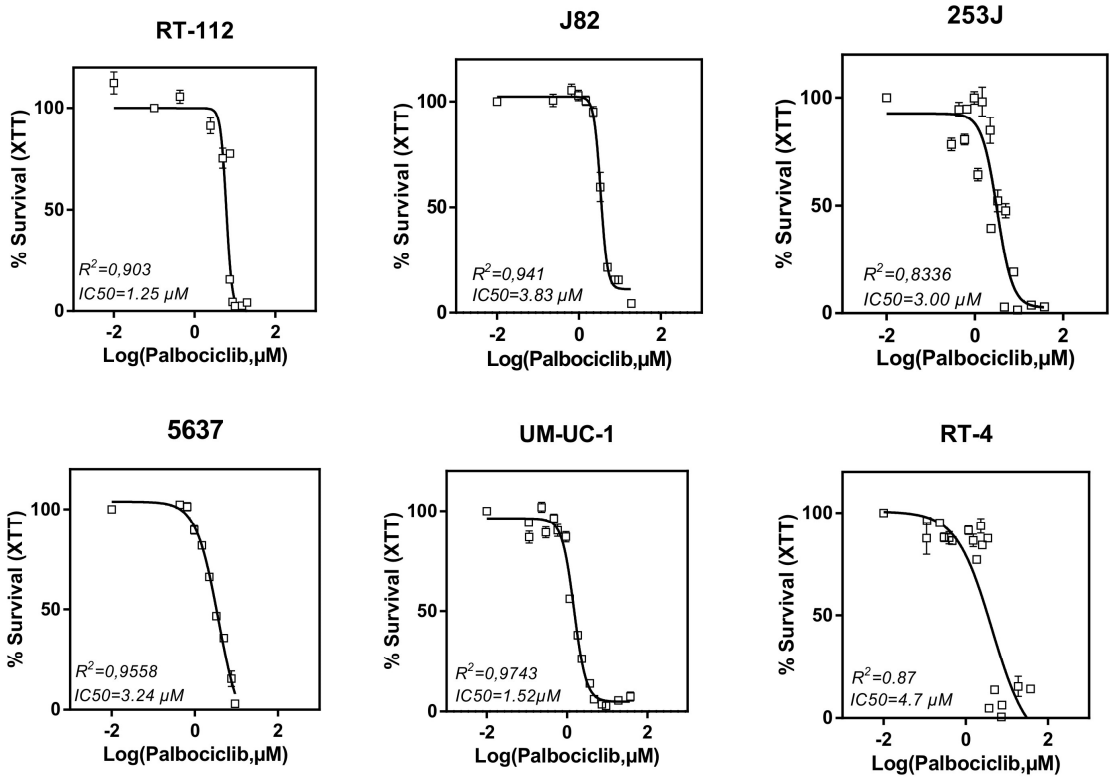
In the case of transgenic mouse samples, micro-dissections of the tumors were carried out before RNA extraction to avoid any possible contamination. Total RNA was extracted using miRNeasy FFPE kit (Qiagen). cDNAs from 12 ng of total RNA were generated, fragmented, biotinylated, and hybridized to the GeneChip Mouse Transcriptome Array 1.0 (MTA 1.0.), now known as Clarion D Assay Mouse (Affymetrix, Thermofisher).

The arrays were washed and stained on a GeneChip Fluidics Station 450 (Affymetrix); scanning was carried out with the GeneChip Scanner 3000 7G; and image analysis with the Affymetrix GeneChip Command Console Scan Control. Expression data were normalized, background and batch corrected using the Guanine Cytosine Count Normalization (GCCN) and Signal Space Transformation (SST), and prior to summarizing using the Robust Multi-Array Average (RMA) algorithm implemented in the Affymetrix Expression Console software. In order to combine the different microarray data sets, possible batch effects were corrected by using the Bioconductor Combat package in R environment (9). Supervised hierarchical clustering was performed using Pearson correlation and average linkage using TMEV software (5). Gene Ontology and Chip Enrichment Analysis were performed using Enrich webtool (http://amp.pharm.mssm.edu/Enrichr/) (6,7). Gene Set Enrichment Analysis (GSEA) was performed using the MSignature and Motif databases (8).

A

Cell Line	Gene									Sensitivity to Palbociclib (IC50±SD)
	TP53	RB1	EZH2	EHMT2	KDM5A	ARID1A	KDM6A	PIK3CA	FGFR3	
253J	wt	wt	wt	wt	wt	wt	wt	E545G	wt	**3±0.5 µM**
RT112	R248Q/S183*	wt	wt	wt	wt	wt	P1191fs	wt	wt	**1.25±0.4 µM**
J82	E271K	I703_splice	wt	wt	wt	wt	C1413Y	P124L	K652E	**3.8±0.8 µM**
5637	R280T	Y325*	wt	wt	W139*	wt	wt	wt	wt	**3.24±0.7 µM**
UMU UC1	P152S	wt	wt	wt	wt	wt	Q1281*	wt	wt	**1.52±0.9 µM**
RT4	wt	wt	wt	wt	wt	3'UTR	wt	wt	wt	**4.7±0.9 µM**

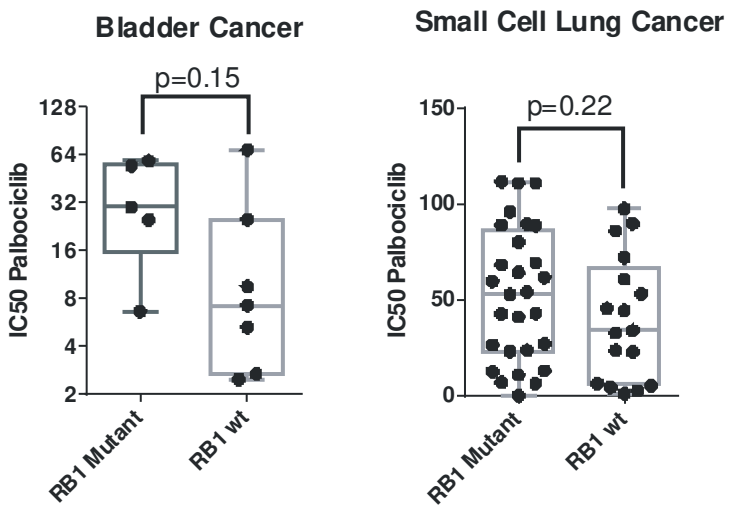
B



Supplementary Fig 1 (relative to Fig 1)

