Supplementary Methods

CDK4/6 and IGF1 receptor inhibitors synergize to suppress the growth of p16INK4A-deficient pancreatic cancers

Andreas M. Heilmann, Rushika M. Perera, Veronika Ecker, Brandon N. Nicolay, Nabeel Bardeesy, Cyril H. Benes, and Nicholas J. Dyson

Cell cycle analysis

Cells were labeled for 1 h with Cell Proliferation Labeling Reagent (Amersham) containing BrdU, which was diluted 1:400 in existing cell culture medium, harvested using trypsin, and fixed with 75% ethanol in phosphate-buffered saline (PBS, Gibco) overnight at -20 °C. DNA was denatured for 30 min at room temperature with 2 N hydrochloric acid, which was neutralized with 0.1 M borate buffer (pH 8.5). BrdU was detected with mouse BrdU antibody (BD Biosciences, clone B44, 1:25) and AlexaFluor® 647 anti-mouse secondary antibody (Molecular Probes, 1:200). DNA was counter-stained with 10 µg/mL propidium iodide (PI) in the presence of 50 µg/mL ribonuclease (Qiagen). Data acquisition and analysis were performed using an LSRFortessa cell analyzer with FACSDiva 6.1.3 software (BD Biosciences).

Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase (SA-β-gal) was detected with a SA-β-gal staining Kit (Cell Signaling Technology) according to the manufacturer's instructions. Pictures were taken on a Nikon Diaphot microscope (Nikon) using a QICAM Fast1394 camera and QCapture software (QImaging). In order to quantify the percentage of SA-β-

gal positive cells, at least 100 cells in two randomly selected fields from two independent replicate experiments each were scored.

Trimethylated Lysine 9 Histone H3 (H3K9me3) staining

Cryosections of the xenograft tumors were stained with anti-H3K9me3 antibody (Millipore, 07-442), followed by secondary antibody (Alexa 568 donkey-anti rabbit, Invitrogen, A10042) at the end point of the treatment study. Sections were counterstained with DAPI and observed with a Nikon Eclipse 90i microscope.

Population doubling assay

Cells were seeded at 100000 cells per well into 6-well plates one day prior to treatment with BMS-754807, PD-0332991, their combination or DMSO. Every four days, live cells as determined by Trypan blue exclusion were counted and reseeded at 100000 cells per well in drug-containing medium.

RNA interference

For transient knockdown experiments, cells were seeded into 12-well plates at 100000 cells per well one day prior to transfection with ON-TARGETplus siRNA SMARTpools against human CCND1, CDK4, CDK6, TSC2 or scrambled (siscbl) control siRNA SMARTpools (Thermo Scientific). The siRNA pools were transfected at a concentration of 50 nM using Lipofectamine® RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Two days later, the cells were seeded into 96-well plates for sensitivity assays or 6-well plates for signaling analysis. MIA-PaCa-2 and HuP-T3 cells stably expressing non-silencing (shscbl) or two different RB1-targeting short hairpin shRNAs (shRB1-1, shRB1-2) were generated by lentiviral transduction with LKO.1 vectors containing the following oligonucleotide sequences:

CCACATTATTTCTAGTCCAAA (shRB1-1, cloneID TRCN0000040163), GACTTCTACTCGAACACGAAT (shRB1-2, cloneID TRCN0000010418).

Western blotting

Western blotting was performed with standard methods. Cells treated as indicated were rinsed with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% [v/v] Igepal CA-630, 0.1% [w/v] sodium dodecyl sulfate (SDS), 0.5% [v/v] sodium deoxycholate) containing EDTA-free Complete Protease inhibitors (Roche) and PhosSTOP phosphatase inhibitors (Roche), sonicated and cleared by centrifugation at 15,000 × g for 10 min. Tumor tissue that had been shock frozen in liquid nitrogen was lysed directly in RIPA and treated as described for cell lysates. Protein concentrations were determined by Bio-Rad Protein Assay (BioRad) and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, blotted onto polyvinylidene difluoride membrane (Immobilon-P, Millipore), and probed according to the antibody manufacturer's specifications. The following antibodies were purchased from Cell Signaling (catalog number) and used at 1:1000 dilution unless otherwise stated: P-pRB S807/811 (9308), pRB (9309), P-p70S6K1 T389 (9206), p70S6K1 (9202), P-AKT S473 (4060), AKT (9272), P-4EBP1 T37/T46 (2855), 4EBP1 (9644), P-ERK1/2 T202/T204 (4370), ERK1/2 (4695), P-IGF1Rβ Y1135/6/ IRβ Y1150/1 (3024), TSC2 (4308), CDK4 (2906, 1:2000), and CDK6 (3136, 1:2000). Tubulin antibody was purchased from Sigma (clone DM1A, 1:10000), IGF1Rβ antibody was from Santa Cruz Biotechnologies (sc-713, 1:200) and Cyclin D1 antibody was obtained from Abcam (clone SP4, 1:400).