A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53

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Through targeted homologous recombination, we developed a panel of matched colorectal cancer cell lines that differ only with respect to their endogenous *TP53* status. We then used these lines to define the genes whose expression was altered after DNA damage induced by ionizing radiation. Transcriptome analyses revealed a consistent up-regulation of polo-like kinase 1 (PLK1) as well as other genes controlling the G_2/M transition in the cells whose *TP53* genes were inactivated compared with those with WT *TP53* genes. This led to the hypothesis that the viability of stressed cells without WT *TP53* depended on *PLK1*. This hypothesis was validated by demonstrating that stressed cancer cells without WT *TP53* alleles were highly sensitive to PLK1 inhibitors, both in vivo and in vitro.

In cancers, the *TP53* tumor suppressor gene is inactivated more frequently than any of the other ≈21,000 protein-encoding genes in the human genome (1–6). *TP53* has been estimated to be altered by point mutation in approximately half of all human cancers (2, 7–9). In cancers without such intragenic mutations, the p53 protein is often functionally inactivated by binding to proteins encoded by viral or cellular oncogenes such as E6 or MDM2, respectively (10–15). In normal cells, the p53 protein is a key node in the network controlling the response to stress, particularly those associated with damage to DNA such as oxidation and irradiation (16–20). One of its major functions is the activation of a transcriptional response to such stresses that determine whether cells arrest and repair the damage or undergo apoptosis. In the absence of *TP53*, both cell cycle arrest and apoptosis are compromised, presumably allowing these cancer cells to proliferate under situations wherein normal cells would die. Several of the transcriptionally activated genes that mediate the cell cycle arrest and apoptotic functions of *TP53* have been identified, including the cyclin-dependent kinase inhibitor p21 and the proapoptotic protein PUMA (21–23).

In light of its extraordinarily high mutation rate across many different tumor types, *TP53* provides a uniquely attractive target for drug development. However, like any tumor suppressor gene, *TP53* is not itself easily "druggable:" Drugs generally inhibit the function of proteins rather than restore normal function to defective proteins. It has thereby been challenging to develop small molecules that restore transcriptional activation to mutant p53 proteins, although some promising compounds of this type have been developed (24–27).

It therefore may be useful to attempt to target elements in the pathways that *TP53* regulates, rather than p53 itself, for therapeutic purposes. Indeed, clever strategies for exploiting the absence of functional p53 in cells have been devised and some are in clinical trials (28). Because loss of G_1 arrest in p53 mutants prevents repair of DNA damage, a number of synthetic lethal strategies have been proposed. Notably, some of these strategies involve compounds that disrupt the G_2/M checkpoint, the major repair checkpoint that remains at least partially functional in cells without normal p53 function (27, 29–36).

In an effort to better understand the pathways regulated by p53, we constructed a variety of human colorectal cancer cell lines that were isogenic except at the *TP53* locus. Human colorectal cancer cells, rather than mouse cells, were chosen for these experiments because the pathways that are regulated by p53 are likely to vary between species and cell types (37–39). We then analyzed the transcriptome of these cells after subjecting them to the stress imposed by DNA damage.

Most similar transcriptome analyses have in the past been performed on cells with overexpressed p53 genes (21–23, 40–42). Overexpression can induce a variety of effects that may play little role under more physiologic conditions (43, 44). Through the use of cells in which the WT or mutant *TP53* genes were under the control of their normal regulatory elements, we hoped to uncover pathways that provided leads for therapeutic development. Indeed, we found that a common signature of cells with inactive *TP53* genes, regardless of the precise nature of the mutant allele, was a relative up-regulation of genes controlling the G_2/M checkpoint. This in turn suggested a specific therapeutic approach for cancers without WT *TP53* genes, as described below.

Results

Generation of a Panel of Isogenic Cell Lines Differing in p53 Status. We used 3 recombinant adenoassociated virus (rAAV) vectors to alter the endogenous alleles of 4 commonly used colorectal cancer cell lines. One vector resulted in the deletion of *TP53* exon 2 in the targeted lines (Fig. 1*A*). The 2 others were "knockin" vectors, resulting in the creation of either mutant (tryptophan) or WT (arginine) codons at amino acid 248 in exon 7 (Fig. 1*B*). The codon 248 mutation is among the most common observed in human cancers. The isogenic cell lines used in this study derived from SW48, DLD-1, RKO, and HCT116 cells, are listed in Table 1. In general, at least 2 independent clones of each knockout or knockin cell line were derived. Independent clones with the same genotype always behaved identically in the assays described below.

