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MEK plus PI3K/mTORC1/2 Therapeutic Efficacy Is Impacted by TP53 Mutation in Preclinical Models of Colorectal Cancer

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Abstract

Purpose: PI3K pathway activation occurs in concomitance with RAS/BRAF mutations in colorectal cancer, limiting the sensitivity to targeted therapies. Several clinical studies are being conducted to test the tolerability and clinical activity of dual MEK and PI3K pathway blockade in solid tumors.

Experimental Design: In the present study, we explored the efficacy of dual pathway blockade in colorectal cancer preclinical models harboring concomitant activation of the ERK and PI3K pathways. Moreover, we investigated if TP53 mutation affects the response to this therapy.

Results: Dual MEK and mTORC1/2 blockade resulted in synergistic antiproliferative effects in cell lines bearing alterations in KRAS/BRAF and PIK3CA/PTEN. Although the on-treatment cell-cycle effects were not affected by the TP53 status, a marked proapoptotic response to therapy was observed exclusively in wild-type TP53 colorectal cancer models. We further interrogated two independent panels of KRAS/BRAF- andPIK3CA/PTEN-altered cell line– and patient-derived tumor xenografts for the antitumor response toward this combination of agents. A combination response that resulted in substantial antitumor activity was exclusively observed among the wild-type TP53 models (two out of five, 40%), but there was no such response across the eight mutant TP53models (0%). Interestingly, within a cohort of 14 patients with colorectal cancer treated with these agents for their metastatic disease, two patients with long-lasting responses (32 weeks) had TP53 wild-type tumors.

Conclusions: Our data support that, in wild-type TP53 colorectal cancer cells with ERK and PI3K pathway alterations, MEK blockade results in potent p21 induction, preventing apoptosis to occur. In turn, mTORC1/2 inhibition blocks MEK inhibitor–mediated p21 induction, unleashing apoptosis. Clin Cancer Res; 21(24); 5499–510. ©2015 AACR.

Translational Relevance

The utmost objective of targeted therapies is to achieve selective cancer cell death by specifically targeting the driver oncogenes. Equally important is the identification of potentially responding patients by means of biomarkers, in order to avoid overtreatment and to maximize response rate. A significant proportion of patients with colorectal cancer carry mutations in both the ERK (Ras/Raf) and PI3K pathways and are therefore potentially susceptible to dual pathway blockade. Nonetheless, half of the tumors harboring alterations in KRAS/BRAF and PIK3CA/PTEN have a TP53 mutation along. In this work, we show that mutant TP53 tumor cells are unable to engage apoptosis upon MEK plus mTORC1/2 blockade. Our results are of interest for the design of future cancer therapies targeting the ERK/MEK and PI3K/mTORC1/2 signaling cascades in colorectal cancer.

Introduction

Metastatic colorectal cancer (mCRC) is the third leading cause of cancer-related deaths worldwide. Currently, the standard of care for mCRC is chemotherapy (fluoropyrimidin and oxaliplatin/irinotecan combinations). Target-specific agents against the EGFR—cetuximab and panitumumab—or against the VEGF pathway—bevacizumab and aflibercept—as well as the multityrosine kinase inhibitor regorafenib, are also approved for the treatment of mCRC (1–3). However, the efficacy of anti-EGFR compounds is often limited by mutations that activate downstream signaling pathways, rendering targeted therapy ineffective (4–6).

The landscape of somatic mutations in colorectal cancer has been defined during the last years (7). According to The Cancer Genome Atlas Network (8), 42% of primary colorectal cancers display activating mutations of the KRAS oncogene, 10% in BRAF and an additional 10% in NRAS, being typically mutually exclusive mutations. Mutations in this pathway result in hyperactivation of the mitogen-activated protein kinase kinase 1 (MAP2K1 or MEK1) and the downstream mitogen-activated protein kinases p42/p44 (p42/p44 MAPK or ERKs; ref. 9). Activation of the phosphatidylinositol-3-kinase/Akt/mammalian target of the rapamycin (PI3K/Akt/mTOR) pathway occurs in 22% of all the primary colorectal cancer either as mutation in PIK3CA (the gene encoding for the catalytic subunit of PI3K, p110α) or by mutation/homozygous deletion of the phosphatase and tensin homolog PTEN (encoding for the phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase; ref. 8), resulting in activation of downstream targets, such as Akt and mTOR. As a whole, mutations in RAS/BRAF and PIK3CA/PTEN frequently coexist, resulting in activation of both cascades (10). Activating mutations in either or both pathways confer resistance to EGFR-targeting therapies (5, 11–13), providing a rationale for dual MEK and PI3K pathway blockade in mCRC. Mutations in TP53, the gene encoding for p53, are frequent within the colorectal cancer tumors bearing mutations in the ERK and PI3K pathways, accounting for 46% of this subset (8).

Combined inhibition of the MEK and PI3K/Akt/mTOR pathways has been preclinically tested in a variety of cancer models, such as lung (14), pancreatic (15), breast (16, 17), and colorectal cancers (18). The tolerability and preliminary efficacy of anti-MEK and anti-PI3K/Akt/mTOR complex 1/2 (mTORC1/2) therapy is being evaluated in several clinical trials, including patients with advanced colorectal cancer (19, 20). The overlapping/synergistic toxicities observed by dual targeting have hampered achieving full maximal tolerated doses of the respective single agents, therefore compromising pathway blockade (19, 21, 22). As a consequence, there is a need to identify which patients are more likely to benefit from these regimens, and to improve the therapeutic schedules to maximize exposure reducing side effects. In the present study, we elucidate the impact of TP53 mutation on the antitumor activity of combined MEK and PI3K/mTORC1/2 inhibition in colorectal cancers harboring concomitant mutations in both signaling pathways.

Materials and Methods

Cell lines and reagents

All the colorectal cancer cell lines were obtained from the ATCC, with the exception of LIM2405, which was obtained from the Ludwig Institute for Cancer Research (Switzerland). All cell lines were authenticated using DNA profiling by the ATCC/Ludwig archive. DLD-1 was maintained in RPMI-1640 (Invitrogen), HT-29 and HCT116 in McCoy's 5A (Modified) Medium (Invitrogen), and SW948, RKO, and LIM2405 in DMEM, all were supplemented with 10% FBS and 2 mmol/L L-glutamine (Life Technologies) at 37°C in 5% CO_2. PD0325901 and MLN0128 were obtained from Takeda California. General laboratory supplies were acquired from Sigma-Aldrich, Invitrogen, or Merck.

Western blot analyses

Cells were grown in 60-mm dishes and treated with PD-0325901 (referred to as PD-901), MLN0128 (formerly known as INK-128), or a combination of both for the indicated concentrations and times. Cells were washed with ice-cold PBS and scraped into ice-cold lysis buffer [Tris–HCl pH 7.8 20 mmol/L, NaCl 137 mmol/L, EDTA pH 8.0 2 mmol/L, NP40 1%, glycerol 10%, supplemented with NaF 10 mmol/L, Leupeptin 10 μg/mL, Na_2VO_4 200 μmol/L, PMSF 5 mmol/L, and Aprotinin (Sigma-Aldrich)]. Lysates were cleared by centrifugation at 13,000 rpm for 10 minutes at 4°C, and supernatants removed and assayed for protein concentration using the Pierce BCA Protein Assay Kit (Thermo Scientific). Thirty micrograms of total lysate was resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were then hybridized using the following primary antibodies: pAkt (S473), Akt, pS6 (S240/244), pS6 (S235/236), p4EBP1 (S65), 4EBP1, pERK (T202/Y204), ERK, cleaved PARP, PARP, cleaved caspase-7 and p53 (Cell Signaling Technology), tubulin (Sigma-Aldrich), c-Myc (Santa Cruz Biotechnology), p21 (Neomarkers, ThermoFisher Scientific Inc.) in 5% BSA in Tris Buffered Saline (Tris 50 mmol/L, NaCl 150 mM) + 0.1% Tween 20 (TBST) (Sigma Aldrich) and GAPDH (Cell Signaling Technology) in 1% nonfat dry milk in TBST. Mouse and rabbit horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences) were used at 1:2,000 in TBS-T 1% nonfat dry milk. Protein–antibody complexes were detected by chemiluminescence with the Immobilon Western HRP Substrate (Millipore), and images were captured with a FUJIFILM LASS-3000 camera system.

