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Modeling the Etiology of p53-mutated Cancer Cells*

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p53 gene mutations are among the most common alterations in cancer. In most cases, missense mutations in one *TP53* allele are followed by loss-of-heterozygosity (LOH), so tumors express only mutant p53. *TP53* mutations and LOH have been linked, in many cases, with poor therapy response and worse outcome. Despite this, remarkably little is known about how *TP53* point mutations are acquired, how LOH occurs, or the cells involved. Nutlin-3a occupies the p53-binding site in MDM2 and blocks p53-MDM2 interaction, resulting in the stabilization and activation of p53 and subsequent growth arrest or apoptosis. We leveraged the powerful growth inhibitory activity of Nutlin-3a to select p53-mutated cells and examined how *TP53* mutations arise and how the remaining wild-type allele is lost or inactivated. Mismatch repair (MMR)-deficient colorectal cancer cells formed heterozygote (p53 wild-type/mutant) colonies when cultured in low doses of Nutlin-3a, whereas MMR-corrected counterparts did not. Placing these heterozygotes in higher Nutlin-3a doses selected clones in which the remaining wild-type *TP53* was silenced. Our data suggest silencing occurred through a novel mechanism that does not involve DNA methylation, histone methylation, or histone deacetylation. These data indicate MMR deficiency in colorectal cancer can give rise to initiating *TP53* mutations and that *TP53* silencing occurs via a copy-neutral mechanism. Moreover, the data highlight the use of MDM2 antagonists as tools to study mechanisms of *TP53* mutation acquisition and wild-type allele loss or silencing in cells with defined genetic backgrounds.

p53 is a stress-responsive tumor suppressor encoded by the *TP53* gene. *TP53* mutations are among the most common alterations in cancer (1). In most cases, missense mutations in one *TP53* allele are followed by loss-of-heterozygosity (LOH),[2] so tumors express only mutant p53. *TP53* mutations and LOH have been linked, in many cases, with poor therapy response, increased tumor aggressiveness, and decreased long term survival (2–5). Despite this, remarkably little is known about how *TP53* point mutations are acquired, how LOH occurs, or the cells involved.

Genomic instability is a hallmark of colorectal cancer (CRC) and is divided into the following two classes: chromosome instability (CIN) and microsatellite instability (MSI) (6–9). Tumors with CIN include most (~85%) of all CRCs and are characterized by gross karyotypic changes, including alterations in chromosome number and structure (8). The CIN phenotype appears to result, at least in part, from truncating mutations in the tumor-suppressor protein adenomatous polyposis coli, a protein that controls proper chromosome segregation in mitosis (6, 8). Tumors with MSI account for the remaining 15% of CRCs. MSI tumors, as the name implies, are characterized by rapid changes (instability) in the length of short repetitive microsatellite sequences in the genome (10). In contrast to CIN, MSI tumors have a relatively stable karyotype, but instead harbor multiple frameshift and missense mutations that disrupt the normal function of proto-oncogenes or tumor suppressors. The MSI phenotype results from a deficiency in DNA mismatch repair (MMR) due to mutation or silencing of one or more MMR genes (6, 8, 11, 12). Under normal conditions, the MMR machinery helps maintain genetic stability by repairing mismatched bases or insertion-deletion loops that arise during DNA replication (13, 14). A deficiency in MMR creates an environment in which cells rapidly accumulate mutations, including those that drive cancer development (15–17). The core MMR machinery in humans consists of three heterodimeric protein complexes involved in either mismatch recognition (hMUTSα and hMUTSB) or repair (hMUTL). hMUTSα (composed of hMSH2 and hMSH6) recognizes and preferentially binds single nucleotide mismatches, whereas hMUTSB (composed of hMSH2 and hMSH3) recognizes and preferentially binds insertion-deletion loops (18–20). Upon mismatch recognition, the hMUTL complex, composed of hMLH1 paired with hPMS2 or hPMS1, is recruited to the lesion where its enzymatic ATPase and endonuclease activities are required to complete the repair process (11, 12, 21). Interestingly, *TP53* mutations in CRC are *less* frequent in MSI tumors (10–20% of cases) than CIN tumors (~50–60% of cases) (22–25), suggesting MMR deficiency does not give rise to *TP53* mutations in MSI tumorigenesis (25–27). An alternative explanation is that other growth regulatory genes may be more susceptible to mutation in an MMR-deficient background than *TP53*, and mutation of these other genes may reduce selective pressure to mutate *TP53* (25).

