SUPPLEMENTARY INFORMATION

p27kip1 expression and phosphorylation dictate Palbociclib sensitivity in KRAS-mutated Colorectal Cancer

Gian Luca Rampioni Vinciguerra, Alessandra Dall'Acqua, Ilenia Segatto, Maria Chiara Mattevi, Francesca Russo, Andrea Favero, Roberto Cirombella, Giorgia Mungo, Davide Viotto, Javad Karimbayli, Margherita Pesce, Andrea Vecchione, Barbara Belletti, and Gustavo Baldassarre.

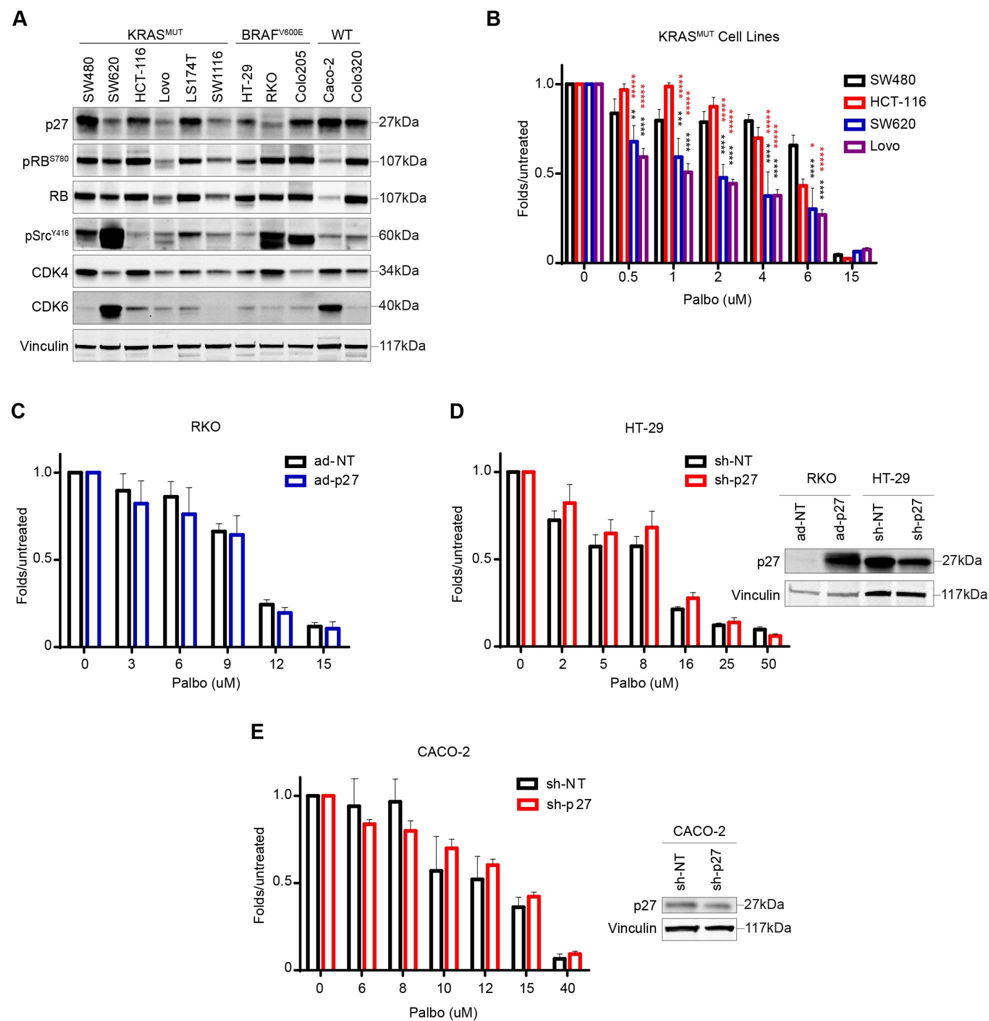
CONTENTS:

Supplementary Figures 1 – 7 Page 2 - 8

Supplementary Materials and Methods Page 9 - 18

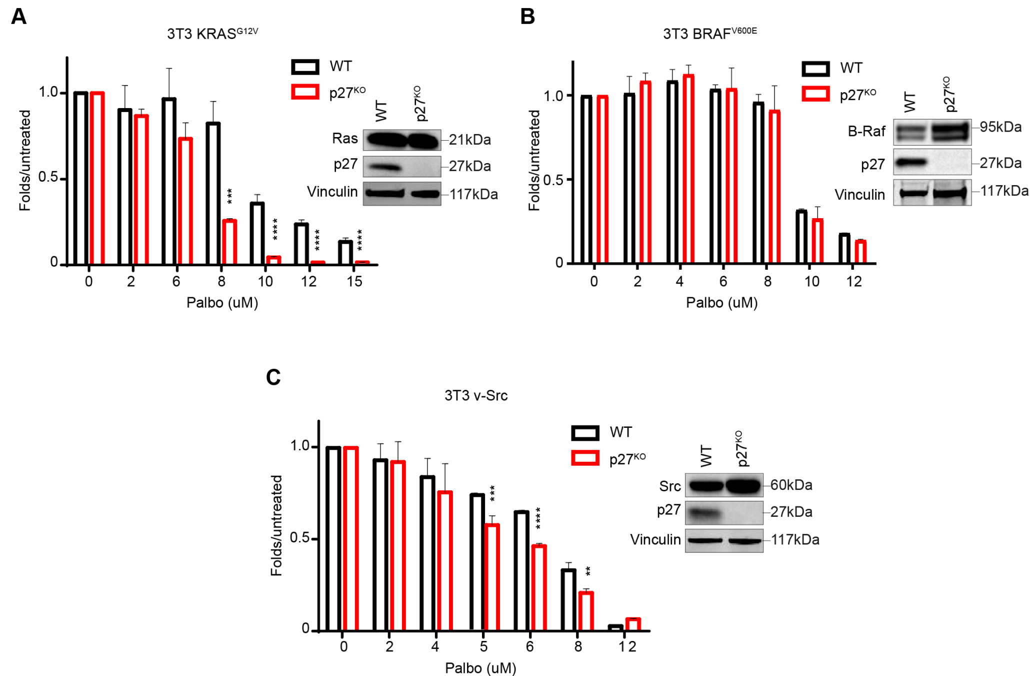
Supplementary References Page 18

SUPPLEMENTARY FIGURE LEGENDS

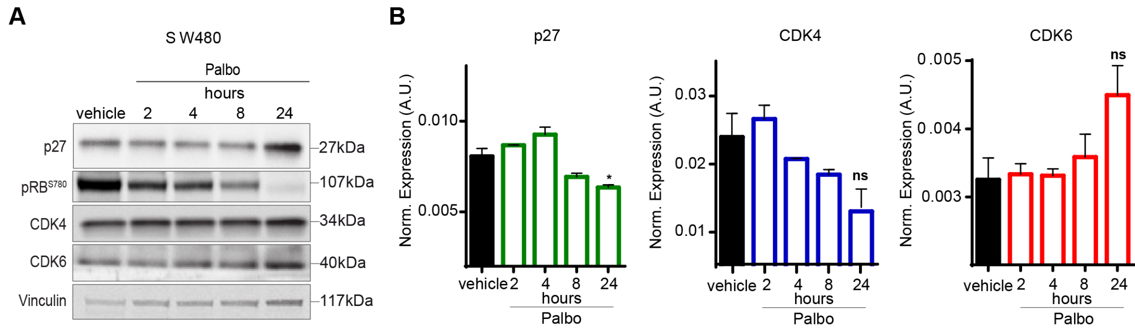


Supplementary Figure 1. p27 modulates Palbo response in KRAS^{MUT} CRC cell lines. A, Western blot analysis evaluating the expression of the indicated proteins in the indicated CRC cell lines grouped according to KRAS and BRAF mutational status. Vinculin was used as loading control. **B,** Dose-response curve evaluating Palbo sensitivity in cell lines with high (SW480, HCT-116) or low p27 expression (SW620, Lovo). Black and red asterisks indicate statistical significance referred to SW480 or HCT-116, respectively. **C**, Dose-response curve of KRAS^{WT}/BRAF^{V600E} RKO cells transduced with control (ad-NT) or p27 (ad-p27) expressing adenoviral particles, treated for 72 hours with increasing doses of Palbo, as indicated. **D**, Left: Dose-response curve of KRAS^{WT}/BRAF^{V600E} HT-29 cells control (sh-NT) and silenced for p27 (sh-p27), treated for 72 hours with increasing dose of Palbo, as indicated. Right: WB analysis of p27 in lysates from cells used for the dose-response curve of **C** and **D**. **E**, Left: Dose-response curve of KRAS^{WT}/BRAF^{WT} Caco-2 cells control (sh-

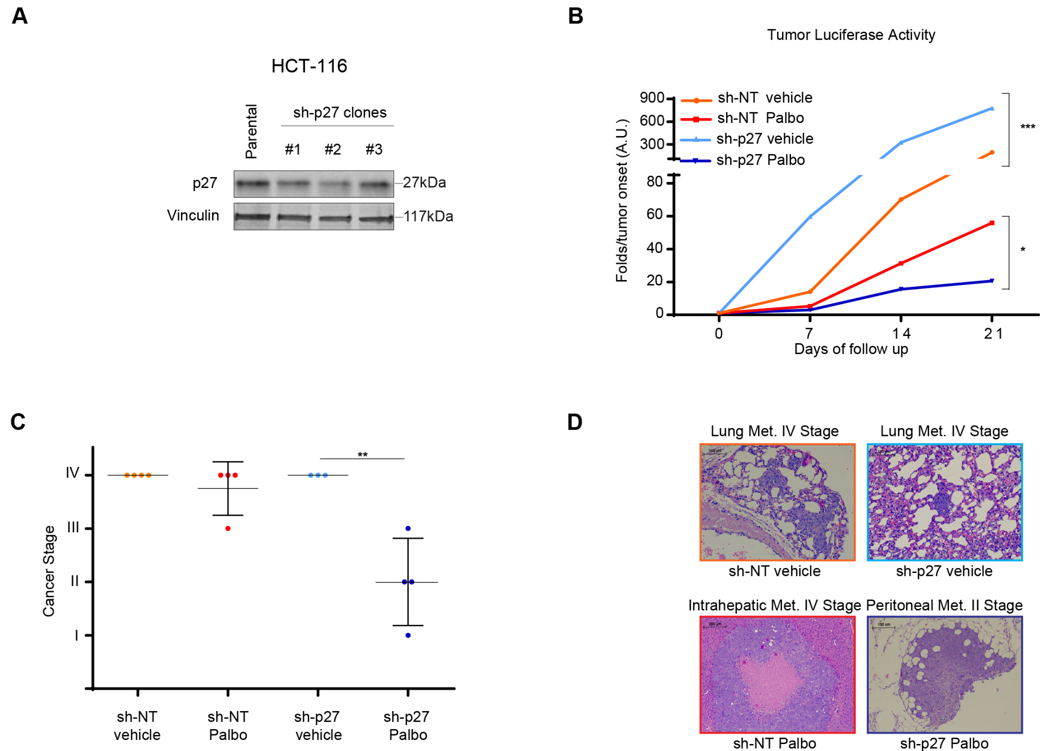
NT) and silenced for p27 (sh-p27), treated for 72 hours with increasing doses of Palbo, as indicated. Right: Western blot analysis of p27 in the same cells used for the dose-response curve. In D and E vinculin was used as loading control. In all dose-response curves, cell viability was measured by MTS assay and data show the percentage of viable treated cells compared to untreated cells. Data are the mean of at least n=3 independent experiments and Student's t-test was used to calculate statistical analysis. Asterisks indicate significant differences. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