Determination of inhibitory concentration 50 and combination index

Cells were seeded in 96-well plates and treated with 1:10 serial dilutions of PD-901 and MLN0128 within the 10 μmol/L to 1 pmol/L range as single agents or in 1:1 combinations. After 4 days of treatment, cell proliferation was analyzed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega) as described by the manufacturer. Proliferation curves were calculated using GraphPad Prism (GraphPad Software), and the combination index (CI) was determined using CompuSyn (ComboSyn Inc.; ref. 23). CI < 1 indicates synergism, CI = 1 indicates additive effect, and CI > 1 indicates antagonism. Experiments were performed in triplicate.

Determination of cell cycle and apoptosis

Cell cycle and hypodiploid (sub-G_1) cells were quantified by flow cytometry. Briefly, cells were washed with PBS, fixed in cold 70% ethanol, and then stained with propidium iodide while treating with RNase (Sigma-Aldrich). Quantitative analysis of sub-G_1 cells was carried out in a FACS calibur cytometer using the Cell Quest software (BD Biosciences). Annexin V–positive cells were quantified using the Guava Nexin Reagent (Millipore) according to the manufacturer's recommendations. Briefly, cells were harvested in 1% BSA/PBS and diluted 1:1 in the Guava Nexin Reagent. After 20 minutes at room temperature, cells were analyzed in the Guava System (Millipore).

RNA extraction and quantitative PCR (qPCR)

RNA was extracted using the PerfectPure RNA Tissue Kit (5Prime), according to manufacturer's instructions. QRT-PCR was performed using TaqMan probes (Applied Biosystems) according to the manufacturer's recommendations. Reactions were carried out in an ABI7000 sequence detector (Perkin Elmer), and results were expressed as fold change calculated by the ΔC_t method relative to the control sample. The β-glucuronidase gene GUSB mRNA was used as internal normalization control.

Overexpression of p53-R248W, p21, and RNA interference

p53-R248W was synthesized and cloned into a pBABE backbone vector (GenScript). The pINDUCER20-HA-p21[Cip1] plasmid was kindly provided by Stephen J. Elledge (Howard Hughes Medical Institute and Department of Genetics, Harvard Medical School, Boston, MA; ref. 24). siRNAs against p21 and nontargeting siRNAs control were synthesized by Sigma-Aldrich. Cells were transfected with the indicated siRNAs at 50 nmol/L during 24 hours using a DharmaFECT1 transfection agent (Dharmacon Research), as described by the manufacturer. The shTP53 construct was obtained from The RNAi Consortium (Broad Institute, MA).

Establishment of cell line–derived tumor xenografts in nude mice

Mice were maintained and treated in accordance with the institutional guidelines of the Vall d'Hebron University Hospital Care and Use Committee. Six-week-old female athymic nude HsdCpb:NMRI-Foxn1[nu] mice were purchased from Harlan Laboratories. Mice were housed in air-filtered laminar flow cabinets with a 12-hour light cycle and food and water ad libitum. HT-29, HCT116, or LIM2405 cells were resuspended in sterile PBS before subcutaneous injection at a final concentration of 2×10^6 cells/100 μL per mouse.

Establishment of colorectal cancer patient–derived xenografts

Human colon carcinoma models were used and grown as described by Puig and colleagues (25). Experiments were conducted following the European Union's animal care directive (86/609/EEC) and were approved by the Ethical Committee of Animal Experimentation of the Vall d'Hebron Research Institute and patient consent. Patient-derived xenograft (PDX) models from passages 5 to 8 were established by implantation of enzymatically digested surgical colon cancer biopsies in NOD/SCID mice (NOD.CB17-Prkdc[scid]/NcrCrl). PDXs recapitulated the same histopathologic and genetic features as the original patients' carcinomas. For drug-efficacy studies, a total of 1×10^5 patient-derived tumor cells were obtained from an established tumor, suspended in PBS 1:1 with Matrigel (BD Bioscience) and injected subcutaneously into both flanks of NOD/SCID mice, as previously described (26, 27). After 3 to 8 weeks, when palpable tumors matched 200 ± 50 mm³, treatments started and tumor size was evaluated twice weekly by caliper measurements.

PDXs from the Candiolo Cancer Institute (18) were established following procedures approved by the local Ethical Commission and by the Italian Ministry of Health. Briefly, 25-mm³ Matrigel-coated tumor material derived from liver metastectomies were implanted in the flank of NOD/SCID mice, as previously described (18). For treatment-efficacy experiments, established tumors (~400 mm³) were treated with 40 mg/kg of BEZ235 (Selleck Chemicals) or 25 mg/kg of AZD6244 (Sequoia Research Products). Tumor size was evaluated once weekly by caliper measurements.

In vivo treatment study

Animals were divided into four groups, consisting of 6 to 10 mice per group. Animals were treated with PD901 6 q.d./week (2 mg/kg in 5% NMP, 95% PEG in water, oral gavage) and/or MLN0128 6 q.d./week (0.3 mg/kg in 5% NMP, 15% PVP in water, oral gavage). Tumors were measured with digital calipers, and tumor volumes were determined using the formula (length × width²) × (π/6). At the end of the experiment, animals were euthanized using CO_2 inhalation. Tumor volumes are plotted as mean ± SE of 6 to 10 mice. For Western blot analysis, whole-protein lysates from two to four different tumors derived from each treatment were processed as described above.

Patient selection and genotyping of tumor samples

All patients with pathologically confirmed mCRC refractory to standard therapy referred to phase I clinical trials at the Molecular Therapeutic Research Unit of Vall d'Hebron Institute of Oncology had archived formalin-fixed paraffin-embedded (FFPE) tumor samples analyzed for targeted molecular aberrations: KRAS/NRAS/BRAF/PIK3CA mutations were identified using the TheraScreen molecular diagnostic assay (DxS) or the OncoCarta Panel v1.0 Sequenom MassARRAY, by the Pathology Service from the Vall d'Hebron University Hospital or the Cancer Genomics Group at Vall d'Hebron Institute of Oncology (VHIO), respectively. For three patients, KRAS was sequenced at the hospital of origin using the commercial PCR kit (DxS). PTEN expression by immunohistochemistry was determined by the

Molecular Oncology Group at VHIO. The three laboratories are UNE-ISO 15189 accredited (28). Patients' informed consent was obtained at baseline. In total, from 2011 to 2013, 14 patients with double pathway aberrations participated in early clinical trials, with MEK plus PI3K pathway inhibitors based on the results of tumor profiling as well as logistic factors, including study availability and eligibility criteria (19). Patients achieved clinical benefit if their cancer was controlled for a minimum of 16 weeks, the time point for the second response evaluation by computerized tomography (28). Tumor specimens from primary colorectal cancer or metastasis and from the established PDX were subjected to capture-based massive parallel sequencing (MiSeq; Illumina). Briefly, to avoid false negatives, only tumor samples with a tumor area above 30% were analyzed. DNA was extracted from 5 × 10 μm slices using the Maxwell FFPE Tissue LEV DNA Purification Kit (Promega). An initial multiplex-PCR with a proofreading polymerase was performed on samples. An in-house–developed panel of more than 600 primer pairs targeting frequent mutations in oncogenes plus several tumor suppressors, totaling 57 genes, was applied (Supplementary Table S1 and primer pairs available upon request). The panel includes the entire coding sequence of TP53 (NM_00546). Indexed libraries were pooled and loaded onto a MiSeq instrument, and paired-end 100-bp-read-length sequencing was performed (2 × 100 bp). Initial alignment was performed with the Burrows–Wheeler Aligner after primer sequence clipping, and variant calling was done with the Genome Analysis Toolkit Unified Genotyper and VarScan2 followed by ANNOVAR annotation. Mutations were called at a minimum 3% allele frequency. SNPs were filtered out with the SNP database (dbSNP; ref.29) and 1,000 genome datasets (30). All detected variants were manually inspected.