Examples of the PCR and sequencing results that were used to confirm the targeting events are illustrated in Fig. S1 *A–D*. Western blot analyses were performed before and after treatment with

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Data deposition: Microarray expression data have been deposited in National Center for Biotechnology Information Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE13886).

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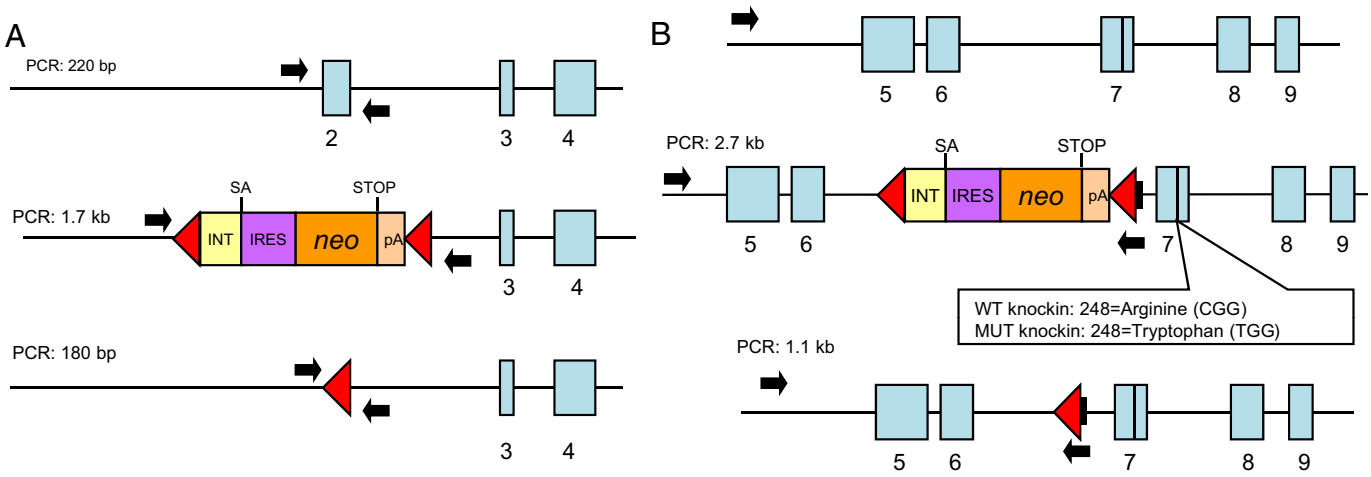

Fig. 1. TP53 locus before and after targeting. (*A*) (*Top*) *TP53* genomic locus including exons 2, 3, and 4 (blue boxes). (*Middle*) Same locus after insertion of the targeting vector (62), resulting in replacement of exon 2 and surrounding intronic sequences with a cassette containing intronic sequences (INT), splice acceptor site (SA), internal ribosomal entry sequence (IRES), neomycin phosphotransferase gene (neo), stop codon (STOP), and polyadenylation site (pA). Red triangles indicate loxP recombination sites. (*Bottom*) The same locus after Cre-mediated excision of the targeting construct. (*B*) (*Top*) *TP53* genomic locus including exons 5 to 9 (blue boxes). (*Middle*) Same locus after insertion of the rAAV targeting vectors, one containing the WT sequence in exon 7 and the other a mutation (R248W) in exon 7. (*Bottom*) The same locus after Cre-mediated excision of the knockin constructs. In both *A* and *B*, arrows indicate the position of the PCR primers used to screen clones for the desired recombination. The sizes of the PCR products generated with these primers are also indicated.

5-FU, a cancer chemotherapeutic drug known to activate p53 (Fig. 2). SW48 parental cells have 2 WT *TP53* alleles. When 1 of them is disrupted by targeted homologous recombination, creating a heterozygote, there was less p53 protein in the cell after 5-FU treatment (Fig. 2*A*). When both alleles were disrupted, there was of course no p53 protein. Identical results were observed in HCT116 and RKO cells, both of which normally have 2 WT alleles of *TP53* (Fig. 2 *B* and *C*). The p21 protein (product of the *CDKN1A* gene) is one of the most well-characterized targets of p53 transcriptional activation (45). This protein was induced in the parental cells after 5-FU activation of p53 but not in the cells with both alleles disrupted (Fig. 2 *A*–*C*). In the heterozygotes with 1 WT p53 allele and 1 disrupted allele, p21 induction by 5-FU was detectable but somewhat variable, perhaps reflecting *TP53*-gene dosage effects that varied among the cell lines.

