Supporting Information

Sur *et al.* 10.1073/pnas.0813333106

Materials and Methods

TP53 Targeting Vector Construction. The *TP53* exon 2 knockout construct was previously described (1). The knockin constructs were made by using the SEPT vector (1, 2). Homology arms for the WT knockin vector were PCR-amplified from genomic DNA of the RKO cell line. To construct the R248W knockin vector, the right homology arm (containing exons 7–9) was removed and replaced with a homology arm that was PCR-amplified from SW837 cells (which bear a homozygous R248W mutation in *TP53*). PCR was performed by using Phusion Taq (NEB), and vector arms were sequenced before viral production and infection.

Cell Lines and Targeting. All lines were cultured in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 25 units of penicillin per milliliter and 25 μg of streptomycin per milliliter. The parental colorectal cancer cell lines HCT116, DLD-1, SW48, and RKO were purchased from the American Type Culture Collection). The HCT116 $p53^{+/-}$ and $p53^{-/-}$ lines were previously described (3) *TP53* rAAV knockout and knockin constructs were infected into parental cells and clones selected in 1 mg/mL geneticin as described (2). PCR primers used to verify homologous recombination and Cre-mediated excision are listed in Table S5.

Compounds and γ-Irradiation. BI-2536 (4), ON01910 (5), VX680 (6), AZD1152 (7, 8), and MLN8054 (9) were synthesized as previously described. Nutlin-3 was synthesized in the following way: Bis (4-chlorophenyl)ethylene diamine (GL Chemtech INT) was treated with 2-methoxy 4-isopropoxy benzaldehyde in dichloromethane, followed by NBS-mediated oxidation to generate the desired imidazoline in a single step in near-quantitative yield. Subsequent treatment with phosgene (Sigma), followed by 2-oxopiperazine (Sigma) afforded Nutlin-3 in nearly quantitative yield (Fig. S6). Nocodazole was purchased from (Sigma). γ-Irradiation was performed on cells grown to 70% confluency in 75-cm^2 flasks by exposing them to a ^{137}Cs source (Gammacell 40) until a dose of 12 Gy was delivered.

Western Blot Analysis. Protein extracts were prepared by washing the cells in PBS and lysing on ice in RIPA buffer [1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2)] supplemented with protease inhibitors (Complete protease inhibitor tablet; Roche). Extracts were briefly sonicated then assayed for total protein content with a BCA (bicinchoninic acid) assay (Pierce). Whole-cell lysates were subjected to SDS/PAGE (20 μg of protein per well) and transferred to Immobilon P membranes (Millipore) by using semidry transfer. The blots were incubated with antibodies directed at p53 (1:1,000; cat. no. SC-126 HRP; Santa Cruz Biotechnology), p21 (1:1,000; cat. no. OP64; Calbiochem) and Cyclophilin B (CypB; 1:2,000; cat. no. AB3565; Abcam). Secondary antibodies were HRP conjugated goat anti-mouse (1:10,000; cat. No. 115-035-008, The Jackson Laboratory) and mouse anti-rabbit (1:10,000; cat. No. 211-035-109, The Jackson Laboratory).

Microarray and qPCR. RNA was extracted by using a Plant RNeasy kit (Qiagen) and used for microarray analysis on the Agilent platform. In brief, 5 μg of total RNA was reverse transcribed by MMLV-RT by using an oligo(dT) primer that incorporated a T7 promoter sequence. The cDNA was then used as a template for in vitro transcription using T7 RNA polymerase and Cy-3- and Cy-5-labeled CTP. Samples from the same cell line with and without γ-irradiation were hybridized to the same slide. Labeled cRNA samples were used for hybridization to Agilent 4×44 K microarrays and scanned as directed by the manufacturer. Data were extracted by using Feature Extraction Software v8.1 (Agilent Technologies) under default settings for normalization. Microarrays with >5% feature or local background region outliers were excluded. Distributions of signal and background intensity of both red and green channels were examined with boxplot and maPlot by using the Bioconductor package. The ratios of each feature were calculated after background-subtraction and normalization. Features exhibiting significant changes in gene expression were selected based on PValueLogRatio calculated from the feature extraction software. For qPCR, 5 μg of total RNA was subjected to first-strand synthesis using SuperScript III reverse transcriptase with random hexamers as primers (Invitrogen). First-strand synthesis products were used as PCR templates. Primers are listed in Table S6.