PDXs from the Candiolo Cancer Institute were genotyped as described (18), by capillary electrophoresis (3730 ABI Applied Biosystems) using exon-specific and sequencing primers, which were designed with Primer3 software.

Immunohistochemistry

Xenograft tumors were fixed immediately after excision in 10% buffered formalin solution for a maximum of 24 hours at room temperature before being dehydrated and paraffin-embedded under vacuum conditions (FFPE). Tissue microarrays (TMA) were constructed, including duplicate cores from each tumor. TMA slides underwent deparaffinization and antigen retrieval using PT Link system (DAKO) following manufacturer's instructions. Immunohistochemical staining against cleaved caspase-3 or p53 (DAKO) was performed as follows: 4-μm sections from FFPE material were deparaffinized and hydrated. Antigen retrieval was performed using a T/T Mega microwave system following manufacturer's instructions and DAKO reagents. After peroxide blocking, slides were incubated with primary antibody, secondary antibody, and developed with freshly prepared 0.05% 30,3-diaminobenzidine and counterstained with hematoxylin. Positive and negative controls were run along with the tested slides per each marker. Images were acquired using Aperio ImageScope software (Aperio), and a pathologist blinded to the identity of the samples quantified the percentage of positively stained cells.

p53 status

We analyzed the p53 status of tumor samples from archival tissue primarily upon the TP53mutation status (MiSeq). Only when tumor sample was scarce (tumor area <30%) or the coverage of the genomic assay was insufficient we used IHC to provide a surrogate ofTP53 mutation. Those tumors that markedly stained for nuclear p53 (≥50% of tumor cell positivity) were considered p53 mutant (31).

Statistical analysis

Two-way ANOVA with a Bonferroni post-test was performed using GraphPad Prism (GraphPad software). Error bars represent the SE. All experiments were repeated at least three times. A log-rank test is performed with the clinical data (R software).

Results

Combined MEK and mTORC1/2 inhibition synergistically suppresses colorectal cancer cell proliferation

To test the biochemical effects of dual MEK and mTORC1/2 blockade in vitro, we treated six colorectal cancer cell lines bearing concomitant alterations in KRAS/BRAF andPIK3CA/PTEN (Supplementary Table S2), three of which were also harboring TP53mutations, with the MEK inhibitor PD901, the catalytic mTOR inhibitor MLN0128, or the combination of both. After 24 hours of treatment, PD901 markedly suppressed ERK phosphorylation (Fig. 1A). Treatment with MLN0128 resulted in a reduction of p-Akt-S473 and downstream mTORC1 targets phospho-S6 ribosomal protein, p-S6-S240/244, and phospho-4E binding protein-1, p-4EBP1-S65, accompanied by minor increases in ERK activation. As expected, the combination of PD901 and MLN0128 suppressed both ERK and PI3K pathways in all the cell lines.
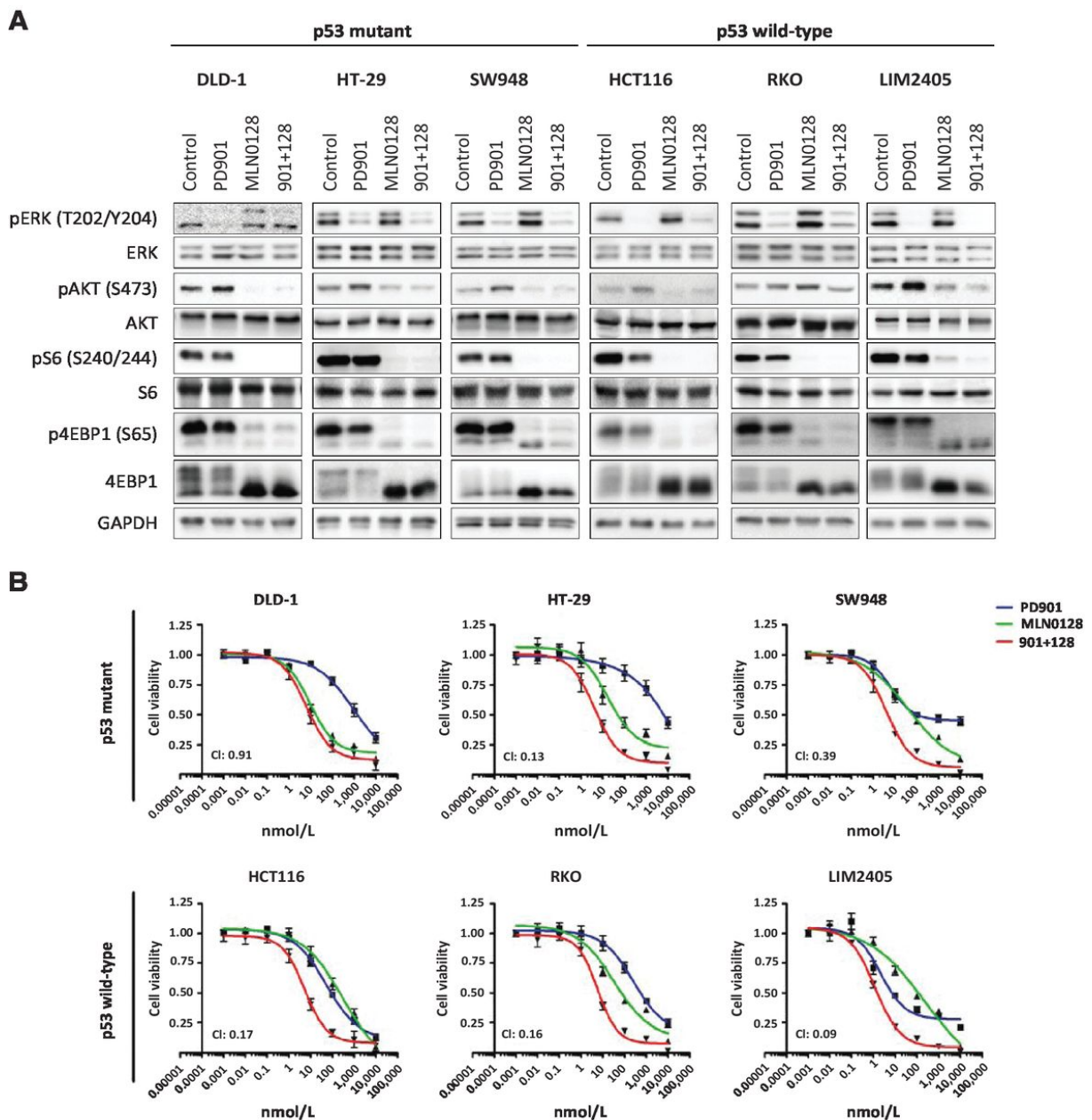

Figure 1.MEK and mTOR inhibition suppresses pathway activation and proliferation irrespective of p53 mutational status. A, the indicated colorectal cancer cell lines were treated with DMSO (control), 50 nmol/L PD901, 50 nmol/L MLN0128, or the combination of both inhibitors (901+128). Whole-cell protein extracts were analyzed by Western blot with the indicated antibodies. ERK and GAPDH protein levels were used as loading controls. Figures are representative of three independent experiments. B, the indicated

colorectal cancer cell lines were treated with 1:10 serial dilutions of PD901, MLN0128, or the combination. Proliferation was measured after 4 days of treatment with a cell viability assay (Cell Titer-Glo; Promega). Proliferation curves were graphed using GraphPad Prism software. Combination index (CI) was calculated using CompuSyn computer software (Fa = 0.5). Data, mean ± SE from three independent experiments.