When 1 WT allele of HCT116 cells was replaced with a mutant (R248W) allele, there was still induction of p53 by 5-FU and consequent activation of p21 (R248W/+ line in Fig. 2*D*). However, when 1 WT allele of HCT116 cells was inactivated by homologous recombination, and the other was replaced with a mutant (R248W) allele, induction of p53 by 5-FU was less marked, and induction of p21 was completely eliminated (R248W/− line in Fig. 2*D*). DLD-1 cells normally have 1 allele that is mutant (S241F) and 1 allele that is not detectably expressed, as assessed by RT-PCR analysis (silent). We detected no mutations of *TP53* in this second allele upon sequencing of all of the exons and intron–exon boundaries of the gene, so the silencing may have been epigenetic. In parental DLD-1 cells (genotype S241F/SIL), there was some increase in p53 upon 5-FU treatment but no induction of p21 (Fig. 2*E*). When the silent allele was disrupted through homologous recombination, there was no change either in p53 or p21 levels, as expected (genotype S241F/−). However, when the mutant S241F allele of DLD-1 was replaced with a WT allele, the level of p53 protein was reduced and p21 was induced upon 5-FU treatment (S241F/+ line in Fig. 2*E*).

As another test of the functionality of the knockin and knockout clones, we evaluated the effects of Nutlin-3, a small molecule that binds to MDM2 and disrupts the interaction between MDM2 and p53 proteins (46, 47). This drug retards the ability of MDM2 to ubiquinate p53 and mark it for degradation. We found that all clones harboring WT p53 were more growth-inhibited by Nutlin-3 than clones without WT p53 (Fig. 3). Clones with completely inactive *TP53* genes had Nutlin-3 sensitivities identical to those with point mutations of p53.

Table 1. *TP53* genotypes of the panel of isogenic cell lines used in this study

Tumor of origin	Genotype	Allele 1	Allele 2
SW48	+/+	WT	WT
SW48	+/−	WT	Inactivated
SW48	−/−	Inactivated	Inactivated
RKO	+/+	WT	WT
RKO	+/−	WT	Inactivated
RKO	−/−	Inactivated	Inactivated
RKO	R248W/+	*R248W*	WT
HCT116	+/+	WT	WT
HCT116	+/−	WT	Inactivated
HCT116	−/−	Inactivated	Inactivated
HCT116	R248W/−	*R248W*	Inactivated
HCT116	R248W/+	*R248W*	WT
DLD-1	S241F/SIL	*S241F*	Silent
DLD-1	−/SIL	Inactivated	Silent
DLD-1	+/SIL	WT	Silent
DLD-1	S241F/−	*S241F*	Inactive

Transcriptional Profile of Cells With and Without WT *TP53*. Ionizing radiation such as that produced by γ-irradiation induces a p53-dependent G_1 arrest via the induction of DNA double-strand breaks (48–50). Using the *TP53* isogenic panel, we first confirmed that the predicted G_1 arrest was observed in the lines containing WT *TP53* genes (Fig. 4). In the isogenic lines in which both *TP53* alleles were inactivated by targeted disruption or point mutation, minimal G_1 arrest was observed (Fig. 4).

We next compared the transcriptional profiles of these cells after γ-irradiation. As expected, a number of known p53 target genes were found to be present at higher levels in the cells containing WT *TP53* alleles than in their isogenic counterparts without WT *TP53* alleles (Table S2). The induced transcripts included those encoded by *CKDN1A*, *BBC3* (*PUMA*), *MDM2*, *FDXR*, *CCNG1*, and *PPM1D*. The differential expression was much more prominent after irradiation than in the absence of irradiation (Table S2).