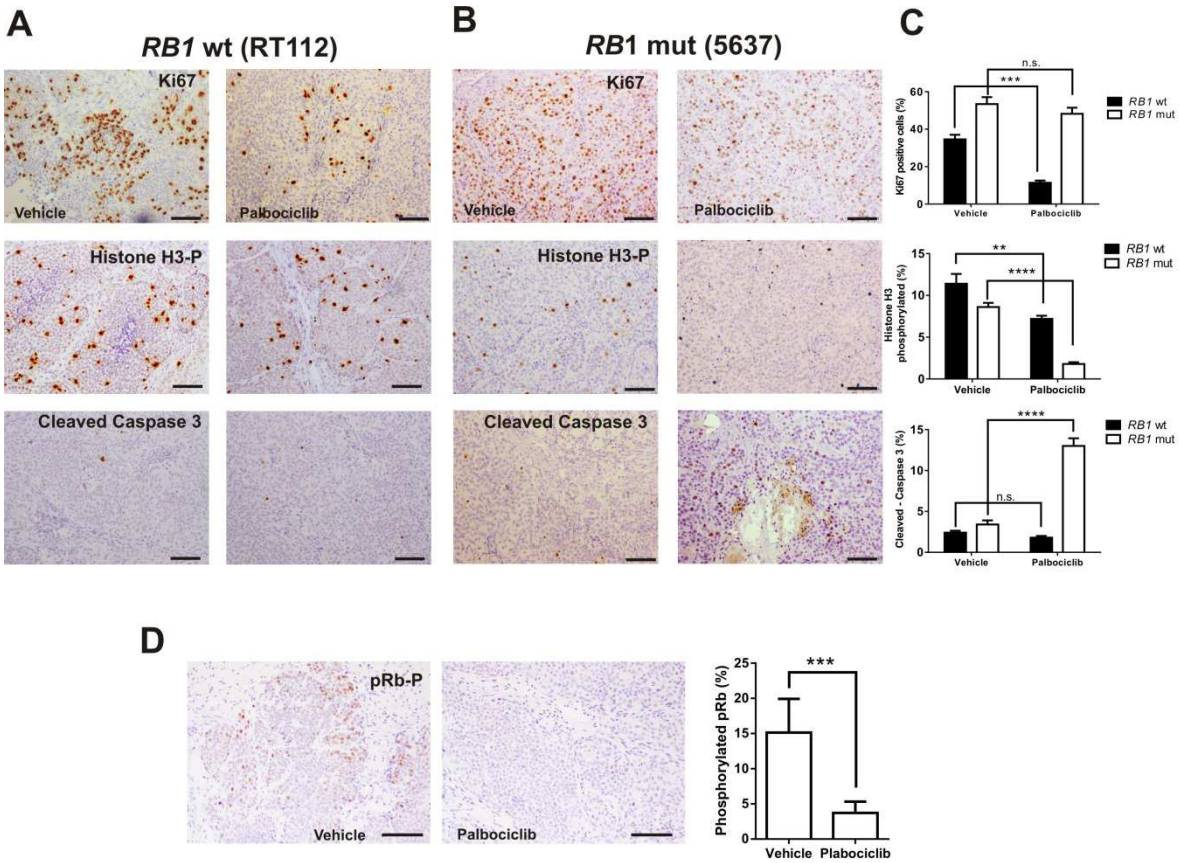
A) Summary of relevant mutations in the BC cell lines used in the study

B) Summary of sensitivity assays (XTT) of quoted BC cell lines to various concentration of palbociclib. Data come from 5 to 10 independent experiments and are shown as mean ± SEM.



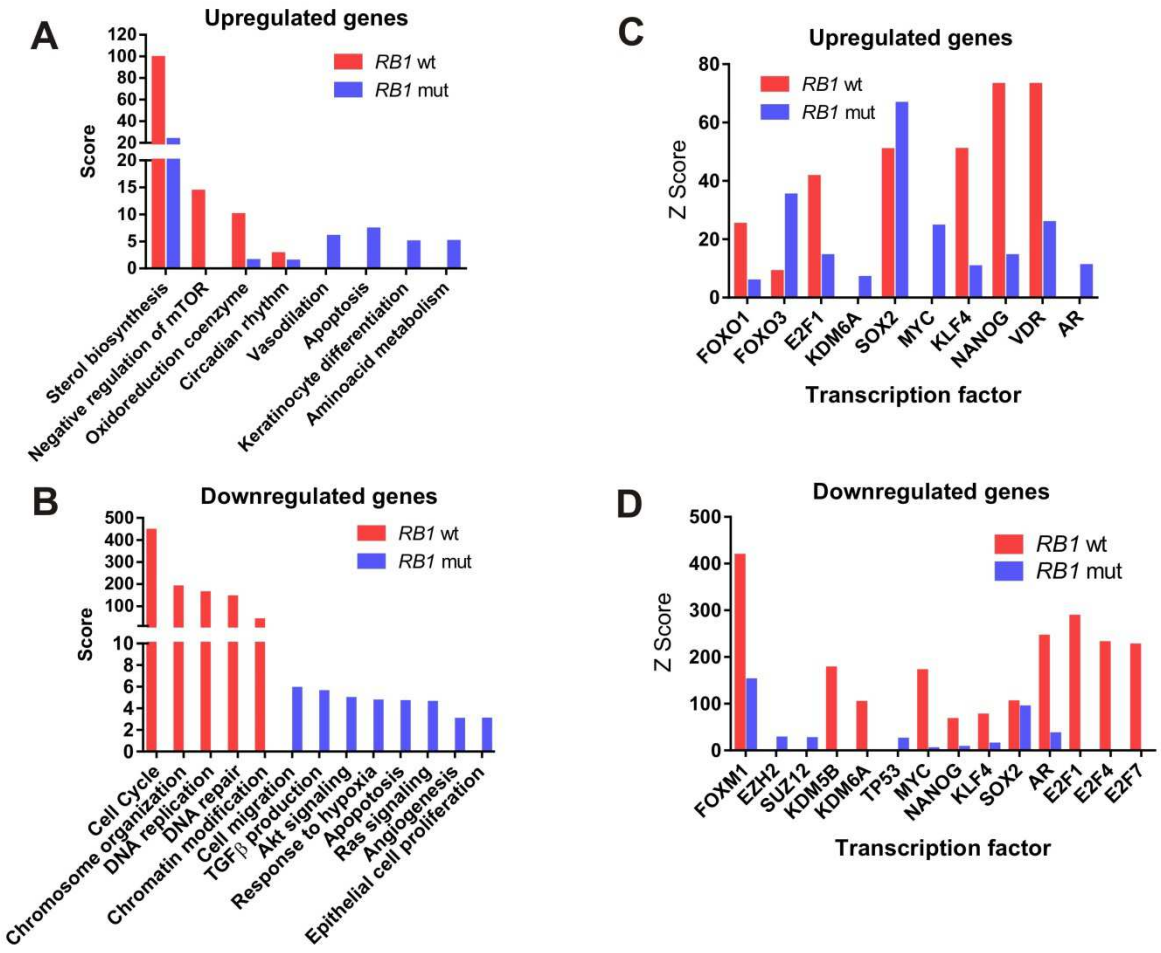
Supplementary Fig 2 (relative to Fig 1)

Analysis of sensitivity to palbociclib in bladder and small cell lung cancer cell lines from the cancer cell line encyclopedia (https://portals.broadinstitute.org/ccle)



Supplementary Fig 3 (relative to Fig 1)