Supplementary Figure 2. p27 affects Palbo response in transformed 3T3 cells. A-C, Left: Dose-response curves of 3T3 cells WT or KO for p27 ($p27^{KO}$), stably expressing $KRAS^{G12V}$ (A), $BRAF^{V600E}$ (B) or v-Src (C), treated for 48 hours with increasing doses of Palbo, as indicated. Right: Representative Western blot (WB) analysis of p27 and each oncogene in the cells used in each dose-response curve. Vinculin was used as loading control. In all dose-response curves, cell viability was measured by MTS assay and data show the percentage of viable treated cells compared to untreated cells. Data are the mean of at least n=3 independent experiments and Student's t-test was used to calculate statistical analysis. Asterisks indicate significant differences. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

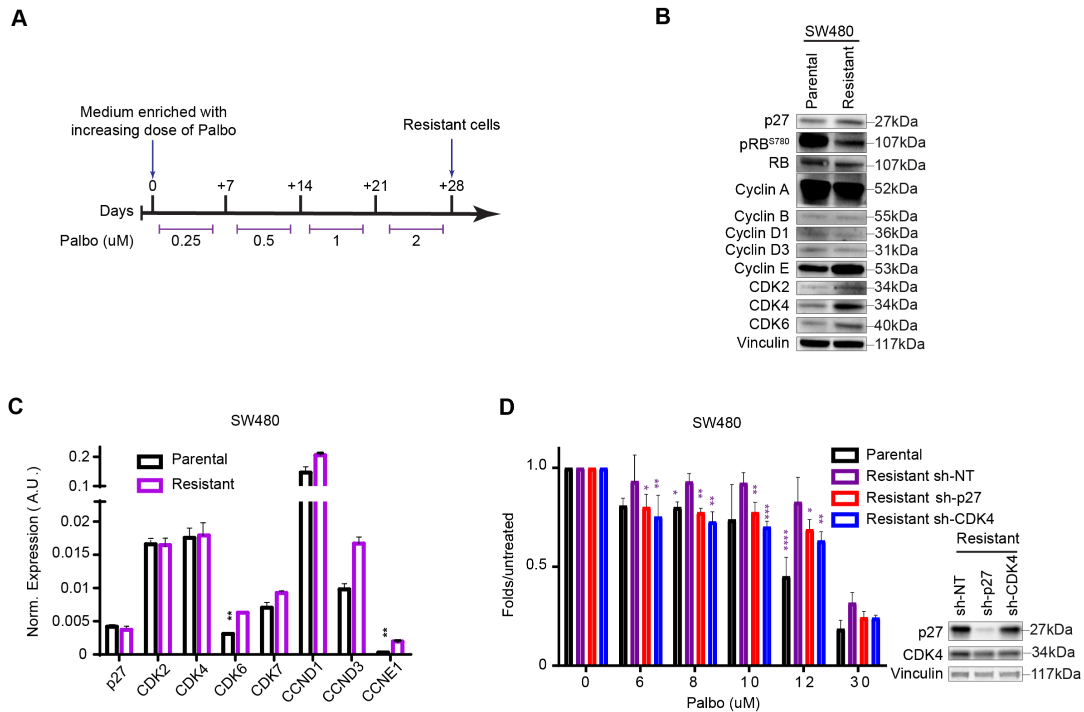


Supplementary Figure 3. Palbo treatment induces an increase of p27. A, Western Blot analysis of the indicated proteins in lysates from SW480 parental cells treated with vehicle or 2μM Palbo for the indicated times. Vinculin was used as loading control. **B,** Graphs report the normalized expression of p27, CDK4 and CDK6 mRNA evaluated by qRT-PCR analysis in SW480 parental cells treated or not with Palbo, as described in A. Data represent the normalized (to ACTB) mean value (±SD) of three different experiments performed in duplicate and are expressed as arbitrary units (AU). Statistical significance is calculated with Student's t-test and expressed as not-significant (ns) or * $p < 0.05$.