The antiproliferative response of combined PD901 and MLN0128 treatment was synergistic (CI < 1) in five out of six cell lines, irrespective of the TP53 mutational status (Fig. 1B).

When examining the cell cycle, we found that the TP53 mutational status did not discriminate the combination response (% of S-phase; Supplementary Fig. S1A). Moreover, cell-cycle biomarkers were markedly reduced in all cell-cycle–sensitive cell lines, independently of the TP53 mutational status (see Supplementary Fig. S1B; transcription factor E2F1 and the phosphorylation levels of the retinoblastoma tumor suppressor, pRb). Thus, the presence of a TP53 mutation did not affect the antiproliferative activity of combined MEK and mTORC1/2 inhibition in colorectal cancer cells harboring activatingRAS/BRAF and PI3K pathway alterations.

Combined MEK and mTORC1/2 blockade promotes apoptosis in TP53 wild-type but not in TP53 mutant colorectal cancer cell lines via activation of BAX

We next investigated whether combined PD901 and MLN0128 induced apoptosis in our panel of colorectal cancer cells. We observed a clear-cut difference in terms of apoptosis induction between colorectal cancer cell lines harboring wild-type or mutant TP53. In wild-type TP53 cell lines (HCT116, RKO, and LIM2405), PD901 or MLN0128 treatment increased cell death, as measured by sub-G_1 DNA accumulation (Fig. 2A). The combination treatment synergistically increased the sub-G_1 population in all three models, reaching profound levels of cell death (45%, 32%, and 79%, respectively) with concomitant caspase-7 and PARP cleavage (Fig. 2B). In contrast, TP53 mutant cell lines (DLD-1, HT-29, and SW948) showed only minor combination response compared with single agents, achieving relatively limited cell death (5%, 15%, and 15%, respectively), with no detectable biochemical readouts of apoptosis. These results were supported by the significantly higher induction of Annexin V in TP53 wild-type versus mutant cells treated with the combination of agents (Fig. 2C).
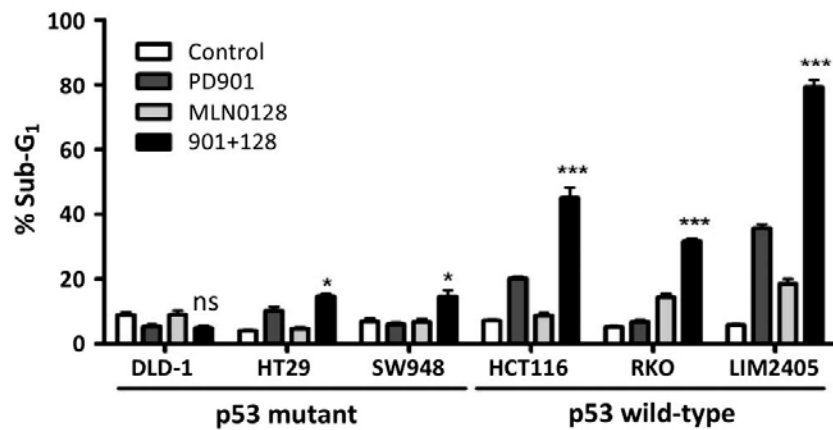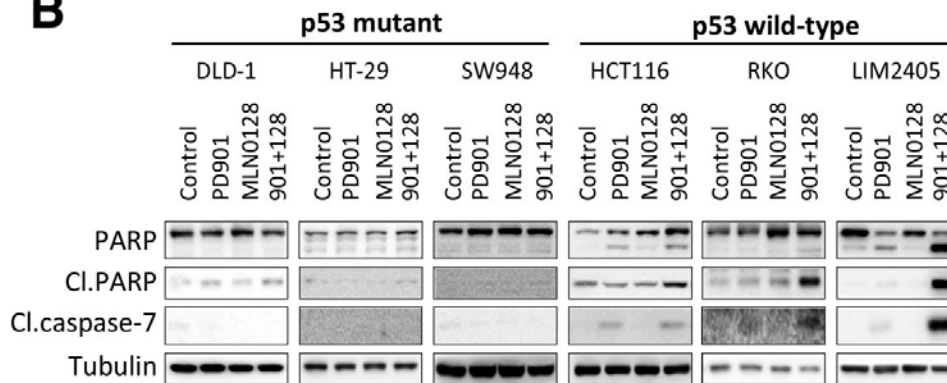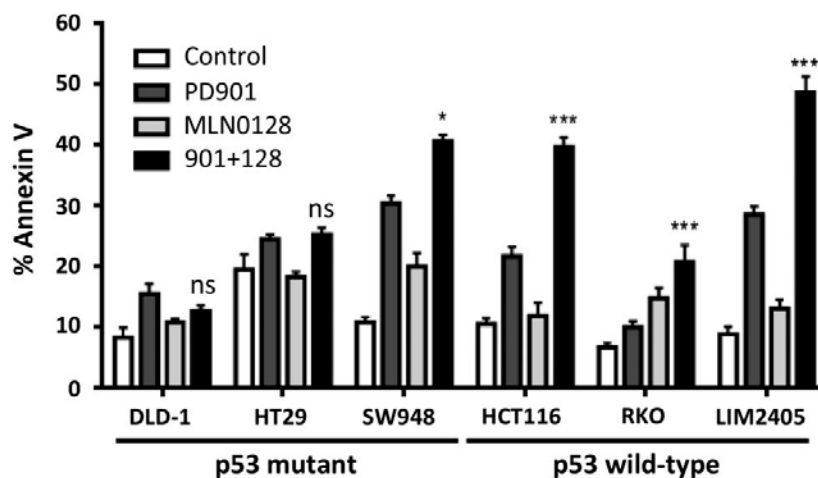
Figure 2. MEK and mTOR inhibition induces apoptosis in p53 wild-type colorectal cancer cells. The indicated colorectal cancer cell lines were treated with DMSO (control), 50 nmol/L PD901, 50 nmol/L MLN0128, or the combination of both inhibitors (901+128). A, apoptosis was measured after 72 hours of treatment as the percentage of cells with sub-G_1 DNA content by flow cytometry and analyzed with FCS Express 4 Flow software. Data, mean ± SE from three independent experiments. B, whole-cell protein extracts were analyzed after 24 hours of treatment by Western blot with the indicated antibodies. Tubulin antibody was used as a loading control. Figures are representative of three independent experiments. C, apoptosis was measured after 72 hours of treatment by quantification of the Annexin V–positive cells (Guava Nexin Reagent; Millipore). n.s., not significant, *, $P < 0.5$; ***, $P < 0.001$.

We further studied which p53 targets are involved in the response to dual MEK and mTORC1/2 blockade. We found that, although the levels of NOXA remained relatively stable across treatments, the levels of PUMA increased in all cell lines upon MEK and/or mTORC1/2 blockade, regardless of the p53 status (Supplementary Fig. S1B and S1C). Interestingly, BAX levels increased following MEK inhibition exclusively in the p53 wild-type cell lines. These results suggest that BAX may be mediating apoptosis in p53 wild-type colorectal cancer treated with MEK and mTORC1/2 inhibitors.