Cellular Proliferation Assays. Cells were seeded in 384-well plates at a density of 100 cells per well (DLD-1 S241F/SIL), 200 cells per well (HCT116 cells, RKO, and SW48 +/+), 300 cells per well (HCT116 R248W/−), or 400 cells per well (DLD-1 +/SIL) in a total volume of 20 μL. Different plating densities were used to control for the different growth rates of cells of various genotype and to permit them to reach similar confluences in endpoint assays. Drugs were added in a volume of 20 μL, and γ-irradiation was performed ≈24 h after plating. Cells were then incubated for 4 days (HCT116 and RKO lines) or 6 days (DLD-1 and SW48) and lysed with 40 μL of a 1:750 dilution of SYBR green I (Invitrogen) in 1% Igepal in PBS (Sigma), then incubated at 37 °C for 24 h. Fluorescence was assayed with a FluoStar Galaxy fluorometer (BMG Technologies). For colony-formation assays, cells were trypsinized at various times after drug treatment and dilutions plated in 25-cm^2 tissue culture flasks. After 14 days of growth, colonies were stained with crystal violet and counted.

Studies in Mice. Five million HCT (p53 −/−) cells were injected s.c. into the flanks of female athymic nu/nu mice and allowed to grow for 3 weeks, reaching 300–400 mm^3 in volume. The animals were then randomly segregated into 4 arms. Over the course of 3 weeks, the first arm received 100 mg/kg BI-2536 (i.v., twice weekly); the second arm received 200 mg/kg Nutlin-3 (oral gavage 4 times weekly, at the same schedule as arm 3); the third arm received both 200 mg/kg Nutlin-3 (oral gavage 4 times weekly, at 24 and 4 h before BI-2536 treatment) and 100 mg/kg BI-2536 (i.v., twice weekly); the fourth arm was an untreated control group. Tumor volume was recorded every 24 h. BALB/c mice were treated with a single i.v. dose of BI-2536 (100 mg/kg) and Nutlin-3 (200 mg/kg administered orally at 4 h as well as 24 h before BI-2536). Approximately 50 μL of blood was obtained by tail vein puncture and analyzed on a Hemavet 950 FS instrument.

1. Topaloglu O, Hurley PJ, Yildirim O, Civin CI, Bunz F (2005) Improved methods for the generation of human gene knockout and knockin cell lines. *Nucleic Acids Res* 33(18):e158.
2. Rago C, Vogelstein B, Bunz F (2007) Genetic knockouts and knockins in human somatic cells. *Nat Protocols* 2(11):2734–2746.
3. Rago C, Vogelstein B, Bunz F, *et al.* (1998) Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. *Science* 282(5393):1497–1501.
4. Hoffmann MG, *et al.* (2004) Dihydropteridinones, method for the production and use thereof in the form of drugs. WO 2004076454.
5. Reddy EP, Reddy MVR (2003) US Patent 6,599,932 B1.
6. Charrier J-DM, Kay D, Miller A (Vertex Pharmaceuticals Incorporated) (2003). Processes for preparing 6-pyrazolylpyrimidines as inhibitors of protein kinase, in particular Aurora kinases, by nucleophilic substitution. WO 2004000833.
7. Heron NMJ, Frederic H, Pasquet GR, Mortlock AA (2004) Preparation of phosphonooxy quinazoline derivatives and their pharmaceutical use. WO 20004058781.
8. Mortlock AA, *et al.* (2007) Discovery, synthesis, and in vivo activity of a new class of pyrazoloquinazolines as selective inhibitors of aurora B kinase. *J Med Chem* 50(9):2213–2224.
9. Claiborne CFP, *et al.* (2005) Preparation of pyrimidoazepine derivatives and methods for inhibiting mitotic progression. US Pat. Appl pp 167.