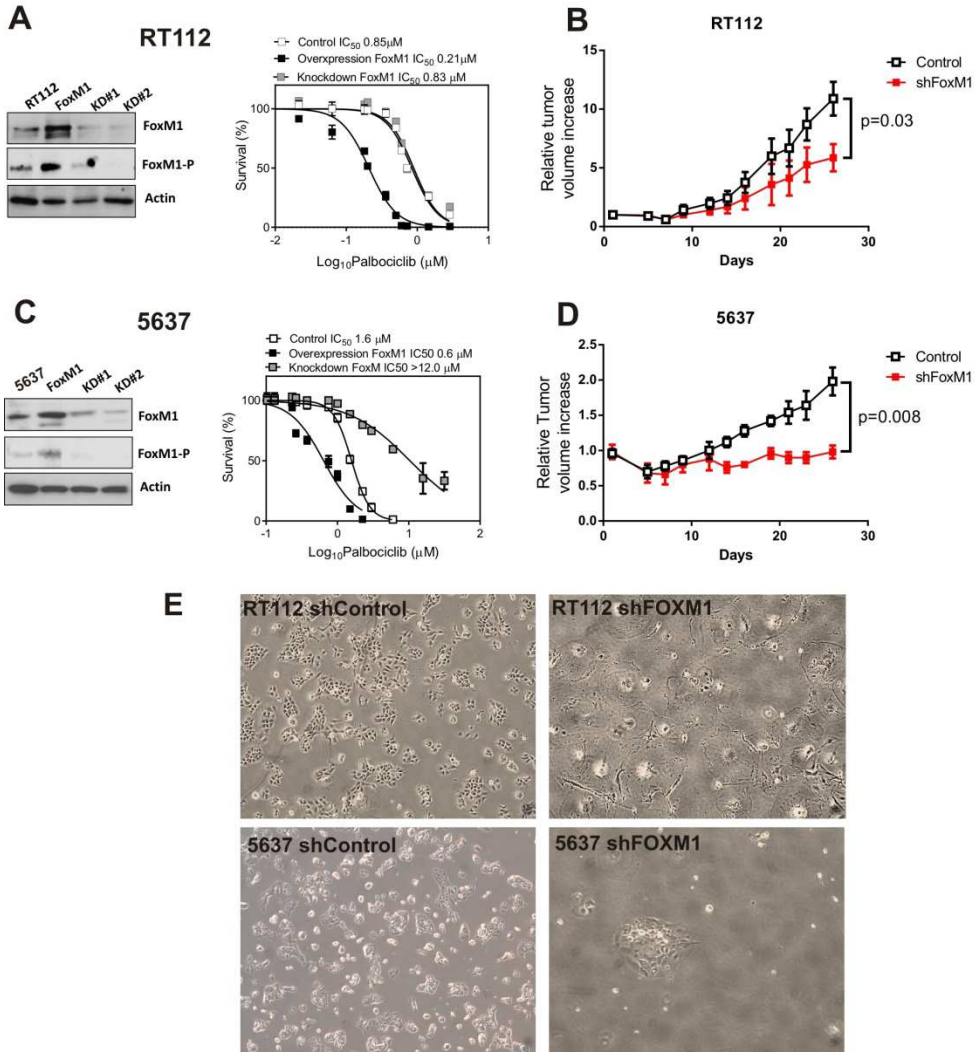
(A,B) Representative examples of control and palbociclib treated tumor xenografts from RT112 (*RB1* wt) (A) and 5637 (*RB1* mut) (B) stained against Ki67, Active caspase 3, and phosphorylated Histone3. Bars = 150 µm. C) Summary of the quantitative analyses of each staining. Data come from 5 different tumors for each cell line and condition (three independent slides) and are shown as mean ± SEM. **** p-value ≤ 0.0001, *** p-value ≤ 0.0005 **, p-value ≤ 0.01 as determined by the Mann-Whitney t-test. D) Representative examples of Control and Palbociclib treated tumor xenografts from RT112 stained against phosphorylated pRb. Bars = 150 µm. Right panel shows the summary of the quantitative analyses of staining. Data come from 5 different tumors for each condition (three independent slides) and are shown as mean ± SEM. *** p-value ≤ 0.0005 as determined by the Mann-Whitney U-test.



Supplementary Fig 4 (relative to Fig 2)

A, B) Summary of Gene Ontology of biological processes showing the relevant functions of the upregulated (A) or downregulated (B) genes in palbociclib treated *RB1* wt or mutant human BC cell lines.

C, D) Summary of putative binding motif enrichment analysis using the Enrich webtool (http://amp.pharm.mssm.edu/Enrichr/) (6,7), showing the relative relevance of various transcription factors in the upregulated (C) and downregulated (D) transcripts in Palbociclib treated *RB1* wt or mutant human BC cell lines.



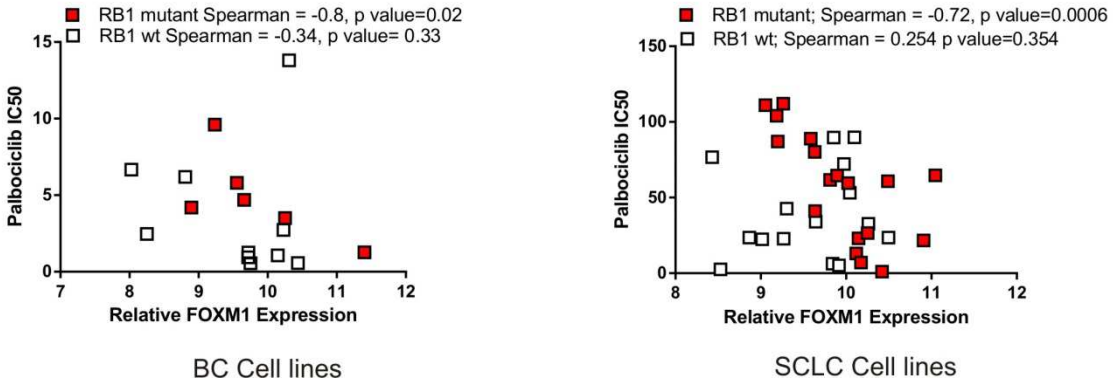
Supplementary Fig 5 (relative to Fig 2)

A) Effects of FOXM1 overexpression or knockdown in the sensitivity to palbociclib in RT112 cells. Left panel immunoblot showing the effects on FOXM1 expression, right panel sensitivity of the quoted cells to palbociclib assayed by XTT. Data come from 5 independent experiments and are shown as mean ± SEM.

C) Effects of FOXM1 overexpression or knockdown in the sensitivity to palbociclib in 5637 cells. Left panel immunoblot showing the effects on FOXM1 expression, right panel sensitivity of the quoted cells to palbociclib assayed by XTT. Data come from 5 independent experiments and are shown as mean ± SEM.

B, D) Tumor growth curves of RT112 (C) and 5637 (D) parental and knockdown cells upon injection in nude mice. Data come from 5 independent mice and are shown as relative mean growth ± SEM. P Values were obtained by ANOVA and Bonferroni multiple comparison test.