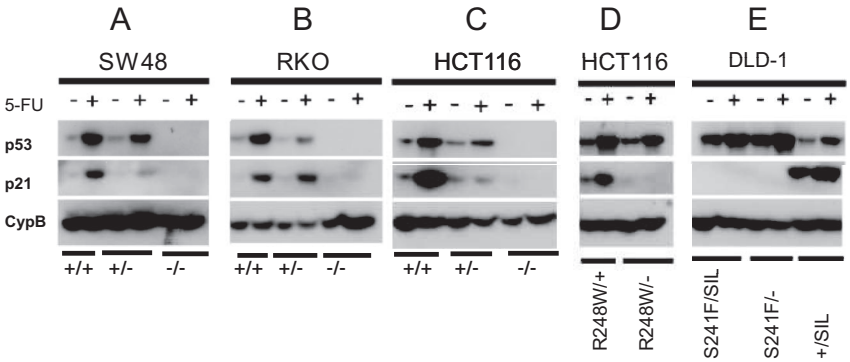

Fig. 2. TP53 and p21 protein expression in isogenic cells lines of various TP53 genotypes. Cells were cultured in the absence or presence of 5-FU. The CypB blots were used as loading controls.

Interestingly, a number of highly up-regulated transcripts that had not been identified in previous studies were identified in these experiments. Among these, one of the strongest up-regulated transcripts was a large noncoding RNA of unknown function (LOC401131 in Table S2).

We were most interested in transcripts that were up-regulated in the cells without WT *TP53* genes, because their identification could lead to therapeutic approaches to inhibit the growth of cancer cells with inactive *TP53* genes. After irradiation, the expression levels of 35 genes were consistently higher (by at least 2-fold) in all lines without WT *TP53* alleles than in those with WT *TP53* alleles (Table S2). In all 35 cases, the differential expression was confirmed by