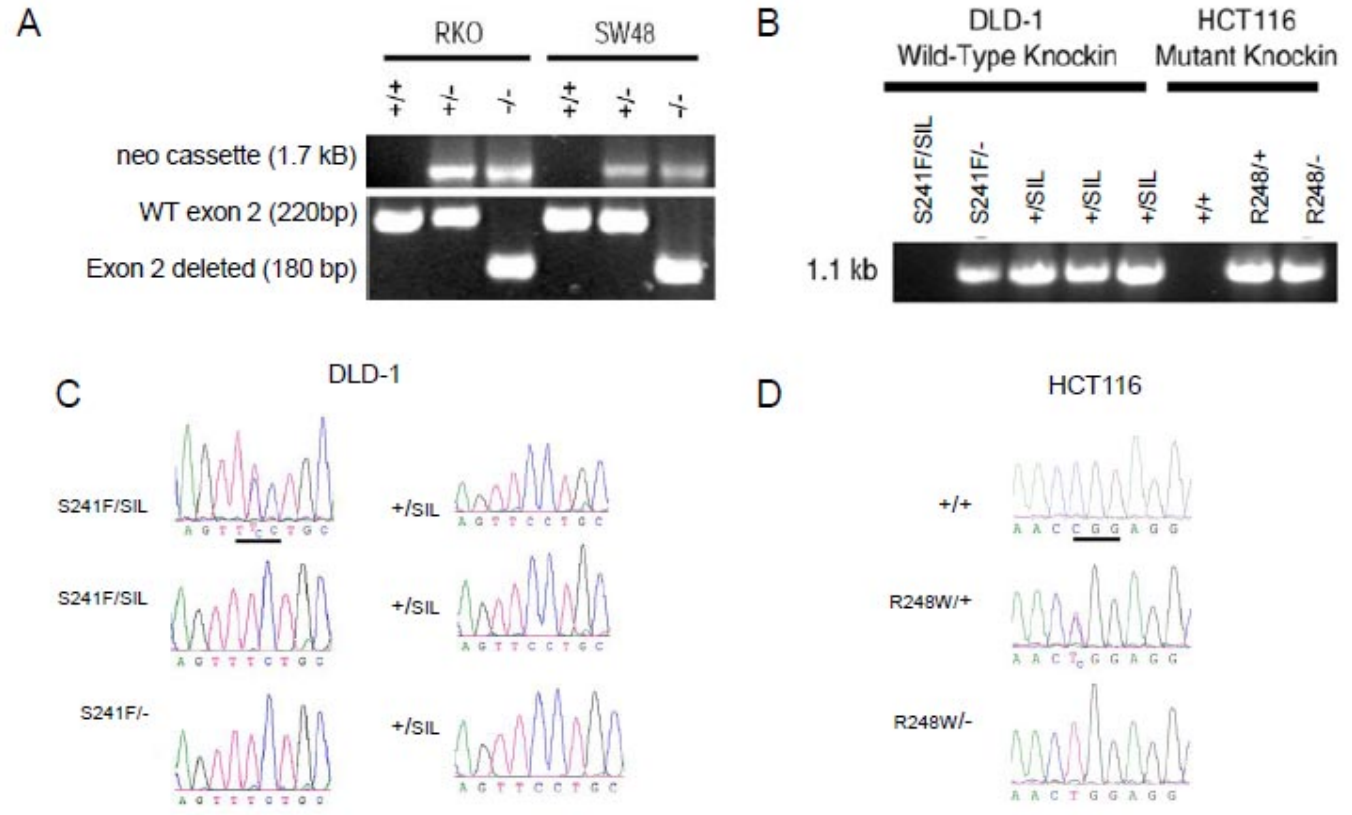

Fig. S1. Examples of targeting event confirmations (*A*) Deletion of exon 2 was confirmed by PCR with the primers shown in Fig. 1*A* and Table S3. (*B*) Integration of the knockin vectors at the *TP53* locus was confirmed by PCR with the primers shown in Fig. 1*B* and Table S3. (*C*) Sequencing chromatograms of DLD-1 cell lines of the indicated genotypes. The chromatogram of the parental line (S241/SIL genotype, upper left) was derived from PCR of genomic DNA, whereas all other sequences correspond to reverse-transcriptase PCR derived from cDNA templates. The underlined nucleotides denote codon 241 [TCC, serine (WT); TTC, phenylalanine]. Three independently derived clones of +/SIL are shown. (*D*) Sequencing chromatograms of HCT116 cell lines of the indicated genotypes. The underlined nucleotides denote codon 248 [CGG, arginine (WT); TGG, tryptophan].