Combined MEK and mTORC1/2 suppression promotes antitumor responses inTP53 wild-type colorectal cancer xenografts

In vivo, xenografts derived from one mutant (HT-29) and two wild-type TP53 (HCC116 and LIM2405) colorectal cancer cell lines exhibited biochemical ERK or PI3K/mTOR signal inhibition with PD901 and MLN0128, respectively, and inhibition of both pathways when treated with the combination of both agents (Fig. 3A). However, detection of PARP and caspase-3 cleavage was evident only in TP53 wild-type tumors upon combination treatment (Fig. 3A and B and Supplementary Fig. S1D). We then expanded our panel of models with five PDXs that harbored alterations in KRAS and PIK3CA/PTEN, in addition to the three cell line–derived models. Single-agent antitumor activity was variable across the eight colorectal cancer models; yet the combination of PD901 and MLN128 resulted in tumor growth stabilization in one out of three TP53 wild-type colorectal cancer models (PDX-T77), and in none of the TP53-mutant ones (Fig. 3C). We confirmed our results with an independent sample set (18), namely, five colorectal cancer PDX models that harbor hotspot mutations in KRAS and PIK3CA, using different MEK and PI3K/mTORC1/2 inhibitors. A combination response of AZD6244 (MEK inhibitor) plus NVP-BEZ235 (PI3K/mTORC1/2 inhibitor) that resulted in tumor growth stabilization was observed in one of two wild-type TP53 PDX and in none of the three TP53-mutant ones (Supplementary Table S3). Altogether, an antitumor combination response was observed in two out of five (40%) TP53 wild-type colorectal cancer models, while there was no such response across the eight TP53-mutant models (0%). These data suggest that TP53 mutational status could affect the antitumor activity of combined MEK and mTORC1/2 inhibition in colorectal cancer.
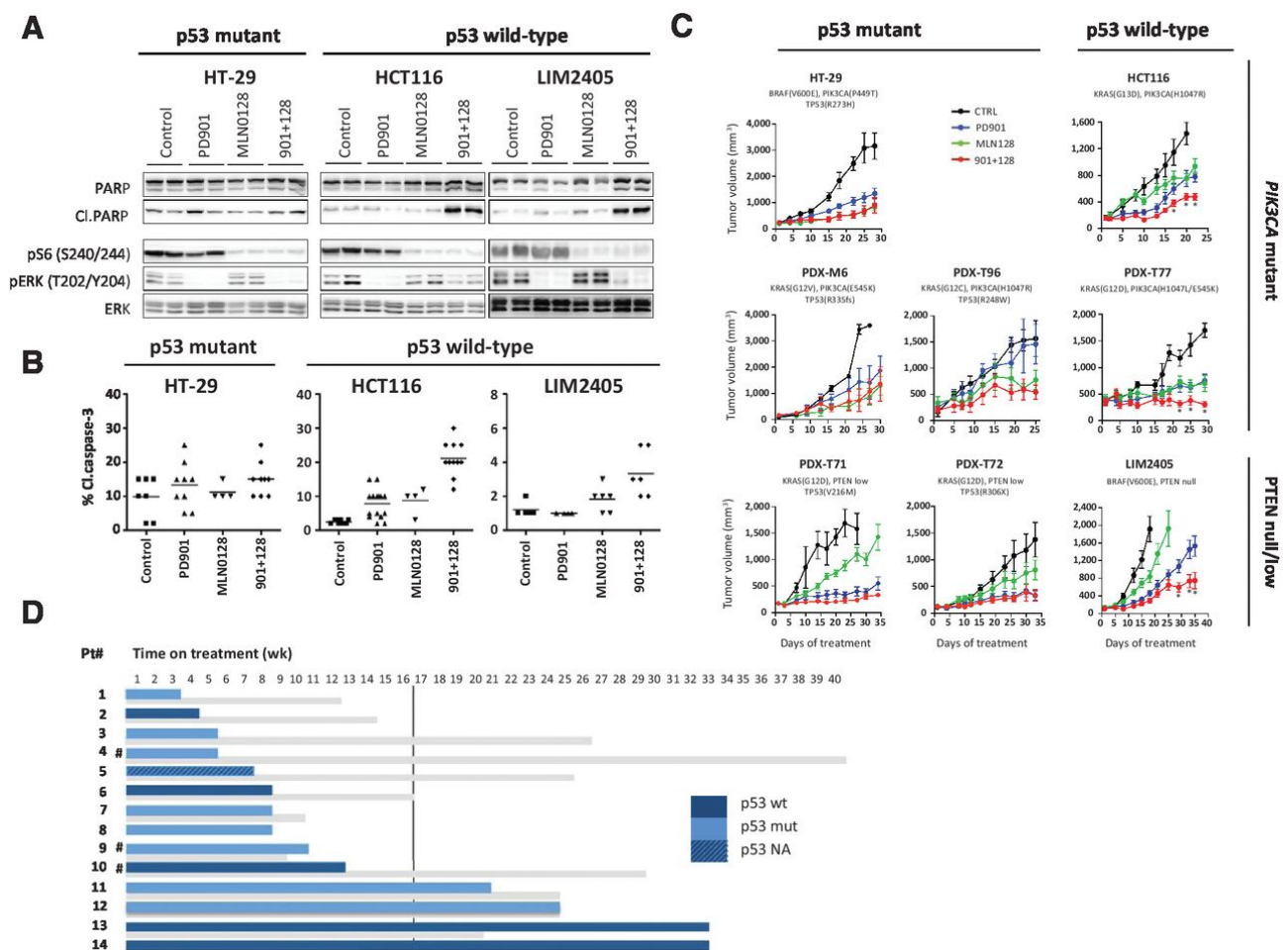

Figure 3. p53 wild-type colorectal cancer tumors benefit from combined MEK and mTOR inhibition. Mice bearing HT-29, HCT116, or LIM2405 tumors were treated with 3 consecutive doses of vehicle control (control, C), PD901 (901, 2 mg/kg, 6 q.d./week), MLN0128 (128, 0.3 mg/kg, 6 q.d./week), or the combination of both inhibitors (901+ 128). A, tumors were collected and analyzed by Western blot with the indicated antibodies. B, tumors were collected and analyzed by immunohistochemistry with cleaved caspase-3 antibody. The images were quantified by a pathologist blinded to the identity of the samples. C, mice bearing HT-29, HCT116,

LIM2405, PDX-M6, PDX-T71, PDX-T72, PDX-T77, and PDX-T96 were treated as indicated for 20 to 60 days. Measurements are displayed as mean ± SE. D, time on MEK plus PI3K therapy of 14 patients (blue) compared with the time on treatment for the previous chemotherapy-containing regimen (gray). Vertical line, 16-week threshold (second response evaluation by computed tomography). The p53 status (sequencing and IHC) is provided in dark blue for wild-type p53, light blue for mutant p53, and shaded blue for nonavailable data. #, patients exiting MEK plus PI3K therapeutic regimen because of toxicity.

p53 status in patients with colorectal cancer treated with MEK plus PI3K/mTORC1/2 therapy

We next investigated whether the p53 status of tumors from patients with colorectal cancer treated with the combination of MEK and PI3K/mTORC1/2 could predict for response to therapy (Fig. 3D). Assessment of the p53 status was possible in 13 cases using DNA sequencing or immunohistochemistry (when the genomic assay failed or when tumor tissue was scarce; Supplementary Table S4 and Supplementary Fig. S2). In this cohort, three patients stopped therapy due to toxicity. For the remaining 10 cases, we observed no significant correlation between p53 mutations and progression-free survival. This was not surprising given the small number of cases and the overall short time of response to therapy in these heavily pretreated patients. However, we noted that the two patients with the longest (32 weeks) responses to dual MEK and mTORC1/2 blockade had tumors with wild-type p53 (Fig. 3D). Intriguingly, the effects of the chemotherapy administered immediately before MEK plus PI3K/mTORC1/2 inhibitors were not long lasting in these two patients, suggesting that these tumors were not indolent (slow growing) and/or overall sensitive to cytotoxic therapies. More data on larger cohorts of patients are required to draw any conclusions about the value of wild-type p53 in predicting response to this therapeutic strategy.

p53 function is necessary for apoptotic and antitumor effects of MEK and mTORC1/2 blockade in colorectal cancer cells