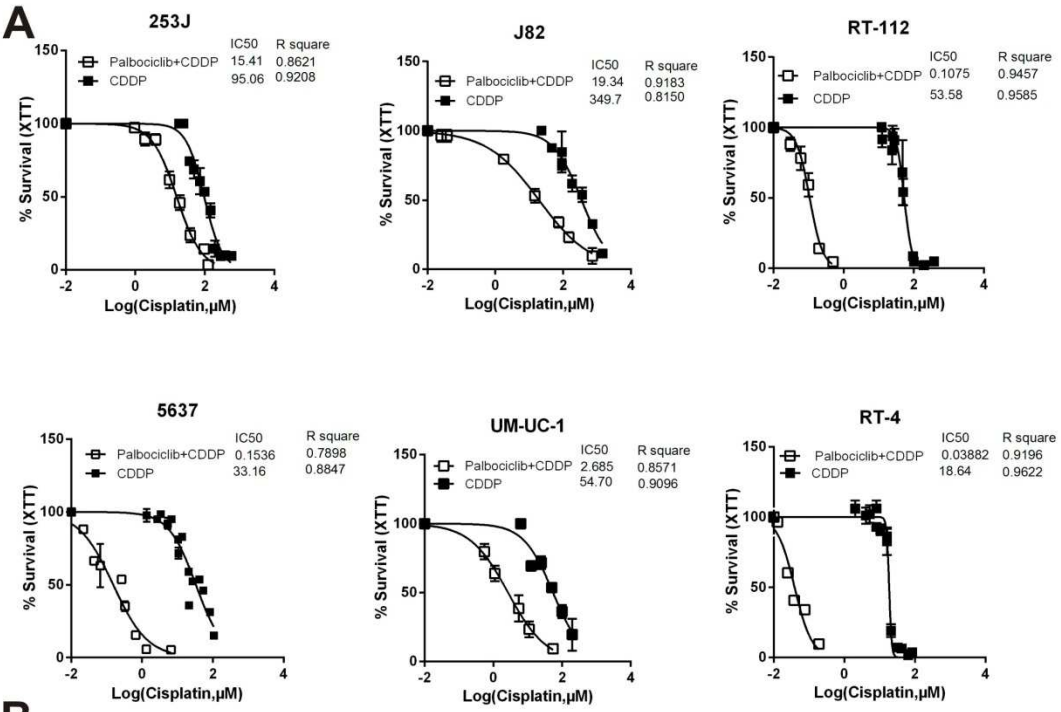
E) Overall aspect of RT112 (upperpanels) and 5637 BC cell lines upon infection with a control (right panels) or shFOXM1 lentivirus. Note the flattened senescence-like features of RT112 cells and the reduction in cell viability with only remaining colonies in 5637 cell line upon knockdown of FOXM1.



Supplementary Fig 6 (relative to Fig 2)

Analysis of the correlation between sensitivity to palbociclib (IC50 values) in bladder and small cell lung cancer cell lines according their *RB1* gene status (mutant cell lines in red squares, *RB1* wt cells in open squares) obtained from from the cancer cell line encyclopedia (https://portals.broadinstitute.org/ccle). Correlation were analyzed by Spearman method providing the rho and *p* values.

A



B

RT-112

Cisplatin (µM) \ Palbociclib (µM)	0	1/4 IC50	1/2 IC50	IC50	2 IC50	4 IC50
0	100 ± 0	94.1 ± 4.7	83 ± 4.8	65.1 ±0.6	1.6 ± 1.7	0.4 ± 1.3
12.5	91.6 ± 1.2	94.3 ± 0.1				
25	93.8 ± 1.5		60.3 ± 8.6			
50	54.1 ± 2.4			2.2 ± 1.3		
100	4.8 ± 0.4				0.4 ± 1.5	
200	2.3 ± 0.8					

253J

Cisplatin (µM) \ Palbociclib (µM)	0	1/4 IC50	1/2 IC50	IC50	2 IC50	4 IC50
0	100 ± 0	99.5 ± 0.35	67.6 ± 5.23	55.6 ± 1.7	6 ± 1.41	3.4 ± 1.02
24.25	99 ± 0.71	98.5 ± 1.06				
48.5	74.2 ± 7.7		54.1 ± 8.44			
97	54.8 ± 2.25			25.4 ± 1.7		
194	6.1 ± 2.17				3.5 ± 1.75	
388	4.5 ± 1.98					

RT-4

Cisplatin (µM) \ Palbociclib (µM)	0	1/4 IC50	1/2 IC50	IC50	2 IC50	4 IC50
0	100 ± 0	94.3 ± 4.61	87.6 ±0.31	67.4 ± 3.98	13.5 ± 2.03	4 ± 0.20
5.25	98.9 ± 7.07	97.9 ± 7.87				
10.5	90.0 ± 1.70		90.7 ± 4.92			
21	20.4 ± 6.63			11 ± 1.25		
42	6.2 ± 0.12				4.1 ± 1.11	
84	3.7 ± 0.03					

UM-UC-1

Cisplatin (µM) \ Palbociclib (µM)	0	1/4 IC50	1/2 IC50	IC50	2 IC50	4 IC50
0	100 ± 0	0.82 ± 1.5	0.81 ± 0.40	0.5 ± 2.1	0.08 ± 0.26	0.01 ± 0.10
12.5	0.73 ± 5.2	0.7 ± 0.15				
25	0.67 ± 3.4		0.63 ± 2.3			
50	0.53 ± 0.7			0.12 ± 1.4		
100	0.41 ± 0.5				0.01 ± 0.88	
200	0.31 ± 0.3					

5637

Cisplatin (µM) \ Palbociclib (µM)	0	1/4 IC50	1/2 IC50	IC50	2 IC50	4 IC50
0	100 ± 0	96.5 ± 0.33	78.7 ± 1.46	51.9 ± 2.39	5.1 ± 2.9	0.1 ± 0.20
12.5	94.5 ± 2.25	80.7 ± 0.98				
25	88.1 ± 4.93		65.8 ± 2.23			
50	75.5 ± 11.79			37.1 ± 0.33		
100	62.7 ± 6.34				1.4 ± 0.66	
200	33.5 ± 1.61					

J82

Cisplatin (µM) \ Palbociclib (µM)	0	1/4 IC50	1/2 IC50	IC50	2 IC50	4 IC50
0	100 ± 0	96 ± 0.6	92.9 ± 0.1	59.2 ± 7.1	17.1 ± 0.7	5 ± 0.2
12.5	54.6 ± 12.5	51.2 ± 8.11				
25	46.3 ± 10.1		46 ± 6.7			
50	54.7 ± 2.6			37.4 ± 2.5		
100	0.5 ± 0.5				0.9 ± 0.6	
200	0.01 ± 1.4					

Supplementary Fig 7 (relative to Fig 3)

A) Summary of sensitivity assays (XTT) of quoted BC cell lines to various concentration of CDDP alone (closed squares) or pretreated with palbociclib at the relative IC50 concentration (open squares) (see Suppl Fig 1). Data come from 5 to 10 independent experiments and are shown as mean ± SEM.

B) Summary of sensitivity assays (XTT) of quoted BC cell lines to combinations of CDDP and Palbociclib used for determination of combination index. Data come from 5 to 10 independent experiments and are shown as mean ± SEM.