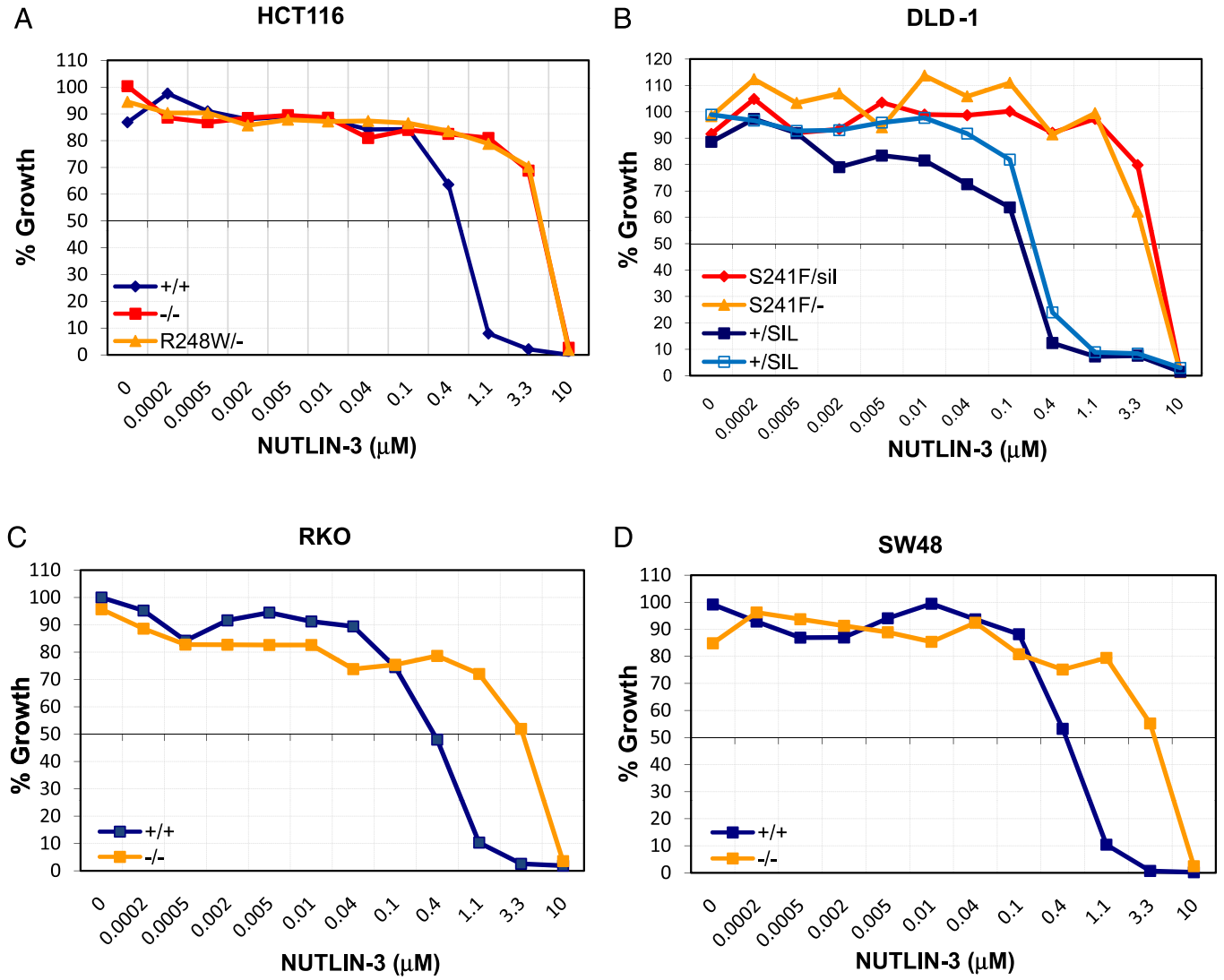

Fig. 3. TP53 genotype-dependent effect of Nutlin-3 on isogenic cancer cell lines. The indicated lines were exposed to increasing doses of Nutlin-3 for 96 h, and their growth was evaluated by assessing cell number in a SYBR green growth assay. All values were normalized to the number of cells of untreated controls. The 2 lines marked +/SIL were independently generated clones.

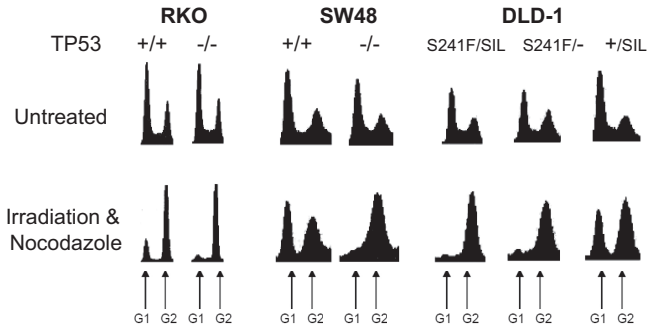

Fig. 4. G_1 arrest in cell lines containing WT *TP53* genes. Flow cytometry profiles before and after exposure of the indicated lines to ionizing radiation are shown. After irradiation, the cells were treated with nocodazole to block them from undergoing mitosis and entering into a subsequent G_1. Peaks corresponding to G_1 and G_2/M are indicated.

quantitative PCR (Table S3). This up-regulation was largely due to a repression of expression in the lines with WT *TP53* (Table S4). Surprisingly, the majority of the up-regulated genes were components of the G_2/M and/or spindle assembly checkpoints (Table S1). Moreover, many other genes associated with the G_2/M and spindle checkpoints were also up-regulated, but less than the 2-fold required for inclusion in Table S1 (Table S4).

Drug-Targeting of Cells with Inactive *TP53* Alleles. Among the G_2/M checkpoint genes up-regulated in the cells without WT *TP53* genes, 2 were particularly intriguing: *PLK1* and *AURKB*. These genes have well-defined, sequential roles in the G_2/M checkpoint, are highly specific in their function, and have enzymatic activities that can be inhibited by previously described small molecules. The expression data (Table S1) coupled with the responses to DNA damaging agents among the isogenic cell line panel (Fig. 4) suggested a therapeutic approach that would exploit the differences in *PLK1* and *AURKB* expression observed in cancer cells without WT *TP53* alleles. In particular, we hypothesized that the up-regulation of *PLK1*and *AURKB* was required for the continued viability of cells without WT *TP53* after stress. Moreover, we expected that a portion of normal (WT *TP53* gene-containing) cells would be specifically arrested in G_1 by the stress and would be partially spared the toxicity of these drugs, which presumably act largely on cells in the G_2 phase of the cell cycle.