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[2] The abbreviations used are: LOH, loss-of-heterozygosity; cnLOH, copy-neutral LOH; CRC, colorectal cancer; MMR, mismatch repair; CIN, chromosome instability; MSI, microsatellite instability; SNP, single nucleotide polymorphism; Aza, 5′-aza-2′-deoxycytidine; h, human; 6TG, 6-thioguanine; HDAC, histone deacetylase; Chr., chromosome; mut, mutant; SAHA, suberoylanilide hydroxamic acid.

The *TP53* gene is located on the short arm of chromosome 17. Following mutation in one *TP53* allele, LOH could result if the remaining wild-type allele or the short arm of chromosome 17 is deleted. In contrast, copy-neutral LOH (cnLOH) refers to the case when there no net change in copy number of the affected allele. Thus, in the case of *TP53*, copy-neutral LOH could occur if the remaining wild-type allele is silenced through epigenetic mechanisms. Examples of *TP53* being silenced through epigenetic mechanisms include glioma cells in which *TP53* is repressed through DNA promoter methylation (28) and senescent keratinocytes in which *TP53* silencing is associated with reduced histone acetylation in the *TP53* promoter (29). Alternatively, cnLOH could also occur if the wild-type *TP53* allele is converted to a mutant allele through a recombination or gene conversion event. In early studies by Vogelstein and others (30–32), *TP53* LOH was examined in CIN CRC tumor sets using a combination of DNA sequencing, restriction fragment length polymorphism analysis, as well as Southern and Northern blotting. The conclusion from these studies was that in the majority of cases, mutation in one *TP53* allele is followed rapidly by deletion/loss of the remaining wild-type *TP53* allele (chromosome 17p deletion). These findings supported perfectly Knudsen's two-hit model for tumor suppressor loss. Recently developed single nucleotide polymorphism (SNP) microarrays have facilitated LOH studies because they allow simultaneous detection of copy number and genotype changes. Melcher *et al.* (33) used SNP arrays to examine and compare genome-wide LOH in CIN and MSI tumors. CIN tumors displayed mostly classic LOH (mutation followed by allele loss) at multiple genomic sites, whereas MSI tumors displayed LOH that was mostly copy-neutral. These findings suggest MSI tumors may be especially prone to cnLOH mechanisms (*e.g.* gene silencing), whereas CIN tumors may be more prone to chromosome-unstable LOH mechanisms (*e.g.* allele or chromosome deletion). To date, the mechanism for *TP53* LOH in MSI CRC tumor cells has not determined.

Wild-type p53 is controlled by MDM2, an E3 ubiquitin ligase that binds p53 and promotes its degradation (34, 35). MDM2 antagonists are being developed as therapeutics (36). Nutlin-3a (Nutlin) occupies the p53-binding site in MDM2 and blocks p53-MDM2 interaction, resulting in the stabilization and activation of p53 and subsequent growth arrest/apoptosis (36, 37). In this study, we leveraged the powerful growth inhibitory activity of Nutlin-3a to select p53-mutated cells and examine how *TP53* mutations arise and how the remaining wild-type allele is lost or inactivated. MMR-deficient CRC cells formed heterozygote (p53 wild-type/mutant) colonies when cultured in low doses of Nutlin-3a, although MMR-corrected counterparts did not. MMR-deficient CRC cells also showed an elevated *TP53* mutation acquisition rate compared with MMR-proficient counterparts. These data indicate MMR deficiency accelerated *TP53* mutation acquisition and gave rise to the initiating mutation in one *TP53* allele. Placing heterozygotes (p53 wild-type/mutant) in higher doses of Nutlin-3a selected clones in which the remaining wild-type *TP53* was silenced. Our data suggest that in all cases silencing of the wild-type allele results from a novel mechanism that does not involve DNA methylation, histone methylation, or histone deacetylation. These data indicate MMR deficiency in CRC can give rise to initiating *TP53* mutations and that *TP53* silencing occurs via a copy-neutral mechanism. Moreover, the data highlight the use MDM2 antagonists as tools to study mechanisms of *TP53* mutation acquisition and wild-type allele loss or silencing in cells with defined genetic backgrounds.