A

	Cell line	
	RT112	5637
Control	50±3	33±5
FoxM1	63±3	98±7
shFoxM1	28±4	17±4
p value C vs OE*	0.004	0.002
p Value C vs KD*	0.005	0.004

B

CDDP IC50 (µM)	Cell line	
	RT112	5637
Control	43.4±1.0	27.7±1.1
shCDK4	42.7±1.2	29.1±1.1

Palbociclib IC50 (µM)	Cell line	
	RT112	5637
Control	1.5±1.0	2.8±1.1
shCDK4	>300	>600

Supplementary Fig 8 (relative to Fig 3)

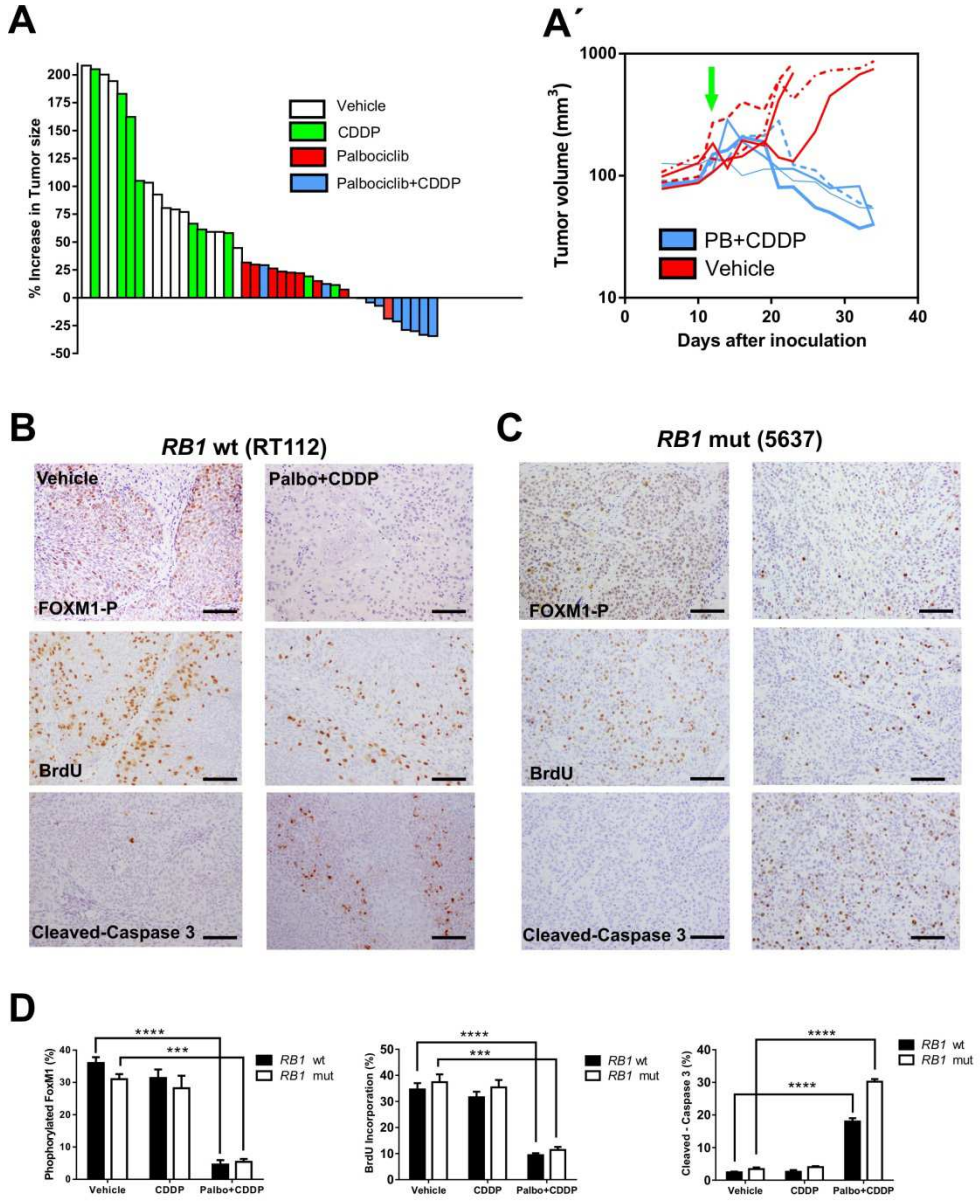
A) Summary of CDDP IC50 data from quoted BC cell lines upon overexpression (indicated by FoxM1 and OE) or knockdown of FOXM1 (indicated by shFoxM1 and KD). p Values were obtained by unpaired Mann Whitney t test for each indicated group of cells.

B) Summary of CDDP IC50 data from quoted BC cell lines upon knockdown of CDK4 (indicated by shCDK4). No significant differences were observed between control (empty vector) and shCDK4 derivatives in each cell line.

Cell Line	Combination index	Type
5637 OE FoxM1	0.83	Synergism
5637 shFoxM1	0.881	Synergism
5637 Cont	0.75	Synergism
RT112 OE FoxM1	0.86	Synergism
RT112 shFoxM1	0.98	Additive
RT112 Cont	0.65	Synergism

Supplementary Fig 9 (relative to Fig 3)

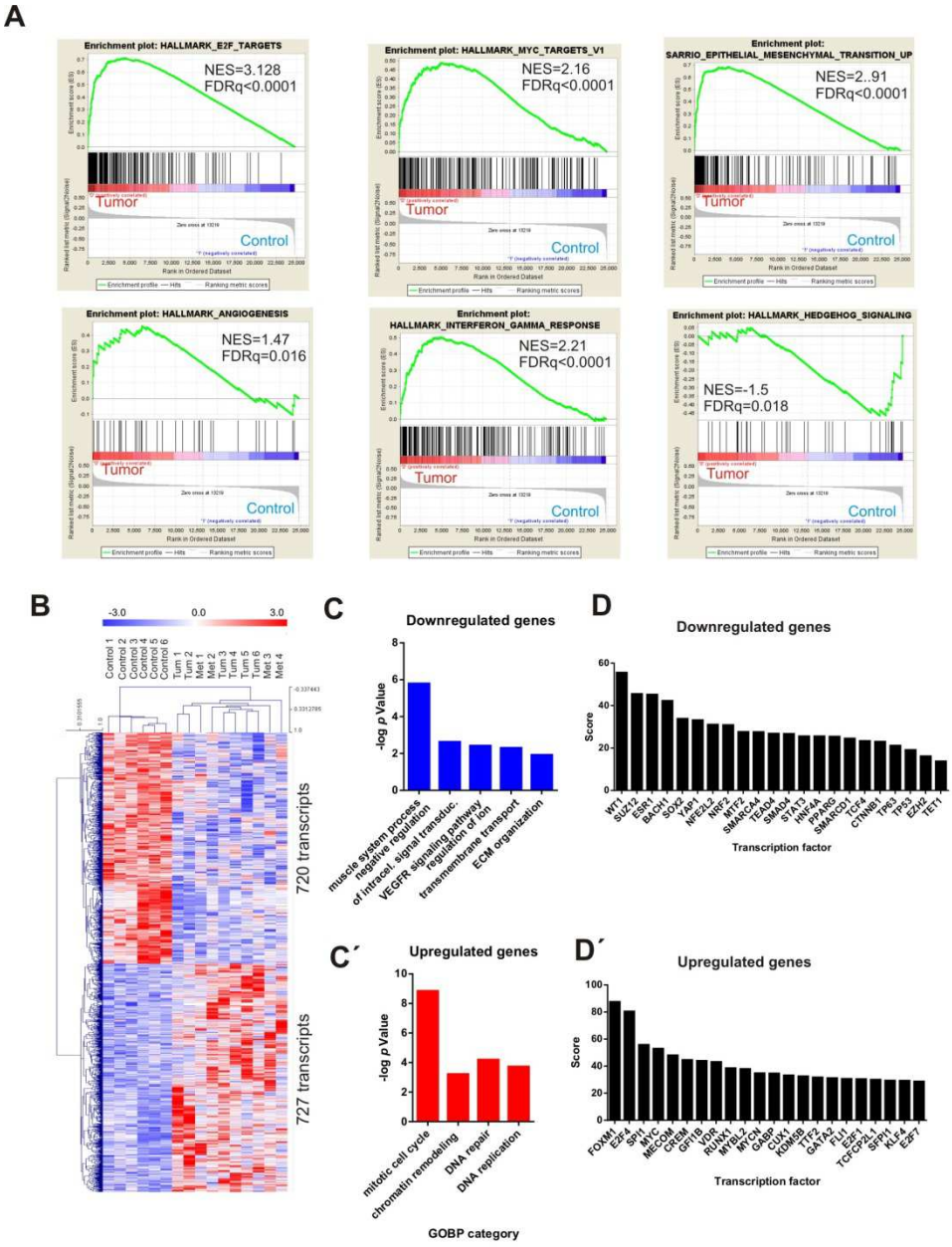
Summary of CDDP and palbociclib Combination index from quoted BC cell lines upon overexpression (indicated by OE FoxM1) or knockdown of FOXM1 (indicated by shFoxM1). The type of cooperativeness between the two compounds for each cell line derivative is also indicated. Combined viability assays were performed according the Chou-Talalay method for 2 drugs (1) after cell viability determination in sextuplicate by XTT Cell proliferation kit analyses.