Because our results in multiple colorectal cancer models supported an association betweenTP53 mutation status and sensitivity to combined MEK and mTORC1/2 inhibition, we sought to further elucidate the role of p53 as a possible determinant of therapy response. We stably overexpressed a mutant variant of TP53 (p53-R248W) or GFP (control) in theTP53 wild-type cell lines HCT116 and LIM2405. The p53-R248W mutation has been described as a dominant-negative variant that represses p53 transcriptional functions by interacting with the endogenous wild-type p53 (32, 33). Upon treatment, both cell line models exhibited the expected reduction in p-ERK and p-AKT/p-S6 upon PD901 and MLN0128, respectively, both in vitro and in vivo (Fig. 4A and Supplementary Fig. S3A and S3B). However, expression of p53-R248W suppressed caspase-3/7 and PARP cleavage induced by combined MEK and mTORC1/2 inhibition (Fig. 4A and Supplementary Fig. S3A and S3B). This resulted in reduced sensitivity to combined PD901 and MLN0128 in p53-R248W xenografts compared with the p53 wild-type counterparts (Supplementary Fig. S3C). Similarly, knockdown of TP53 in HCT116 cells resulted in significant attenuation of the proapoptotic response upon combined treatment with PD901 and MLN128 (Fig. 4B). These data support the hypothesis that p53 function is necessary for the induction of apoptosis and antitumor response to combined MEK and mTORC1/2 inhibition in colorectal cancer.
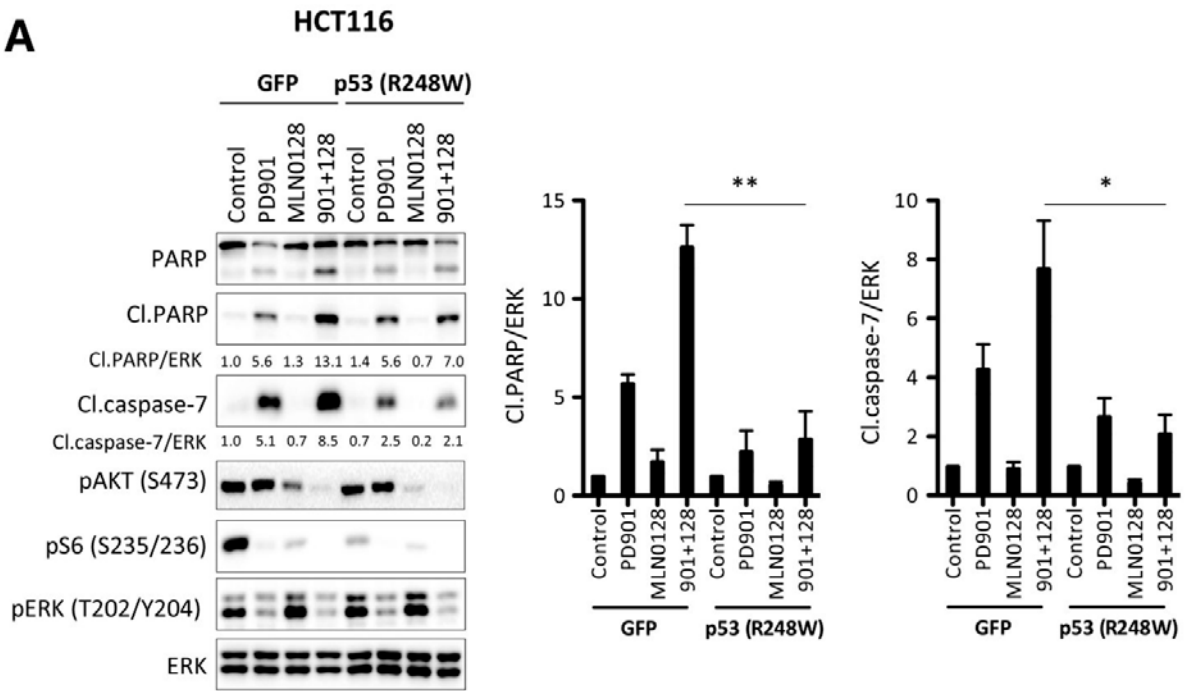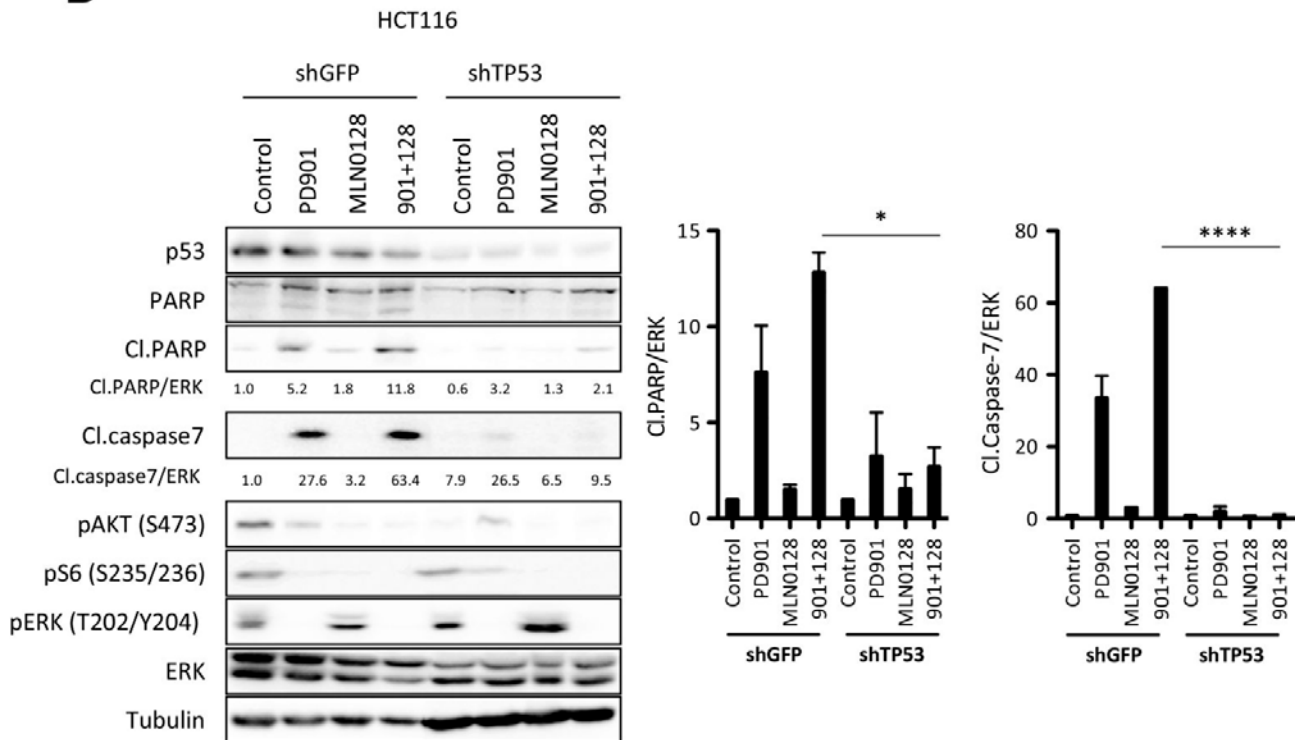
Figure 4. p53 proficiency is required to induce apoptosis in colorectal cancer tumors upon anti-MEK- and mTOR-inhibition. A, stable GFP or p53(R248W) HCT116 cells were treated with DMSO (control), 50 nmol/L PD901, 50 nmol/L MLN0128, or both inhibitors (901 + 128) for 24 hours. Whole-cell protein extracts were analyzed with the indicated antibodies. Figures are representative of three independent experiments, and quantified by densitometry (ImageJ). B, stable shGFP or shTP53 HCT116 cells were treated with DMSO (control), 50 nmol/L PD901, 50 nmol/L MLN0128, or both inhibitors (901 + 128) for 24 hours and resolved as in (A). Figures are representative of three independent experiments and quantified. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

MEK blockade promotes p53-dependent upregulation of p21 in TP53 wild-type colorectal cancer