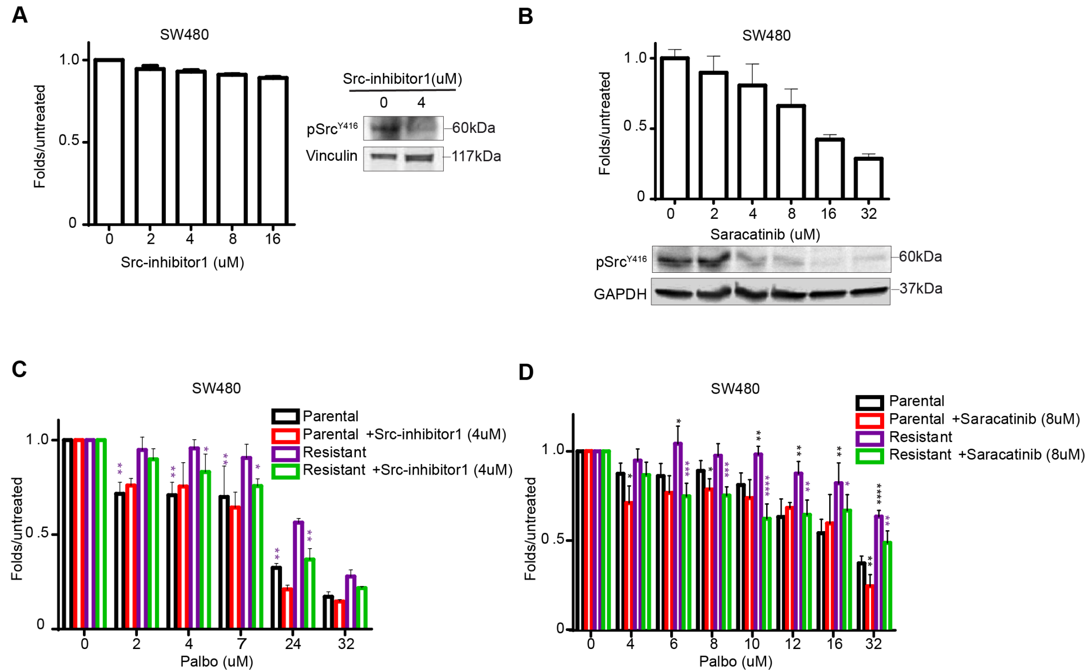


Supplementary Figure 4. ***In vivo*****, Palbo treatment induces tumor downstaging in p27-silenced CRC. A,** Western blot analysis of p27 in lysates from HCT-116 parental cells and clones stably silenced for p27 (sh-p27). Clone#2 was chosen for the *in vivo* experiments. Vinculin was used as loading control. **B,** Graph reports the luciferase activity in control and p27-silenced tumors in mice treated with vehicle or Palbo at different days of follow-up as described in **Figure 3A**. Data represent the mean of Luc activity in each group (n=3 or 4) folded on the one recorded at tumor onset. **C**, Dot plot reports tumor staging of CRC in mice evaluated as reported by others[39], indicating the tumor spreading from the bowel (I Stage) to the peritoneum (II Stage), to mesenteric lymph node or pancreatic foci (III Stage) and the involvement of intrahepatic or lung metastasis (IV Stage). Each dot represents a different mouse from the indicated groups. **D**, Representative images of H&E analysis showing common findings of metastatic distant organs for each mice group as described in **C.**

In all graphs, Student's t-test was used to calculate statistical analysis and asterisks indicate significant differences. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

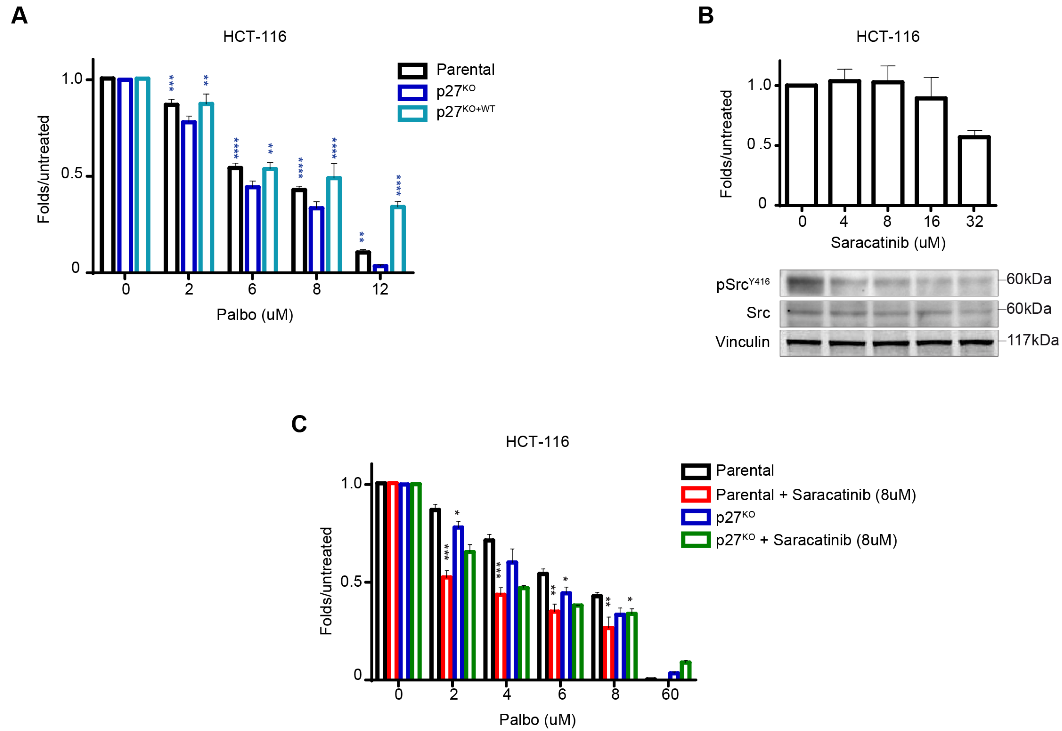


Supplementary Figure 5. p27/CDK4 interaction mediates Palbo-resistance. A, Schematic representation of the experimental workflow used to induce Palbo-resistance in SW480 cells. **B**, Western blot analysis evaluating the expression of the indicated proteins in lysates from parental and resistant SW480 cells. Vinculin was used as loading control. **C**, qRT-PCR analysis of a panel of cell cycle related genes in SW480 parental and resistant cells. Data represent the normalized (to ACTB) mean value (±SD) of three different experiments performed in duplicate and are expressed as arbitrary units (AU), statistical significance is calculated using Student's t-test (** p<0.01). **D**, left: Dose-response curve of SW480 parental and resistant cells transduced with control sh RNAs (sh-NT) or with shRNAs specific for p27 (sh-p27) or CDK4 (sh-CDK4) and then treated for 72 hours with increasing doses of Palbo, as indicated. Cell viability was measured by MTS assay and data are the mean of three experiment and report the percentage of viable cells compared to untreated cells. Asterisks indicate the statistical significance respect to the resistant cells, calculated using the Student's t-test. * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001. Right: Western blot analysis of indicated proteins of lysates from the same cells used for the dose-response curve. Vinculin was used as loading control.