To test this hypothesis, we synthesized 3 AURKB inhibitors (VX-680, AZD1152, and MLN8054) and 2 PLK1 inhibitors (BI-2536 and ON01910) and evaluated them in isogenic lines derived from HCT116 and DLD-1 cells. In each case, we compared a cell line with 2 WT *TP53* alleles to its isogenic twin containing 2 inactivated *TP53* alleles. In the presence of any of the 4 tested drugs alone, only minor differences in the sensitivities of the isogenic cell lines were observed (examples in Fig. 5 *A* and *D*). However, when the cells were first irradiated to induce a G_1 arrest, the cells without WT *TP53* genes proved much more sensitive to the 2 PLK1

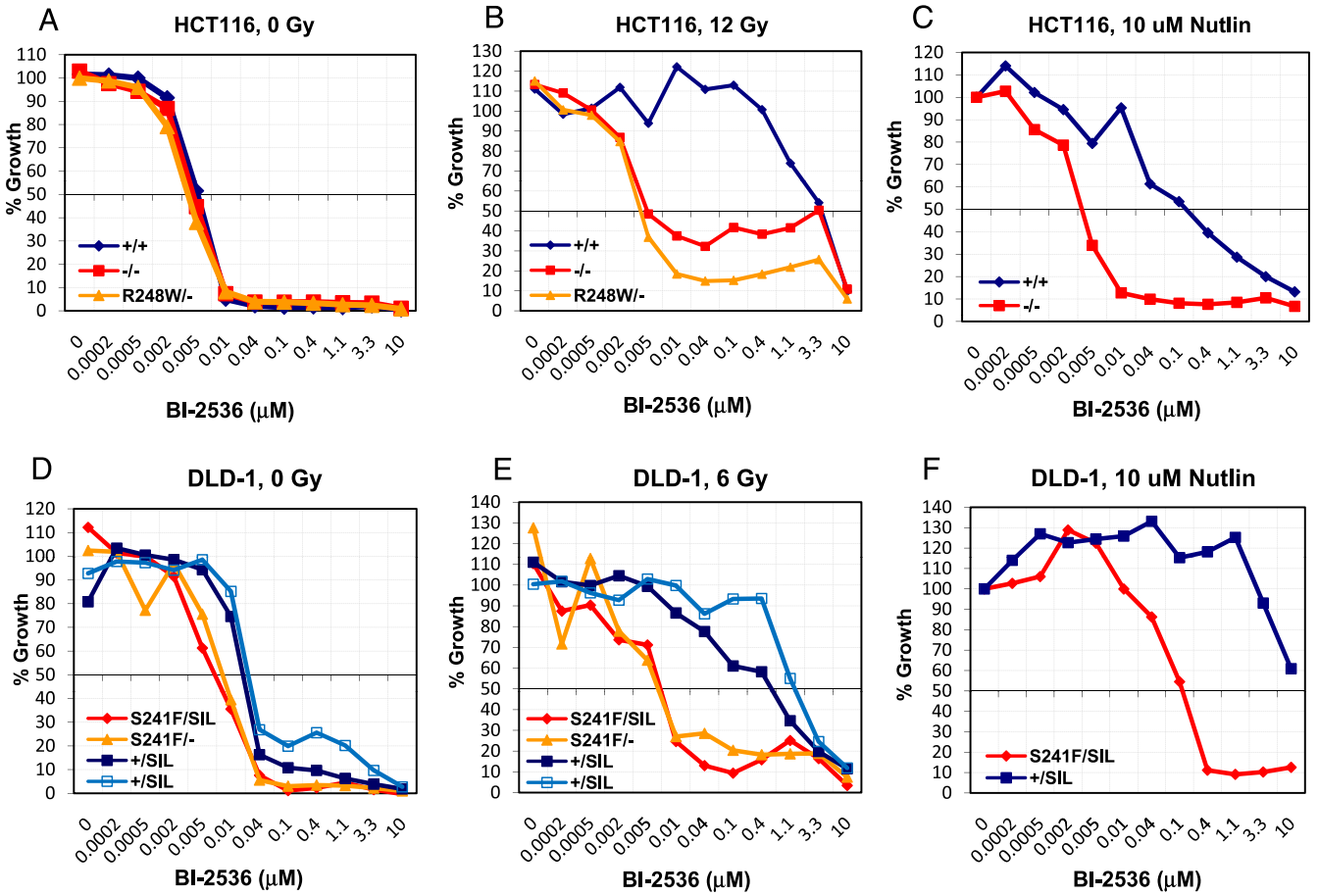

Fig. 5. Evaluation of BI-2536 on the growth of isogenic cell lines with or without WT *TP53* genes. Growth of the indicated lines to increasing doses of the PLK1 inhibitor BI-2536 alone, in the presence of ionizing radiation, or in the presence of Nutlin-3. The growth was assessed by a SYBR green-based growth assay and was normalized to the growth of untreated controls (*A* and *D*), in the presence of irradiation (*B* and *E*) or the presence of Nutlin-3 (*C* and *F*). The 2 lines marked +/SIL were independently generated clones.