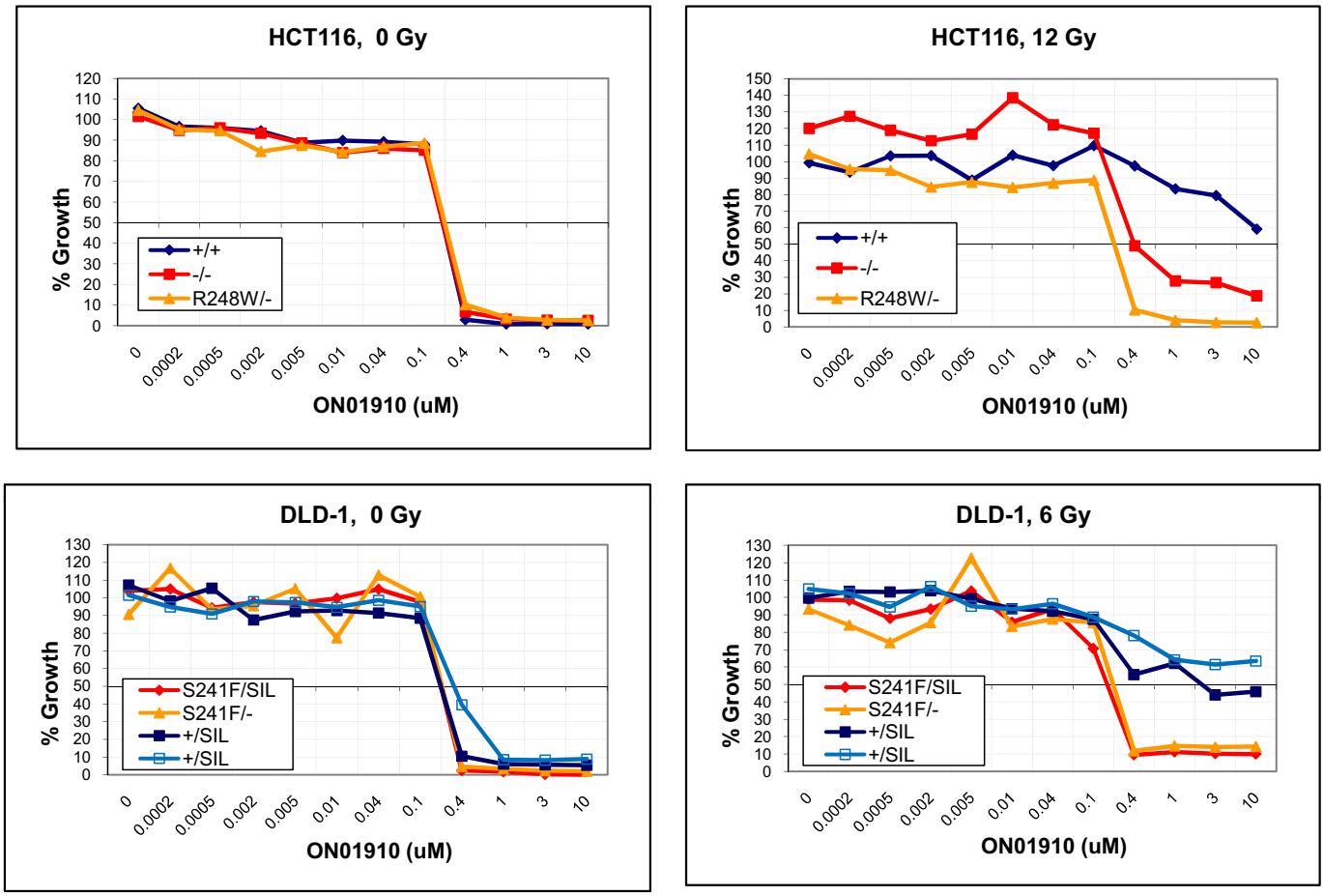

Fig. S2. Response of the indicated lines to increasing doses of the PLK1 inhibitor ON01910 in the absence or presence of ionizing radiation. The 2 lines marked +/SIL were independently generated clones.