Supplementary Fig 10 (relative to Fig 3)

A) Waterfall plot showing the individual tumor growth of xenograft experiments of RT112 and 5637 cells treated with vehicle, CDDP, palbociclib and Palbociclib + CDDP combination. A') individual growth of RT112 cell tumor xenografts treated for extended time. Each line denotes individual tumor growth. In red vehicle-treated mice, in blue mice treated with palbociclib plus CDDP. B, C) Representative examples of immunohistochemistry staining of phosphorylated FOXM1(Thr600), BrdU incorporation and active caspase 3 in RT112 (B) and 5637 (C) xenografts treated with vehicle or the combination with palbociclib + CDDP. Bars=150 µm

D) Summary of the quantitative analyses of staining in B, C. Data come from 5 different tumors for each condition (three independent slides) and are shown as mean ± SEM. **** p-value ≤ 0.0001, *** p-value ≤ 0.0005 as determined by the Mann-Whitney t-test.



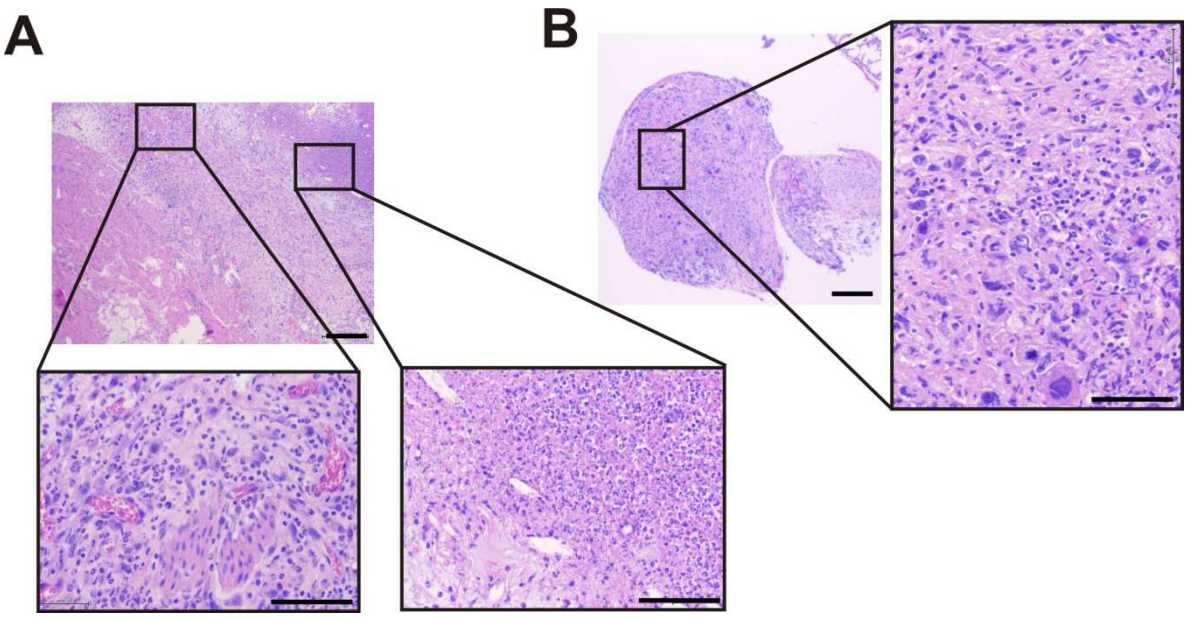
Supplementary Fig 11 (relative to Fig 4). **Genomic Characterization of metastatic mouse bladder cancer model**.

A) Summary of Gene Set Enrichment Analyses showing the relative enrichment in various pathways in tumors vs normal control bladder.

B) Heatmap showing global transcriptome changes in mouse control bladder vs tumor samples.

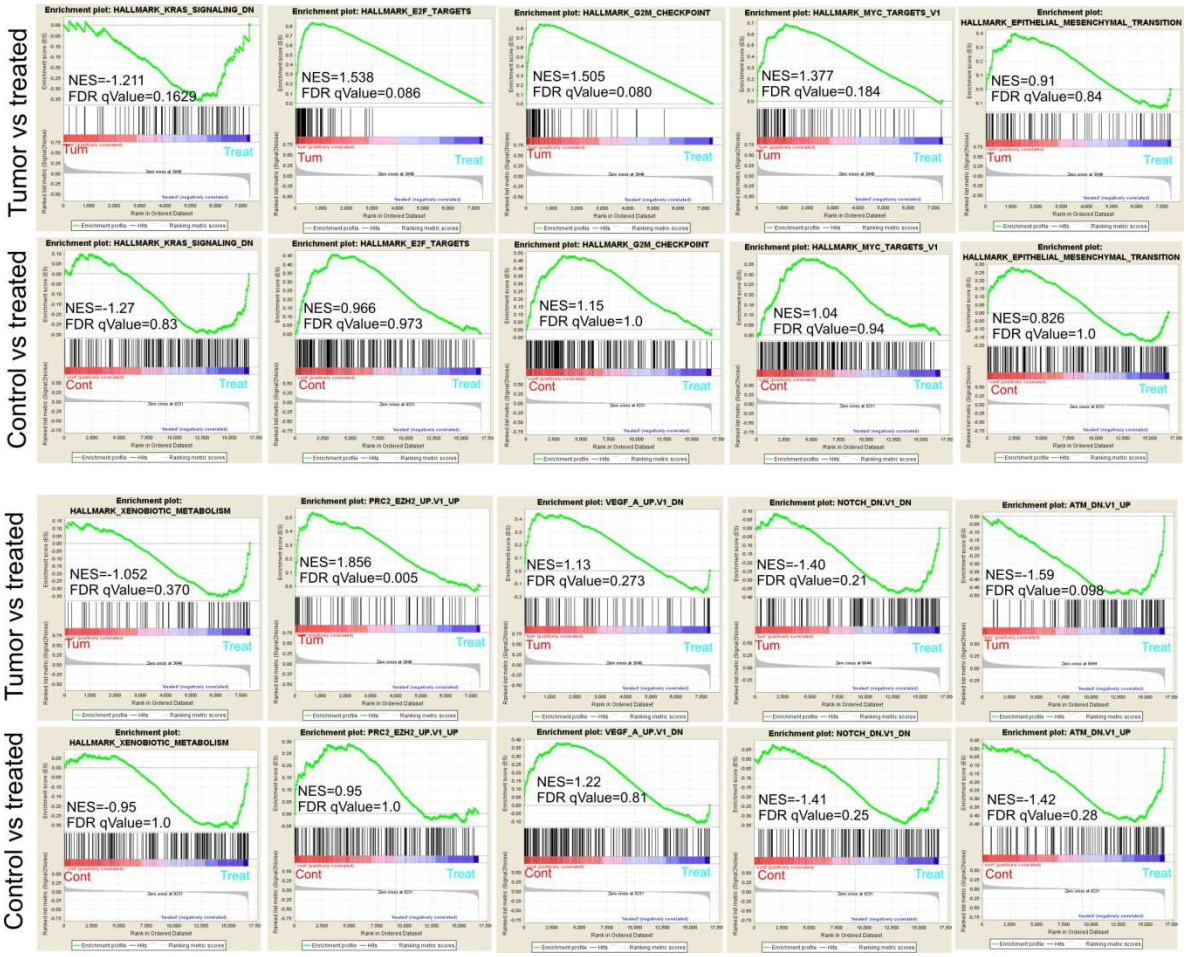
C, C') Summary of Gene Ontology of biological processes showing the relevant functions of the downregulated (C) or upregulated (D) transcripts in mouse bladder tumor samples.