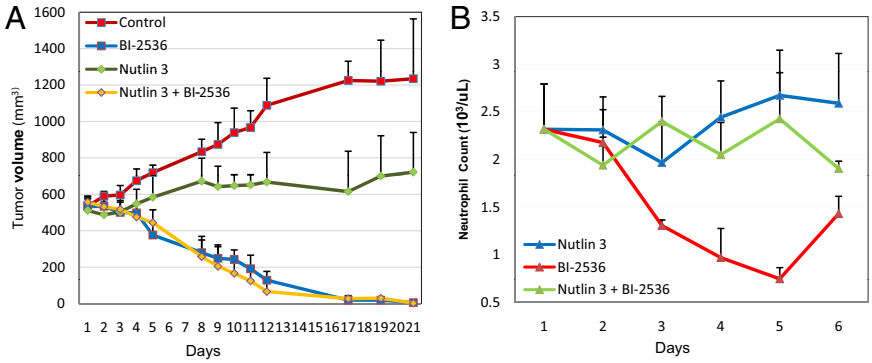

Fig. 6. In vivo effects of BI-2536 and Nutlin-3. (*A*) BI-2536 treatment of nude mice bearing HCT116 *TP53*$^{-/-}$ xenografts results in regression of the tumors. The mice were treated for 3 weeks as follows: twice a week with 100 mg/kg BI-2536 and twice a day 2 times a week with 200 mg/kg Nutlin-3 before BI-2536 treatment. The volume of the tumor was measured on the days indicated. (*B*) BALB/c mice were given 200 mg/kg Nutlin-3 4 and 24 h before administering a single i.v. dose of 100 mg/kg BI-2536. Neutrophils were counted before initial treatment and every 24 h thereafter just before BI-2536 administration. Means and SD of the counts from 5 mice in each treatment arm are shown.

inhibitors, particularly BI-2536 (Fig. 5 *B* and *E* and Fig. S2). The 3 AURKB inhibitors did not appreciably alter the growth of cells with WT *TP53* alleles compared with those with inactive *TP53* alleles (Fig. S3).

To explore the generality of WT *TP53*-mediated protection from PLK inhibitor toxicity, we used Nutlin-3 instead of γ-irradiation to induce a stress-like state, i.e., to block a portion of cells with WT *TP53* genes in G_1. Nutlin-3 in combination with BI-2536 was indeed able to selectively inhibit the growth of *TP53* mutant cell lines in a fashion comparable with that observed with irradiation plus BI-2536 (Fig. 5 *C* and *F*). This selective toxicity was observed in clones derived from all 4 parental colorectal cancer cell lines (SW48, RKO, HCT116, and DLD-1) and was independent of the nature of the mutation that inactivated *TP53* [missense mutation vs. targeted disruption (Fig. 5 and Fig. S4)].

Finally, we attempted to determine whether this therapeutic approach would be efficacious in an experimental animal model. Treatment with BI-2536 at 100 mg/kg twice a week resulted in dramatic tumor regression in nude mice harboring relatively large xenografts of HCT116 cells in which the WT *TP53* genes were disrupted (Fig. 6*A*). Notably, oral administration of Nutlin-3 (200 mg/kg) did not decrease the efficacy of BI-2536. This was expected from our in vitro experiments on the same cell line, wherein we found that Nutlin-3 could not rescue cells without WT *TP53* genes from the effects of BI-2536 (Fig. 5*C*). However, unlike the situation in vitro, we could not protect WT *TP53* HCT-116 tumor cells from BI-2536-mediated cell death with Nutlin-3 in vivo. Whether this was because of inadequate concentrations of Nutlin-3 in vivo for prolonged periods is not known. On the other hand, the major toxicity of BI-2536 in Phase I trials has been hematopoietic, with dangerous levels of neutropenia observed after treatment with this agent in the majority of patients (51). To determine whether Nutlin-3 could rescue this toxicity, we administered the combination of oral Nutlin-3 (200 mg/kg) and BI-2536 (100 mg/kg) to BALB/c mice. BALB/c rather than nude mice were used in these experiments because BI-2536 caused bone marrow toxicity in BALB/c mice at doses that did not cause such toxicity in nude mice. BALB/c mice treated with BI-2536 (100 mg/kg) developed neutropenia within 48 h after treatment with BI-2536. Oral administration of Nutlin-3 (200 mg/kg) efficiently protected the mice from this neutropenia (Fig. 6*B*).