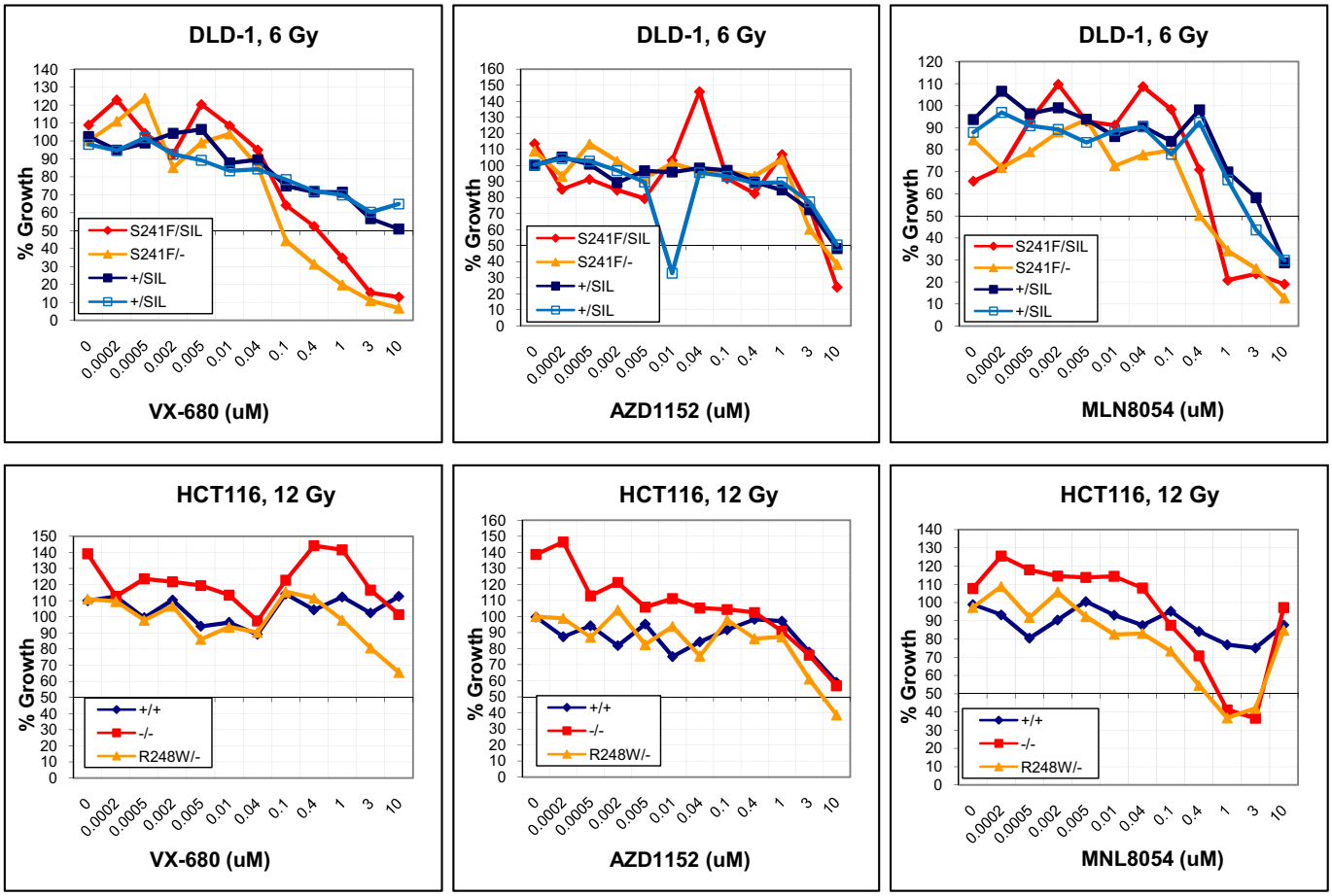

Fig. S3. Response of the indicated lines to increasing doses of the AURKB inhibitors VX680, AZD1152, or MLN8054 in the absence or presence of γ-radiation. The 2 lines marked +/SIL were independently generated clones.