D, D') Summary of putative binding motif enrichment analysis using the Enrich webtool (http://amp.pharm.mssm.edu/Enrichr/) (6,7), showing the relative relevance of various transcription factors in the downregulated (D) or upregulated (D') transcripts in mouse bladder cancer samples.



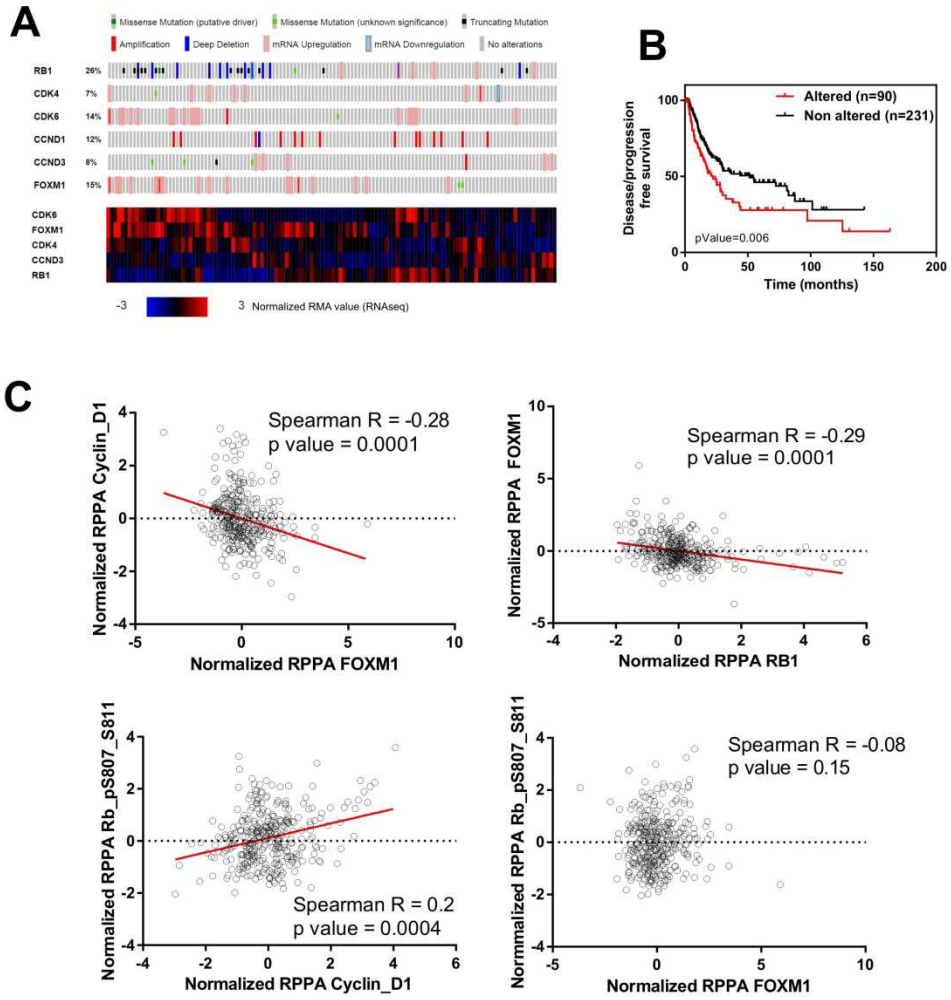
Supplementary Fig 12 (relative to Fig 4).

Representative histology of invasive bladder tumor (A) or visceral metastasis (B) upon palbociclib + CDDP combined treatment showing massive immune cell infiltrates and necrotic areas. Bars =100 µm.



Supplementary Fig 13 (relative to Fig 4)

Summary of representative Gene Set enrichment analyses showing the relative enrichment in various pathways in vehicle vs palbociclib + CDDP treated mouse tumors and in normal bladder vs palbociclib + CDDP treated mouse bladder tumors.



Supplementary Fig 14 (relative to Fig 5)

A) Mutation plot (upper panel) and heatmap (lower panel) shwing the alterations found in TCGA database for each gene denoted on the left. The percentage of bladder tumors showing alteration for each gene and each type of alterations is provided. Data were obtained from cBioportal (http://www.cbioportal.org/)

B) Kaplan-Meier graph showing Disease-Specific/Progression free survival in patients showing alterations in any of the quoted genes in (A) compared with those without alterations. Data were downloaded from TCGA (http://www.cbioportal.org/). p Value was obtained by log rank test.

C) Correlation between the expression of the quoted proteins as downloaded from RPPA data from TCGA (http://www.cbioportal.org/). Data were analyzed by and Spearman correlation, Rho coefficient and p-values are provided for each pair of proteins.