Discussion

Our observations on the isogenic cell lines generated for this study are consistent with previous work (16–19, 46) showing that (*i*) mutant p53 proteins are more stable than the normal p53 protein (Fig. 2*E*); (*ii*) p21 is induced by 5-FU only when WT p53 is present in the cell (Fig. 2); and (*iii*) Nutlin-3 activates WT *TP53* and results in growth arrest (Fig. 3). The data also indicate that this large and varied isogenic panel provides a valuable model for determining chemical sensitivities based on *TP53* status. One advantage of the approach taken in this work, involving homologous recombination to delete or insert specific sequences in endogenous genes, is that the only major difference between cell line pairs is in the sequence of *TP53*. The fact that several independently derived lines with the same *TP53* genotype behaved identically in the assays supports this conclusion. Although we cannot exclude differential expression of other p53 isoforms, they are therefore not likely to play a role in the phenotypes observed in this study.

One of the most interesting observations made in this study was that the majority of the genes whose expression was higher in cells lacking WT *TP53* than in those with WT *TP53* genes were involved in the G_2/M transition. Although we do not know the basis for the up-regulation of these genes in cells with inactivated p53 genes, there are at least 3 possibilities. First, these genes could be directly repressed by WT p53 binding to their promoters. There is indeed evidence that p53 can directly repress genes rather than activate them (52). Second, it is possible that the relatively higher expression of these genes simply reflects a larger fraction of cells arrested in G_2/M after DNA damage when p53 is inactivated. However, the data in Fig. 4 show there is only a modest increase in the fraction of cells in G_2/M in cells with inactive *TP53* genes compared with those with WT *TP53*; the major difference in the cell cycle profiles is in the presence or absence of a G_1 block that generally affects only a minority of the cell population. The third possibility, and the one we favor, is that the up-regulation represents an indirect downstream effect of the altered regulatory pathways resulting from the absence of WT *TP53*. Regardless of the explanation, the differential expression of specific G_2/M checkpoint genes such as *PLK1* and *AURKB* provides a rationale for developing new therapeutic approaches.

The approach described in Figs. 5 and 6, employing an inhibitor of PLK1 together with an agent that protects cells with WT *TP53* genes, builds on previous work in basic and applied research. In particular, previous studies have shown that *TP53* is required for the G_1 arrest after DNA damage and that the CDK2NA gene is essential for this arrest (53). The potential to exploit the defective checkpoint status of cells with inactive *TP53* genes has also been widely recognized and in part stimulated the discovery of drugs that can inhibit PLK1, AURKB, and other proteins that regulate the G_2/M checkpoint (27, 29–36, 51, 54–60). Our results expand on these seminal observations in several ways. First, we confirm the requirement for WT *TP53* in isogenic pairs derived from 4 different human cancer cell lines. The biochemical and functional distinctions between these lines, which differ in some cases by only a single base pair, are remarkable. Second, we show that the relatively high expression of G_2/M checkpoint genes is a consistent feature of the cancer cell lines without WT *TP53*. Although many previous studies have uncovered genes up-regulated in cells with WT *TP53* genes after stress, the discovery of a large class of functionally related genes that is specifically up-regulated in cells without WT *TP53* genes is unique. Third, our studies suggest that coupling BI-2536, a powerful therapeutic, with an agent that protects a portion of

normal cells from entering G_2 is a promising strategy for reducing the toxicity associated with PLK1 inhibitors. Although this strategy might appear to be applicable to any protein controlling the G_2/M checkpoint, our data suggest otherwise. In particular, inhibitors of *AURKB* showed no differential effects on lines with WT vs. without WT *TP53* genes (Fig. S4) even though *AURKB* functions in the same pathway as *PLK1* (61). The reason for this difference between the cytotoxic effects of AURKB and PLK1 inhibitors is unknown. Regardless of the reason, our data suggest that coupling BI-2536 or related drugs with agents that normally induce G_1 arrest is worthy of further investigation.

Material and Methods

For detail of targeting vector construction, cell lines and targeting, compounds and γ-irradiation, Western blot analysis, microarray and qPCR procedures, and mouse studies, see *SI Materials and Methods*.

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