Experimental Procedures

Cell Lines and Culture Conditions—HCT116 $p53^{+/+}$ and HCT116 $p53^{+/-}$ cells (38) were obtained from Dr. Bert Vogelstein (the Johns Hopkins University). HCT116 cells that were MMR-corrected by transfer of chromosome 3 (HCT116+Chr.3) or transfer of chromosomes 3 and 5 (HCT116+Chr.3+5) were gifted by Ajay Goel (39). All cells were grown in McCoy's 5A medium with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were plated 24 h before being exposed to the indicated dose of Nutlin-3 (Sigma), BIX-01294 (Santa Cruz Biotechnology), GSK126 (Active Biochem), 5′-aza-2′-deoxycytidine (Sigma), and AR42 or SAHA (Selleckchem).

Immunoblotting—Whole cell extracts were prepared by resuspending cell pellets in lysis buffer (150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 50 mM Tris, pH 7.5), resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (ThermoFisher). The following antibodies were used (catalogue number, lot number, and the dilution used): rabbit anti-p21 (sc-756, lot no. E2208, 1:500), mouse anti-p53 (sc-126, lot no. C1815, 1:500), and mouse anti-β-actin (sc-47778, lot no. K2713, 1:500) were from Santa Cruz Biotechnology; rabbit anti-acetylated histone H3 (K9) (9649, lot no. 11, 1:1000), rabbit anti-dimethyl-histone H3 (K9) (4658, lot no. 5, 1:1000), and rabbit anti-trimethyl-histone H3 (K27) (9733, lot no. 8, 1:1000) were from Cell Signaling. Primary antibodies were detected with goat anti-mouse (62-6520, lot no. QG215721, 1:10,000) or goat anti-rabbit (65–6120, lot no. QK229568, 1:10,000) secondary antibodies conjugated to horseradish peroxidase (Life Technologies, Inc.), using Clarity chemiluminescence (Bio-Rad).

cDNA and Genomic DNA Isolation and Sequencing—Total RNA was isolated from cells using the RNeasy® mini kit (Qiagen) according to manufacturer's protocol. RNA (1 μg) was reverse-transcribed into single-stranded cDNA using SuperScript III First-Strand Synthesis SuperMix kit (Life Technologies, Inc.) according to the manufacturer's protocol. *TP53* was amplified using the following primers: p86, 5′-GTGACACGCTTCCCTGGATTGG-3′, and p1447, 5′-AATGGAAGTCCTGGGTGCTTCTGA-3′. The PCR product was isolated using the GeneJET PCR purification kit (Thermo Scientific) and sent out for Sanger sequencing. The following primers were used for sequencing: forward primers p86, 5′-GTGACACGCTTCCCTGGATTGG-3′; p479, 5′-ATTCTGGGACAGCCAAGTCTGTGA-3′; and p774, 5′-ACATAGTGTGGTGGTGCCCTATGA-3′; reverse primers p630, 5′-CTGCTTGTAGATGGCCATGGCG-3′, and p1447, 5′-AATGGAAGTCCTGGGTGCTTCTGA-3′.

Genomic DNA was isolated using the Maxwell® 16 Cell DNA purification kit (Promega). Exons 1–11 were sequenced with the following primers: exon 1 forward 5′-CACAGCTCTGG-

CTTGCAGA-3′ and reverse 5′-AGCGATTTTCCCGAGCTGA-3′; exons 2 and 3 forward 5′-TCTCATGCTGGATCCCCACT-3′ and reverse 5′-AGTCAGAGGACCAGGTCCTC-3′; exon 4 forward 5′-TGAGGACCTGGTCCTCTGAC-3′ and reverse 5′-AGAGGAATCCCAAAGTTCCA-3′; exons 5 and 6 forward 5′-TGTTCACTTGTGCCCTGACT-3′ and reverse 5′-TTAACCCCTCCTCCCAGAGA-3′; exon 7 forward 5′-CTTGCCACAGGTCTCCCCAA-3′ and reverse 5′-AGGGGTCAGCGGCAAGCAGA-3′; exons 8 and 9 forward 5′-TTGGGAGTAGATGGAGCCT-3′ and reverse 5′-AGTGTTAGACTGGAAACTTT-3′; exon 10 forward 5′-CAATTGTAACTTGAACCATC-3′ and reverse 5′-GGATGAGAATGGAATCCTAT-3′; and exon 11 forward 5′-GTCATCTCTCCTCCCTGCTT-3′ and reverse 5′-CAGTCTCCAGCCTTTGTTCC-3′. *TP53* promoter was sequenced using the following primers: Pr1 forward 5′-GTTAGCCCCAGTCCTTACCA-3′ and reverse 5′-GGGAGATGAAGTGTGAGGT-3′; Pr2 forward 5′-GAAGTTCTCAGGTTGGGTGC-3′ and reverse 5′-AGAAGAGGAGGGAAGCACAG-3′, and Pr3 forward 5′-GTGGCATCAGTTCAGAGTCC-3′ and reverse 5′-GCCTGGAGAATGAGATGCAG-3′.