Supplementary Figure 6. Targeting Src restores Palbo-sensitivity in resistant cells. A, Left: Dose-response curve of SW480 cells treated for 72 hours with increasing doses of Src-inhibitor1, as indicated. Right: Western blot analysis evaluating of $pSrc^{Y416}$ expression in SW480 cells untreated and treated with 4µM of Src-inhibitor1. Vinculin was used as loading control. **B,** Dose-response curve of SW480 cells treated for 72 hours with increasing doses of Saracatinib, as indicated. On the bottom**,** representative Western blot analysis evaluating of $pSrc^{Y416}$ expression in SW480 cells lysates from the same experiment. GAPDH was used as loading control. **C,** Dose-response curve of SW480 parental and resistant cells treated for 72 hours with increasing doses of Palbo in combination or not with Src-inhibitor1 used at the fixed dose of 4µM, as indicated. **D**, Dose-response curve of parental and resistant SW480 cells treated for 72 hours with increasing doses of Palbo, in combination or not with Saracatinib 8µM, as indicated. In **C** and **D**, Black and purple asterisks indicate statistical significance referred to parental or resistant cells, respectively.

In all dose-response curves, cell viability was measured by MTS assay and data report the percentage of viable treated cells compared to untreated cells. Data are the mean of three experiments and Student's t-test was used for statistical analysis. Asterisks indicate significant differences. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



Supplementary Figure 7. Src-inhibition does not enhance Palbo-sensitivity in p27^{KO} cells. A, Dose-response curve of HCT-116 cells parental and p27^{KO} transduced with adenoviral particles expressing p27 (p27^{KO+WT}) or not (p27^{KO}), treated for 72 hours with increasing dose of Palbo, as indicated. Blue asterisks indicate the statistical significance respect to p27 ^{KO} cells. **B,** Top: Dose-response curve of HCT-116 cells treated for 72 hours with increasing dose of Saracatinib, as indicated. Bottom: Western blot analysis of Src and pSrc^{Y416} in HCT-116 cells lysates from the same experiment. Vinculin was used as loading control.

C, Dose-response curve of HCT-116 parental and p27^{KO} cells treated for 72 hours with increasing doses of Palbociclib in combination or not with Saracatinib 8μM, as indicated. Asterisks indicate the statistical significance respect to the parental cells. * p <0.05; ** p <0.01; *** p <0.001.

1 **MATERIALS AND METHODS**

2

3 **Histological analysis and Immunofluorescence**

4 Histological sections (4 µm thick) were cut from the paraffin blocks, deparaffinized
5 with xylene, and stained with hematoxylin and eosin, according to standard
6 procedures. For immunohistochemical analysis, histological sections were
7 deparaffinized and rehydrated through serial ethanol treatments. Slides were
8 immersed in EnVision flex target retrieval solution high pH (Agilent) to enhance
9 antigen retrieval. Endogenous peroxidase was blocked with 3% hydrogen peroxide
10 for 15 minutes (min). Detection of p27 was performed with primary antibody clone
11 57 (cat. # 556049, BD Transduction Laboratories, dilution, 1:100); Src with primary
12 antibody clone 36D10 (cat. #2109, Cell Signaling Technology, dilution 1:300);
13 $pSrc^{Y416}$ with primary antibody clone D49G4 (cat. #6943, Cell Signaling
14 Technology, dilution 1:300), followed by Envision FLEX/HRP (Agilent) according
15 to manufacturer's protocol and standard procedures.

16 For Immunofluorescence analyses on cultured cells, cells were plated on coverslips,
17 fixed 20 min in 4% paraformaldehyde (PFA) permeabilized with 0.2% Triton X-100
18 (Sigma-Aldrich) for 5 min and then incubated with primary antibodies, as reported
19 above and previously described [36]. Briefly, incubation with primary antibodies was
20 performed over-night (ON) at 4°C, followed by 1 hour (h) at room-temperature (RT)
21 with secondary antibody (AlexaFluor® 488-, 546-, 568- or 633-conjugated,
22 Invitrogen). Nuclei were counterstained using TO-PRO-3 (Invitrogen) for 30 min at
23 RT. Staining for F-Actin (AlexaFluor® 546 Phalloidin Invitrogen) was performed
24 1h at RT after the incubation with secondary antibodies. Primary antibody used was:
25 p27 (BD Transduction Laboratories # 556049). Samples were analyzed using a

confocal laser-scanning microscope (TSP8, Leica) interfaced with a Leica fluorescent microscope. Collected images were analyzed and p27 expression was quantified using the LAS (Leica) and the Volocity® (PerkinElmer) softwares.

Cell culture and generation of Palbociclib resistant cells

Caco-2, HCT116, HCT-116-Luc2, HT29, Lovo, RKO, SW480 and SW620 colorectal cancer cell lines were obtained from the American Type Culture Collection (ATCC) and were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Carlo Erba) and 1% penicillin and streptomycin (PS, Lonza).

SW480 Palbociclib resistant cells were generated by continuous treatment with increasing dose (from 0.1 to 2 μM) of Palbociclib (PD-0332991 hydrochloride, Clinisciences) for 4 weeks and isolating a resistant population that was then maintained in medium containing 2μM Palbociclib, as described [14]. Resistant phenotype was confirmed by Kinase assay in presence of Palbociclib as described above.