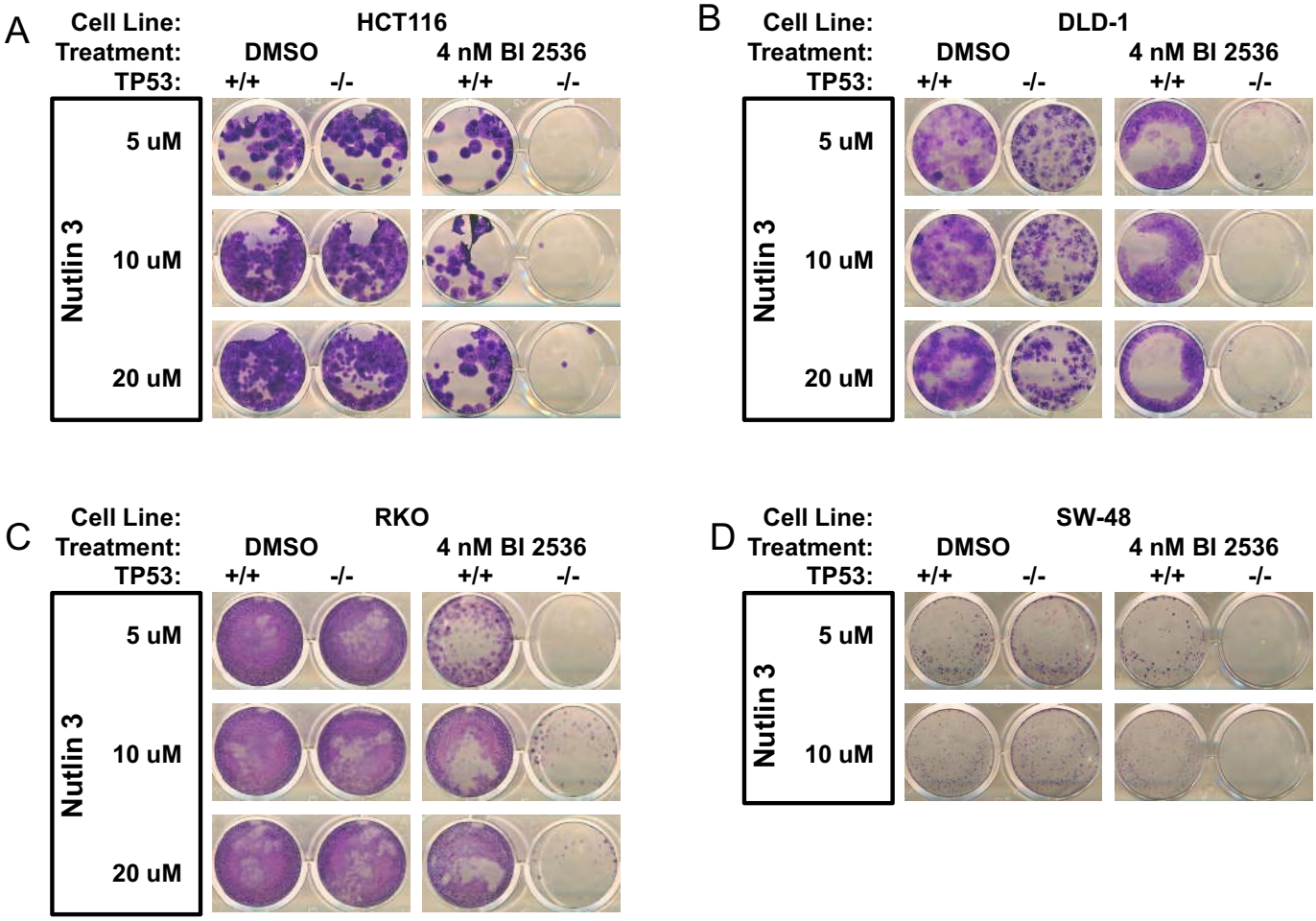

Fig. S4. Colony-formation assays in the indicated lines after exposure to BI 2536 and various doses of Nutlin-3. BI 2536 was dissolved in DMSO, and equivalent concentrations of DMSO were used as controls.