Supplementary Table 1

List of antibodies

Primary Antibodies for Western Blot and Immunohistochemistry				
Target	Clone	Source	Dilution of Western Blot	Dilution of Immunohistochemistry
Akt1/2	N-19	Santa Cruz SC 1619	1:500	
AKT-P (S473)	EP2109Y	Abcam 81283	1:2500	
AKT-P (S473)	D9E	Cell Signaling 4060	1:500	1:50
BrdU	BMC9318	Roche 11170376001		1:20
CDK2	M2	Santa Cruz sc-163	1:500	
CDK4	-	Santa Cruz sc-260	1:500	
CDK8	-	Abcam 2955	1:500	
CDK9	-	Abcam 6544	1:500	
Cleaved Caspase-3	-	Cell Signaling #9661		1:200
Cyclin A	BF683	Santa Cruz sc-239	1:500	
Cyclin B1	-	Santa Cruz sc-55	1:500	
Cyclin D1	SP4	NeoMarkers RM- 9104 -S1	1:500	
Cyclin E	HE12	Santa Cruz sc-247	1:500	
E2F1	KH95	Santa Cruz sc-251	1:2000	
E2F3(a-b)	C-18	sc-878X	1:1000	
ERK	K-23	Santa Cruz SC-94	1:500	
ERK1/2-P (Thr202/Tyr204)	D13.14.4E	Cell Signaling 4370	1:500	1:400
ERK-P	E-4	Santa Cruz sc-7383	1:1000	
EZH2	6A10	Abnova MAB9542	1:2000	1:200
EZH2(Thr487)	EPR1410	Abcam 109398	1:500	
FOXM1	D12D5	Cell Signaling 5436	1:500	
FOXM1-P(Ser35)	-	Cell Signaling 14170	1:500	
FOXM1-P(Thr600)	D9M6G	Cell signaling 14655S	1:500	1:100
Histone H3-P (Ser10)	-	Millipore. Cat. # 06-570		1:500
Ki67	MM1	Novocastra. NCL-Ki67p	1:500	
p107	C18	Santa Cruz sc-318	1:500	
p130	10/Rb2	BD Transduction 610262	1:500	
p16	M-156	Santa Cruz sc-1207	1:500	
p27	-	abcam ab7961	1:500	
p53	CM5	Novocastra NCl-p53_CM5p	1:1000	
p-Rb Ser 807/811	-	Cell Signaling 9308	1:200	
PTEN	N-19	Santa Cruz SC 6818	1:500	
Rb total	Clone G3-245	BD Pharmigen 554136	1:500	
Stat3	79D7	Cell Signaling 4904	1:500	
Stat3-P (Tyr705)	-	Cell Signaling 9131	1:1000	1:500
β-Actina	I-19	Santa Cruz sc-1616	1:1000	

Secondary antibody for Western Blot and Immunohistochemistry				
Target	Clone	Conjugated	Source	Dilution
Anti-Rabbit IgG	Donkey	HPR	GE Healthcare NA934	1:5000
Anti-Mouse IgG	Donkey	HPR	JACKSON Nº 715-035-151	1:5000
Anti-Goat IgG	Donkey	HPR	Santa Cruz sc-2020	1:10000
Anti-Rabbit IgG	Donkey	Biotin-SP	JACKSON Nº 711-065-152	1:1000
Anti-Mouse IgG	Donkey	Biotin-SP	JACKSON Nº 715-035-151	1:1000

Supplementary Table 2

Baseline characteristics of the patients and clinicopathological results in the series

Patients (n)	68
Age median (range)	72 yr (49–90)
Sex	M=53 F=15
Smoker status	No=12 Currently smoker=22 Ex smoker=32 ND=2
Stage	Papilloma =1 Ta=27 T1=31 T2=9
Grade	Papilloma =1 Low=35 High=29 PUNLMP=3
Alterations in normal mucosa	Dysplasia=5 Glandular cystitis=1
Intravesical Instillation	BCG=2 (1 Recurrent) Mitomycin=1 (1 Recurrent)
Recurrence events	ND = 1 Recurrence=22 Non recurrence=36
Stage of recurrence	Ta=6 T1=9 T2=2
Grade of recurrence	Low=7 High=10 PUNLMP=1

Supplementary Table 3

List of RTqPCR primers

Primer sequences for RT-qPCR

Primer name	Sequence
TBP-F	5'-AGTGAAGAACAGTCCAGACTG-3'
TBP-R	5'-CCAGGAAATAACTCTGGCTCAT-3'
FOXM1-F	5'-ACTTTAAGCACATTGCCAAGC-3'
FOXM1-R	5'-CGTGCAGGGAAAGGTTGT-3'

Supplementary Table 7

Distribution of the EPICURO study subjects according to the phosphorylated FOXM1 status and the clinical variables

Var	ALL N=832	p-FOXM1 + N= 130	p-FOXM1 – N=702	p-value
Age				
T1[19-63]	261 (31.4%)	34 (26.2%)	227 (32.5%)	
T2(63-71]	290 (34.9%)	51 (39.2%)	239 (34.2%)	0.326
T3 (71-80.5]	281 (33.8%)	45 (34.6%)	233 (33.3%)	
Gender				
Males	736 (88.5%)	112 (86.2%)	623 (88.9%)	0.458
Female	96 (11.5%)	18 (13.8%)	78 (11.1%)	
Stage				
Ta	498 (59.9%)	63 (48.5%)	435 (62%)	
T1	107 (12.9%)	28(21.5%)	79 (11.2%)	
T2	116 (13.9%)	24 (18.5%)	92 (13.1%)	**0.004**
T3	67 (8.1%)	10 (7.7%)	57 (8.1%)	
T4	44 (5.3%)	5 (3.8%)	39 (5.6%)	
Grade				
G1	209 (25.1%)	12 (9.2%)	197 (28.1%)	
G2	241 (29%)	41 (31.5%)	200 (28.5%)	**1.8e-05**
G3	382 (45.9%)	77 (59.2%)	305 (43.4%)	
Stage&Grade				
LR-NMIBC	417 (50.2%)	45 (34.6%)	372 (53.1%)	
HR-NMIBC	175 (21.1%)	44 (33.8%)	131 (18.7%)	**5e-05**
MIBC	239 (28.8%)	41 (31.5%)	198 (28.4%)	

Supplementary Table 8

Bladder cancer outcome adjusted hazard ratios (HR) and 95% confidence intervals (CI) for the phosphorylated FOXM1+ in the EPICURO study subjects.

	p-FOXM1 +	p-FOXM1 -
TOTAL		
Recurrence		
Outcome (%)	32 (24.6%)	172 (24.5%)
HR (95% CI)	1.1 [0.76-1.62]	
Progression		
Outcome (%)	44 (33.8%)	151 (21.5%)
HR (95% CI)	**1.7[1.18-2.32]**	
Disease Specific		
Outcome (%)	150 (18.1%)	681 (81.9%)
HR (95% CI)	1.3[0.87-1.96]	
TOTAL *ADJUSTED**		
Recurrence		
Outcome (%)	32 (24.6%)	172 (24.5%)
HR (95% CI)	1.2[0.82-1.78]	
Progression		
Outcome (%)	44 (33.8%)	151 (21.5%)
HR (95% CI)	**1.4 [0.96-1.9]**	
Disease Specific		
Outcome (%)	150 (18.1%)	681 (81.9%)
HR (95% CI)	1.1[0.7-1.58]	
LR-NMIBC		
Recurrence		
Outcome (%)	150 (36%)	267 (64%)
HR (95% CI)	1 [0.6-1.76]	
Progression		
Outcome (%)	24 (5.8%)	393 (94.2%)
HR (95% CI)	**3.9[1.6-9.31]**	
Disease Specific		
Outcome (%)	16 (3.8%)	401 (96.2%)
HR (95% CI)	**3.1[0.99-9.6]**	
HR-NMIBC		
Recurrence		
Outcome (%)	51 (29.1%)	124 (70.9%)
HR (95% CI)	1.4[0.8-2.6]	
Progression		
Outcome (%)	35 (20%)	140 (80%)
HR (95% CI)	1.3[0.65-2.71]	
Disease Specific		
Outcome (%)	19 (10.9%)	156 (89.1)
HR (95% CI)	1[0.35-2.74]	
MIBC		
Progression		
Outcome (%)	136 (56.9%)	103 (43.1%)
HR (95% CI)	1.2[0.75-1.77]	
Disease Specific		
Outcome (%)	115 (48.1%)	124 (51.9%)
HR (95% CI)	0.9[0.58-1.53]	

*Age-, gender-, stage- and grade-adjusted hazard ratios (HR) and 95% confidence intervals in brackets

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