3T3 WT or $p27^{KO}$ fibroblasts were obtained from primary mouse embryonic fibroblasts (MEF) following up the 3T3 immortalization protocol, as previously described [34,35] and cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Carlo Erba) and 1% PS (Lonza). 293-FT cells were obtained from Invitrogen (#R70007, Thermo Fisher Scientific), and maintained in DMEM high glucose (Sigma-Aldrich), supplemented with 10% FBS (Carlo Erba), 2 mM Non-Essential Amino Acids (NEAA, Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 2 mM Sodium Pyruvate, and 500 μg/ml G418 (Sigma-Aldrich). Cell lines were grown at

50 37 °C, under 5% CO2, in humidified incubators and routinely tested using
51 MycoAlert detection Kit (Lonza) for mycoplasma contamination. Only mycoplasma
52 negative cells were used for experiments. Human cell lines were authenticated by
53 STR analysis in 2018, according to PowerPlex® 16 HS System (Promega) protocol
54 and using GeneMapperTM software 5 (ThermoFisher) to identify DNA STR
55 profiles.

56

57 **Transfection and generation of stable clones**

58 293FT cells (Invitrogen, Thermo Fisher Scientific) were used for lentiviral
59 production and grown in DMEM supplemented with 10% FBS (Carlo Erba). p27
60 and CDK4 silenced cells were generated by lentiviral system. pLKO vectors
61 encoding for human shRNAs of the MISSiON system (pLKO lentiviral vector,
62 Sigma-Aldrich) were used as described elsewhere [14]. Briefly, 293FT cells were
63 transfected with Gag-Pol and VSV-G (Invitrogen recombinant lentivirus producing
64 system) plus pLKO shRNA (p27: TRCN0000039930 and TRCN0000356318;
65 CDK4: TRCN0000196698 TRCN0000010520) by standard calcium phosphate
66 transfection protocol (Promega). After 48 and 72 hrs, conditional medium
67 containing lentiviral particles was harvested and used to transduce target cells. HCT-
68 116-Luc sh-p27 clones were selected in complete medium supplemented with
69 1.5µg/ml puromycin.

70 3T3 fibroblasts transformed with K-Ras^{G12V}, pM-vSrc were described previously
71 [34,35], BRAF^{V600E} transformed cells were generated using the pBabe-BRAFV600E
72 plasmid obtained by Dr. W Han through Addgene and selected in puromycin for one
73 week. Selected clones were analysed for BRAF^{V600E} expression and used in dose

74 response curve. Recombinant adenoviruses for p27 overexpression ($p27^{WT}$, $p27^{CK-}$

75 and $p27^{1-170}$) were produced with the Adeno-X ViraTrak ZsGreen1 Expression

76 System 2 (Clontech), according provider's instructions. Adenovirus production,

77 titration and characterization were performed as previously described [50].

78

79 **Generation of stable $p27^{KO}$ cell clones**

80 To generate HCT-116 p27 KO clones, CRISPR/Cas-9 technology was used

81 essentially as described [51]. Briefly, First, using Cas-9 lentiviral particles (LV-

82 Ef1aCas9Blst vector, Sigma-Aldrich), stable pool of HCT-116 Cas-9 was produced.

83 Clones were selected in complete medium supplemented with Blasticidin 8 μM.

84 Stable HCT116 Cas-9 p27 KO pool was obtained by transduction with guide RNA

85 (gRNA) lentiviral particles (LV04U6-gRNA:hPGK-puro-2A-tBFP, Sigma-Aldrich).

86 The gRNA designed using Sigma-Aldrich tool to target CDKN1B was

87 GTCCCGGGTTAACTCTTCG. Clones were selected after 72 hrs from transduction

88 in complete medium supplemented with 1.5 μg/ml of puromycin. In order to analyze

89 the Cas9 activity pool, DNA was isolated with Maxwell® 16 Mouse Tail DNA kit in

90 Maxwell® 16 Instrument (Promega) to performed Next Generation Sequencing

91 (NGS) analysis (MySeq, Illumina). To obtained p27 KO HCT-116 clones single-cell

92 was seeded into 96-well plates. Selected, clones were analyzed by NGS and the

93 clones resulted KO were next confirmed by Western Blot analysis of p27

94 expression.

95

96 **Cell viability and kill curve**

97 For dose-response curve, colorectal cancer cells were transduced with lenti- or
98 adenoviral particles to obtain p27 silencing or overexpression and after 24 hours
99 (hrs) seeded in 96- well culture plates at concentration of $3x10^3$ cells/well.
100 Transformed 3T3 WT or $p27^{KO}$ cells were seeded at concentration of $1x10^3$-$3x10^3$,
101 depending on cell type.

102 After 24 hrs, cells were treated with increasing doses of Palbociclib (PD-0332991
103 hydrochloride Clinisciences), Saracatinib (AZD0530, SelleckChem) and SRC-
104 inhibitor-1 (S2075, Sigma-Aldrich) alone or in combination, as indicated.

105 Cell viability was determined at the end of treatment (48 or 72 hrs depending on the
106 experiment, as indicated) using the MTS assay (CellTiter 96 AQueous One Solution
107 Cell Proliferation-MTS-Assay, Promega) following manufacturer's instructions.