Given that the cell cycle and apoptosis inhibitor p21 is a downstream target of MEK, mTORC1/2, and p53, we hypothesized that expression of p21 could limit cell death mediated by the combination of PD901 and MLN128 (34, 35) in our preclinical models. As expected, p21 mRNA and protein levels were lower in TP53 mutant cells than in TP53wild-type cells (Fig. 5A and B and Supplementary Fig. S3D and S3E). Moreover, MEK blockade inhibited the expression of c-Myc and increased p21 mRNA and protein levels preferentially in p53 wild-type cells (HCT116, RKO, and LIM2405). This effect was reverted (posttranscriptionally) by mTOR blockade (Fig. 5A and B). These data are in agreement with p21 being negatively regulated by c-Myc, a downstream target of MEK (36), and with mTORC1 regulating p21 stability at the translational level (34). In contrast, p21 mRNA and protein levels remained unaffected in the TP53-mutant models (DLD-1, HT-29, and SW948) treated equally.
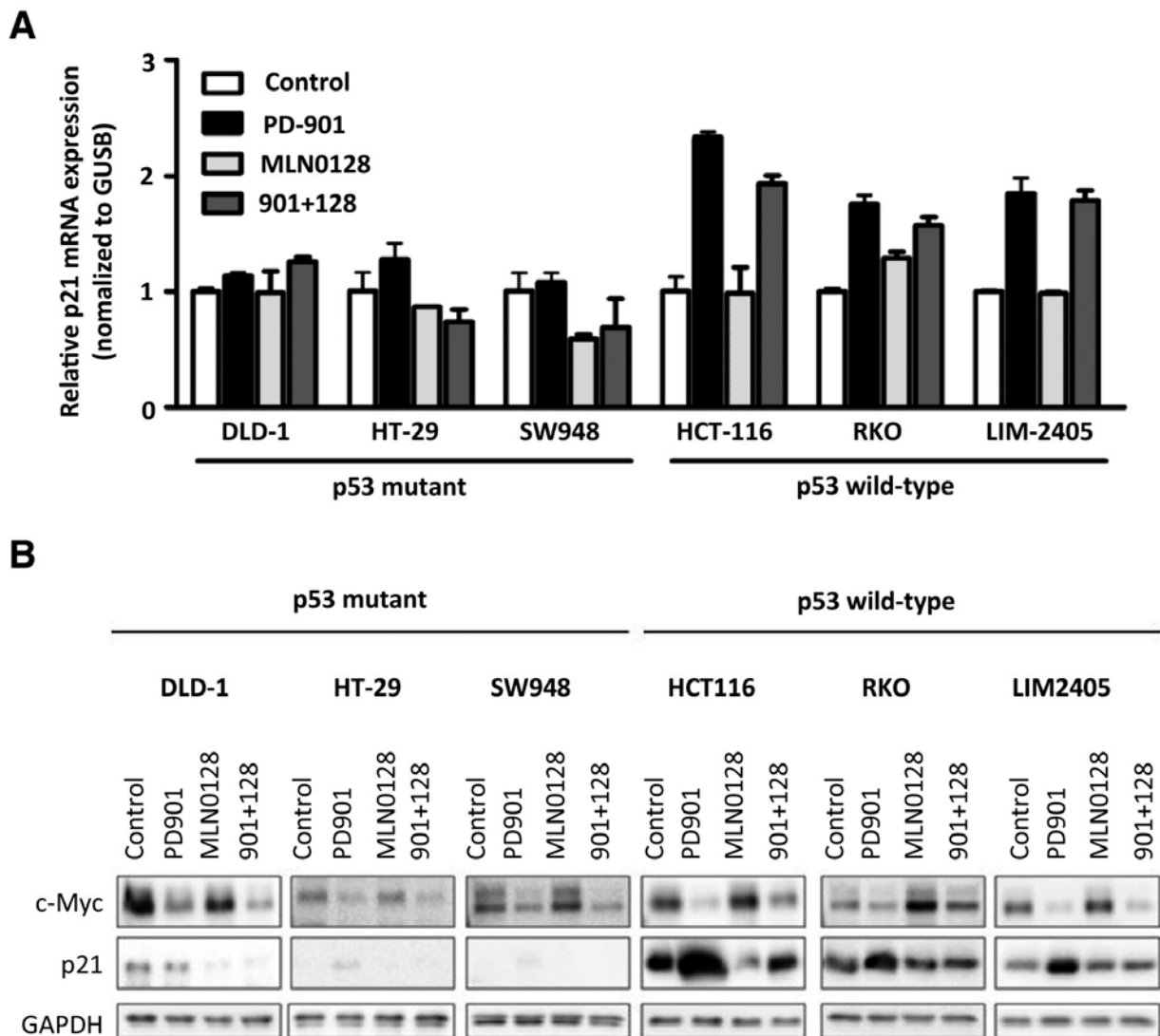

Figure 5. MEK inhibition induces p21 upregulation in p53 proficient colorectal cancer cells. A, colorectal cancer cell lines were treated with DMSO (control), 50 nmol/L PD901, 50 nmol/L MLN0128, or the combination of both for 16 hours. p21 mRNA levels were analyzed by qRT-PCR, normalized to GUSB mRNA levels, and expressed as fold change compared with control. B, colorectal cancer cell lines were treated with DMSO (control), 50 nmol/L PD901, 50 nmol/L MLN0128, or the combination of both for 24 hours and whole-cell protein extracts were analyzed by Western blotting with the indicated antibodies. GAPDH antibody was used as a loading control. Figures are representative of three independent experiments.

To confirm the role of wild-type p53 in modulating p21, we made use of the HCT116-GFP and HCT116-p53-R248W cell lines described above. In GFP-transfected cells, treatment with PD901 resulted in induction of p21. In agreement with the previous data, expression of p53-R248W prevented p21 upregulation consequent to MEK inhibition (Supplementary Fig. S3F). We next confirmed that p21 upregulation upon MEK inhibition was p53 dependent by downregulating p53 in two TP53 wild-type cell lines, HCT116 and LIM2405 (Supplementary Fig. S3G). In both models, p53 knockdown counteracted PD901-mediated induction of p21. In summary, we show that MEK blockade results in increased p21 mRNA and protein expression in TP53 wild-type colorectal cancer cells.

p21 upregulation prevents apoptosis mediated by MEK blockade in TP53 wild-type colorectal cancer cells