Synthetic scheme for Nutlin 3

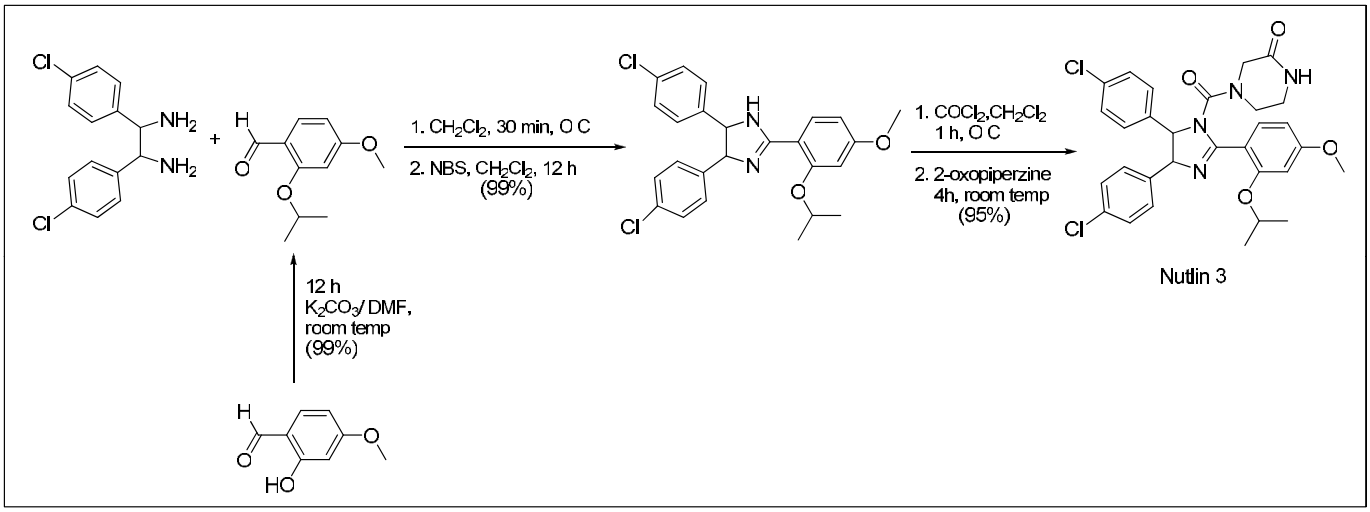

Fig. S5. Scheme for synthesis of Nutlin-3.

Other Supporting Information Files

Table S1 (PDF)
Table S2 (PDF)
Table S3 (PDF)
Table S4 (PDF)
Table S5 (PDF)
Table S6 (PDF)