108

109 **Preparation of protein lysates and Western blot analysis**

110 Protein lysates and Western Blot were performed essentially as previously described
111 [34-36]. Proteins were separated in 4-20% SDS-PAGE (Criterion TGX Precast Gel,
112 Bio-Rad) and transferred to nitrocellulose membranes (GE Healthcare). Membranes
113 were blocked with 5% non-fat dried milk (NFDM) in TBS-0.1% Tween-20 or in
114 Odyssey Blocking Buffer (LI-COR, Biosciences) and incubated at 4°C overnight
115 (ON) with primary antibodies. Primary antibodies used were: p27 (BD Transduction
116 Laboratories #556049), p27 C-19 (Santa Cruz Biotechnology, #sc-528), CDK2
117 (Santa Cruz Biotechnology, #sc-6248), CDK4 (Santa Cruz Biotechnology, #sc-260),
118 CDK6 (Santa Cruz Biotechnology, #sc-53638), Cyclin A B8 (Santa Cruz
119 Biotechnology, #sc-271682), Cyclin B1 GNS1 (Santa Cruz Biotechnology, #sc-245)
120 Cyclin-D1 (Calbiochem, #CC12), Cyclin-D3 (Cell Signaling Technology, #2936),

121 Cyclin-E1 (Santa Cruz Biotechnology, #sc-481), pY (BD Transduction
122 Laboratories, #610000), pY1068 EGFR (Cell Signaling Technology, #3777), EGFR
123 (Santa Cruz Biotechnology, #sc-03), pY416 SRC (Cell Signaling Technology,
124 #2101), SRC (Santa Cruz Biotechnology, #sc-19), pS780 Rb (Santa Cruz
125 Biotechnology, #sc-12901), panRAS (Calbiochem, #OP40), B-RAF (Santa Cruz
126 Biotechnology, #sc-5284), PSF (Santa Cruz Biotechnology, #sc-101137), tubulin
127 (Sigma-Aldrich, #T5168), Vinculin (Santa Cruz Biotechnology, #sc-73614),
128 GAPDH (Cell Signaling Technology, #5174). p-p27^{Y88} was a kind gift from Ludger
129 Hengst (Medizinische Universitat Innsbruck, Innsbruck, Austria).

130 Following incubation with primary antibody, membranes were washed in TBS-0.1%
131 Tween 20 and incubated 1 hour (h) at room temperature (RT) with secondary
132 antibodies IRconjugated (Alexa Fluor 680, Invitrogen; IRDye 800, Rockland) for
133 infrared detection (Odyssey Infrared Detection System, LI-COR) or with the
134 horseradish peroxidaseconjugated secondary antibodies (GE Healthcare) for ECL
135 detection (Clarity Western ECL Substrate, Bio-Rad). The Re-Blot Plus Strong
136 Solution (Millipore) was used to strip the membranes, when re-blotting was needed.

137

138 **Quantitative Real-Time PCR (qRT-PCR)**

139 RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific)
140 following manufacturer's instructions. Total RNA was quantified using the
141 QuantiFluor RNA System (Promega). RNA was retro-transcribed (RT) using the
142 GoScript Reverse Transcriptase (Promega) and RT reactions were run in an Opticon
143 qRT-PCR Thermocycler (Bio-Rad). qRT–PCR analyses were performed essentially
144 as described [36]. cDNAs were amplified using SYBR green dye-containing reaction

145 buffer SSoFast (Bio-Rad) and the CFX384 Touch Real-time PCR Detection System
146 (Bio-Rad). Normalization of the data was performed using two different
147 housekeeping genes. The following primers (Sigma-Aldrich) were used:

148 **CDKN1B**: **FW** AGATGTCAAACGTGCGAGTG; **REV** TCTCTGCAGTGCTTCTCCAA.
149 **CDK2**: **FW** CCTCCTGGGCTGCAAATA; **REV** CAGAATCTCCAGGGAATAGGG.
150 **CDK4**: **FW** GTGCAGTCGGTGGTACCTG; **REV** TTCGCTTGTGTGGGTTAAAA.
151 **CDK6**: **FW** TGATCAACTAGGAAAAATCTTGGAC; **REV** GGCAACATCTCTAGGCCAGT
152 **CDK7**: **FW** CCATGTGCTCGAATTACGG; **REV** CTTGGCAGCTGACATCCAG.
153 **CCND1**: **FW** AGAAGGAGGTCCTGCCGTCC; **REV** GGTCCAGGTAGTTCATGGCC.
154 **CCND3**: **FW** GGAAGATGCTGGCTTACTGG; **REV** AGACAGGTAGCGATCCAGGT.
155 **CCNE1**: **FW** ACAGCTTGGATTTGCTGGAC; **REV** TCTTTGGTGGAGAAGGATGG.
156 **ACTB**: **FW** CCAGAGGCGTACAGGGATAG; **REV** CCAACCGCGAGAAGATGA.
157 Relative expression was calculated using the comparative Ct method.
158

159 **Immunoprecipitation and Kinase assay**

160 Immunoprecipitation (IP) experiments were performed using 0.7 mg of total lysate
161 with the primary antibody in HNTG buffer (20 mM HEPES, 150 mM NaCl, 10%
162 Glycerol, 0.1% Triton X-100) supplemented with 1 mM Na3VO4, 10 mM NaF
163 (Sigma-Aldrich), 1 mM DTT (Sigma-Aldrich), gently rocking ON at 4°C. Protein A
164 or Protein G Sepharose™ 4 Fast Flow (GE Healthcare), was added during the last 2
165 hrs of incubation. For p-p27^{Y88}, antibody was previously mixed to Protein G
166 Sepharose™, gently rocking ON at 4°C, to allow the conjugation and then added to
167 total lysates as described. IPs were then washed six times in HNTG buffer and
168 resuspended in 3X Laemmli sample buffer (5X Laemmli buffer composition: 50
169 mM TrisHCl pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue and 125 mM