Finally, we sought to investigate whether p21 upregulation following treatment with PD901 alone precludes the induction of apoptosis in TP53 wild-type colorectal cancer cells. Specific downmodulation of the mRNA of CDKN1A (the gene encoding for p21) in two TP53 wild-type cell lines, HCT116 and LIM2405, resulted in increased induction of apoptosis by PD901, as shown by caspase-7 and PARP cleavage as well as accumulation of the sub-G_1 cell fraction (Fig. 6A and B). In addition, overexpression of p21 in p53 wild-type cells mimicked the effect of MEK inhibition and prevented PARP cleavage upon the combination of PD901 and MLN0128 treatment (Fig. 6C). In summary, our results show that the apoptotic response following MEK and mTORC1/2 blockade in colorectal cancer models is p53 dependent. As a consequence of mTOR blockade, PD901-induced upregulation of p21 is blunted, which likely precipitates apoptosis.
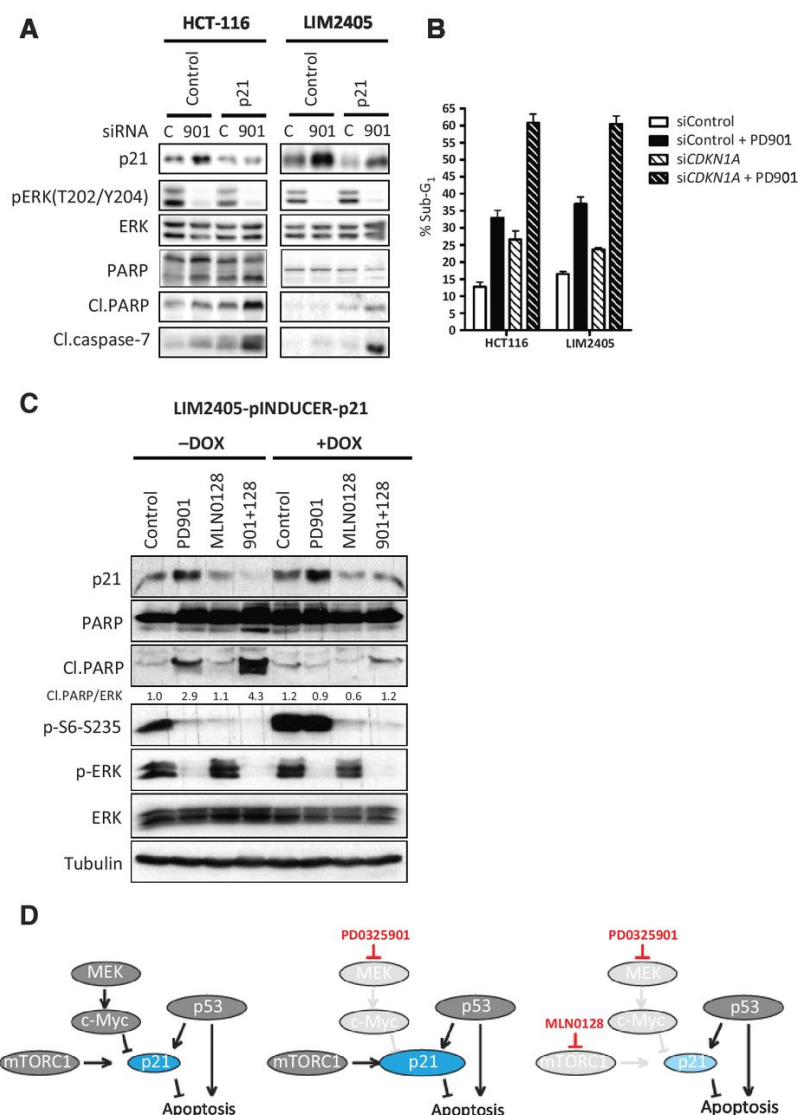
Figure 6. Induction of p21 by MEK blockade inhibits apoptosis in p53 wild-type colorectal cancer cells. A, HCT116 and LIM2405 cell lines were transfected with either a siRNA oligonucleotide targeting p21 (CDKN1A) or a control siRNA oligonucleotide as described. After 24 hours of treatment with DMSO (C) or 50 nmol/L PD901 (901), whole-cell protein extracts were analyzed with the indicated antibodies. Figures are representative of three independent experiments. B, apoptosis was measured after 72 hours of treatment as a percentage of cells with sub-G_1 DNA content by flow cytometry and analyzed with FCS Express 4 Flow software. Data, mean ± SE from three independent experiments. C, The LIM2405 cell line was stably transfected with an inducible p21 vector or with control EGFP. Cells were treated as indicated, and after 24 hours, whole-cell protein extracts were analyzed with the indicated antibodies. D, model depicting the proposed mechanism of action for the combination of anti-MEK and mTOR therapy inKRAS/BRAF- and PI3K/PTEN-mutated colorectal cancer in p53 wild-type cells. In wild-type p53 backgrounds, p21 is under transcriptional and translational control of p53, c-Myc, and mTORC1. PD0325901 blocks the negative transcriptional control of p21 by MEK/c-Myc and enhances the p21 levels, which inhibit apoptosis induction. Concomitant blockade of mTORC1 prevents translation of p21, thereby enabling apoptosis.

Discussion

Agents targeting MEK have shown limited activity used as monotherapy in colorectal cancer (37), with PI3K pathway activation being a potential bypass mechanism (38, 39). Several clinical trials were initiated to ask whether concomitant MEK and PI3K/mTORC1/2 blockade is efficacious in various diseases (19, 20), including colorectal cancer. In this study, we investigated the effectiveness of these agents in colorectal cancer and whetherTP53 mutational status affects the antitumor response.

We focused on colorectal cancers with concomitant alterations of RAS/BRAF andPIK3CA/PTEN, which entitles for approximately 8% of the overall colorectal cancer population (8), to show that wild-type TP53 is associated with preferential response to combined MEK and PI3K/mTOR inhibition. In our in vitro and in vivo models, wild-typeTP53 mediates an apoptotic outcome following MEK plus mTORC1/2 blockade. These preclinical observations need to be validated in a larger cohort of patients.

The inhibition of MEK and mTORC1/2 has previously been explored in other settings, including lung (14), pancreatic (15), breast (16, 17), and colorectal tumors (18). However, the present study is the first one in which TP53 status has been associated with treatment outcome. Particularly, the study by Migliardi and colleagues (18) highlighted the limitations of simultaneously targeting MEK and PI3K/mTORC1/2 in colorectal cancer PDXs, with limited antitumor responses across a panel of 40 models. Our study focused onKRAS/BRAF and PIK3CA/PTEN double mutants, and we similarly observed that minor response was the best therapeutic outcome in PDX, or resulted in disease stabilization in patients. In addition, our study identified TP53 mutation as a potential predictive marker, providing a clinically relevant tool to maximize the efficacy from this therapeutic combination while avoiding unnecessary treatment of the patients. As suggested by Migliardi and colleagues, we presume that triple therapy with approved anti-EGFR agents would provide further therapeutic advantage in BRAF mutant colorectal cancer harboring PI3K pathway alterations (40).

Mechanistically, our data provide insight into the antiapoptotic role of p21 in TP53 wild-type tumors. p21 upregulation precluded the induction of apoptosis upon MEK therapy, an effect that was reverted adding mTORC1/2 inhibitors (Fig. 6D).

TP53 mutations are among the most frequent mutations in colorectal cancer and occur late during colorectal tumorigenesis (41). Although many studies have tried to elucidate the prognostic and predictive value of p53 alterations in colorectal cancer, it remains unclear whether its mutational status could affect outcome and therapy response (42–45). One potential drawback is the limited specificity or sensitivity of the techniques used to determine the p53 status. Two assays have been mainly used in the clinic for the determination of p53 status, namely, mutations in the coding sequence of the TP53 gene (46) and elevated nuclear p53 protein, as assessed by IHC (47). IHC has been generally used as a surrogate marker of TP53 mutations, but this assumption may not always be correct because many genetic changes do not result in p53 overexpression, and positive immunohistochemistry analysis of p53 may occur in the

absence of TP53 mutations (31). Nevertheless, when tissue is scarce or quality is compromised, as in some of our specimens, IHC remains as the unique test available to assess p53 status.

Disappointingly, MEK plus PI3K/Akt/mTORC1/2 inhibitor combinations have shown limited clinical activity even in retrospectively selected, molecularly defined populations (19, 20,48). The overlapping dose-limiting toxicities of these combinations have prevented the achievement of dose levels similar to the single-agent maximum tolerated doses, which compromises pathway inhibition and precludes substantial clinical benefit over single-agent strategies (48). Alternative scheduling, with noncontinuous/pulsatile dosing of either agent, might be an option to increase tolerability of these regimens and likely to induce a proapoptotic response (49). Currently, clinical trials explore further combinations, namely, blockade of mutant BRAF with selective BRAF[V600E] inhibitors (±MEK inhibitors) and EGFR inhibitors in patients with colorectal cancer harboring BRAF[V600E] mutations or targeting MEK in combination with anti-IGF-1R (in KRAS mutant colorectal cancer). It remains to be understood if these strategies will be efficacious in colorectal tumors with concomitant activation of the PI3K pathway. Our work prompts to investigate if patients with wild-type p53 colorectal cancers obtain higher benefit than those with mutant p53 tumors, from therapeutic combinations that directly or indirectly target Ras/Raf and PI3K. This interrogation should be feasible, as the institutions conducting these studies have implemented prescreening strategies that allow massive parallel capture-based sequencing of many cancer-related genes of many cancer-related genes, including TP53.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Footnotes

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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