170 beta-mercaptoethanol). Immunoblot analysis was performed as described above. For
171 kinase assay, cell lysates were immunoprecipitated using anti-CDK2 or control
172 antibody, as described above. After 7 washes in HNTG buffer, two tenth of the IP
173 was resuspended in kinase buffer solution (20 mM TrisHCl pH 6.8, 10 mM $MgCl_2$).
174 Then, a kinase reaction solution containing the sample plus 50 μM ATP, 1μCi of γ-
175 P^{32} ATP and specific substrate (1 μg of H1-Histone for CDK2) in kinase buffer
176 solution was prepared. The reaction was carried out at 30°C for 30 min and then 5X
177 Laemmli sample buffer and 50 mM of DTT was added. After denaturation at 95 °C
178 for 10 min, proteins were loaded on a 4–20% SDS-PAGE. The gel was then dried at
179 80°C for 3 hours under vacuum and exposed on an autoradiographic film (GE,
180 Amersham-Hyperfilm MP) at RT and developed after different time intervals.

181 **Animal experimentation**

182 Animal experimentation was reviewed and approved by the Centro di Riferimento
183 Oncologico di Aviano (CRO) Institutional Animal Care and Use Committee
184 (OPBA). All animal experiments were conducted in adherence to international and
185 institutional committees' ethical guidelines. NOD.CB17-PrkdcSCID (NSG) male
186 mice (Charles River Laboratories, 4 to 6 weeks old, n=47) were xenografted with
187 1×10^6 HCT-116-Luc2 (Caliper) cells either stably silenced (n=7) and not for p27
188 (n=8), or wild-type (n=16) and $p27^{KO}$ (n=16), as described above. Cells were
189 resuspended in 50μl of red-phenol free RPMI and injected intra-cecum under
190 anesthesia, as described by others [39]. To establish the tumor onset after intracecal
191 injection, luciferase activity was measured every 3 days by intraperitoneal injection
192 of luciferin (CycLucI, Merck #5306500001, 100mg/kg) in anesthetized mice using
193 IVIS Spectrum In vivo imaging system by PerkinElmer.

194 After tumor onset, in Palbociclib experiment, sh-NT and sh-p27 injected animals
195 were randomly divided into two groups (4 mice/group; 3 mice/group for shp27
196 control group) and treated with Palbociclib (PD-0332991 hydrochloride,
197 Clinisciences, 100 mg/kg) or with vehicle (Ringer's Lactate solution) 5 days/week
198 for 4 weeks by oral gavage.

199 In Palbociclib/Saracatinib experiment, wild-type and $p27^{KO}$ injected animals were
200 randomly divided into four groups (4 mice/group; 6 mice for wild-type control
201 group, 3 mice for wild-type Palbociclib and Saracatinib groups) and treated with
202 vehicle (Ringer's Lactate solution) or with Palbociclib (PD-0332991 hydrochloride,
203 Clinisciences, 100mg/kg), or with Saracatinib (Selleckchem #S1006, AZD0530,
204 25mg/kg) or a combination of Palbociclib and Saracatinig, 5days/week for 4 weeks
205 by oral gavage.

206 Luciferase activity was measured every week to monitor tumor growth. At the time
207 of sacrifice, all the organs collected were formalin fixed and processed for H&E
208 staining.

209

210 **Statistics and data reproducibility**

211 Statistical significance, means, median, standard deviation were determined by
212 using GraphPad PRISM software (version 6.01), using the most appropriate test, as
213 specified in each figure. A minimum of three biologically independent samples was
214 used for statistical significance. The number and type of replicates used in each
215 experiment is specified in the figure legend. When not otherwise specified, mean
216 and standard deviation are shown in all graphs. Significance was calculated by

217 Student's t-test, Fisher's exact test or Mann-Whitney two-sided test, as appropriate,
218 and indicated by a P<0.05.

219

220 **SUPPLEMENTARY REFERENCES**

221 14 Haines E, Chen T, Kommajosyula N, Chen Z, Herter-Sprie GS, Cornell L et
222 al. Palbociclib resistance confers dependence on an FGFR-MAP kinase-mTOR-
223 driven pathway in KRAS-mutant non-small cell lung cancer. Oncotarget 2018; 9:
224 31572–31589.

225 34 Fabris L, Berton S, Pellizzari I, Segatto I, D'Andrea S, Armenia J et al.
226 p27kip1 controls H-Ras/MAPK activation and cell cycle entry via modulation of
227 MT stability. Proc Natl Acad Sci USA 2015; 112: 13916–13921.

228 35 Pellizzari I, Fabris L, Berton S, Segatto I, Citron F, D'Andrea S et al.
229 p27kip1 expression limits H-Ras-driven transformation and tumorigenesis by both
230 canonical and non-canonical mechanisms. Oncotarget 2016; 7: 64560–64574.

231 36 Dall'Acqua A, Sonego M, Pellizzari I, Pellarin I, Canzonieri V, D'Andrea S
232 et al. CDK6 protects epithelial ovarian cancer from platinum-induced death via
233 FOXO3 regulation. EMBO Mol Med 2017; 9: 1415–1433.

234 50 Schiappacassi M, Lovisa S, Lovat F, Fabris L, Colombatti A, Belletti B, *et*
235 *al* (2011) Role of T198 modification in the regulation of p27(Kip1) protein stability
236 and function. PLoS One. 6(3):e17673.

237 51 Sonego M, Pellarin I, Costa A, Vinciguerra GLR, Coan M, Kraut A, *et al*
238 (2019) USP1 links platinum resistance to cancer cell dissemination by regulating
239 Snail stability. Sci Adv 5(5):eaav3235