FISH Analysis—Cells were harvested and washed with PBS and then incubated with hypotonic solution (2 parts of 0.075 M KCl and 1 part of 0.7% sodium citrate) for 20 min at 37 °C. The cells were then fixed in fixative (3 parts methanol and 1 part acetic acid). The slides were prepared and aged at 55 °C for 3 min on a thermobrite. The slides were incubated with 0.05% Pepsin (Fisher catalogue no. P53-100) for 16 min at 37 °C and then washed with PBS and dehydrated in 70, 85, and 100% ethanol for 1 min each at room temperature. The probe Vysis LSI TP53 Spectrum Orange/CEP 17 Spectrum Green (Abbott Molecular Inc. catalogue no. 01N17-020) was added and hybridized at 73 °C for 5 min and 37 °C for 18 h. Slides were washed with SSC buffer (Gibco catalogue no. 15557-044) with 0.5% Nonidet P-40 (Fisher catalogue no. P53-100) for 2 min at 73 °C and counterstained with DAPI II (Abbott Molecular Inc. catalogue no. 30-804818). Slides were examined under fluorescent microscope.

Treatment of Cell Lines with 5′-Aza-2′-deoxycytidine—Colon cancer cells were seeded in 60-mm dishes and incubated in culture media with 10 μM 5′-aza-2′-deoxycytidine (Aza) (Sigma) for 3 days. *TP53* reactivation was verified by Sanger sequencing.

Real Time PCR—Following cDNA synthesis, real time PCR was performed using EvaGreen quantitative PCR MasterMix-ROX (Midwest Scientific, St. Louis, MO) on an ABI® 7300 real time PCR system. The following primers were used: *ESR1* forward 5′-GGGAAGTATGGCTATGGAATCTG-3′ and reverse 5′-TGGCTGGACACATATAGTCGTT-3′; *TKTL1* forward 5′-TGGACAATCTTGTGGCAATCTT-3′ and reverse 5′-CAGCGCCTCTGATAGATGTTTAT-3′; *p21* forward 5′-ACTCTCAGGGTCGAAAACGG-3′ and reverse 5′-CCTCGCGCTTCCAGGACTG-3′; *p53* forward 5′-GAGGTTGGCTCTGACTGTACCC-3′ and reverse 5′-TCCGTCCCAGTAGATTACCAC-3′; and β-actin forward 5′-TCGTGCGTGACATTAAGGAG-3′ and reverse 5′-GTCAGGCAGCTCGTAGCTCT-3′.

Bisulfite Sequencing—HCT116, heterozygotes, and Mutant p53/WT*silenced* clones' genomic DNA were modified by sodium bisulfite as described previously with minor changes (40). Briefly, genomic DNA (1 μg) was digested using EcoRI overnight and then denatured with 0.3 M NaOH for 10 min at 37 °C. DNA was modified with 0.5 mM hydroquinone (Sigma) and 3.1 M sodium bisulfite (Sigma) at 50 °C for 6 h. The modified DNA was then purified using the Wizard DNA-Clean Up kit (Promega Corp., Madison, WI) according to the manufacturer's protocol and treated with 0.3 M NaOH for 20 min at 37 °C. DNA was precipitated overnight with a mixture of glycogen, ammonium acetate, and absolute ethanol at −80 °C. Nested PCR was performed to amplify the *TP53* promoter region. The following primers were used: first reaction, forward 5′-TGttt-TtAtAGtTtTGGtTTGtAGAATTT-3′ and 5′-TaACTCAaAa-AaaACTCATCAAaTTCAaT-3′, and second reaction, 5′-GtA-GAATTTTttAttttAAAATGTTAGTATt-3′ and 5′-CAAaTT-CAaTCAaaAaCTTACCCAATCCAa-3′; lowercase letters indicate bisulfite-converted nucleotides. The amplified DNA fragment was cloned into the pCR4-TOPO TA cloning vector (Life Technologies, Inc.), and 9–10 individual clones for each cell line were sequenced.

Long Range PCR and Verification of the TP53 Promoter and Genomic Sequences Are Intact—Genomic DNA from HCT116, WT/R267W, and WTsilenced/R267W was isolated using genomic DNA mini kit (IBI Scientific). Three different regions of the *TP53* gene were amplified using various different primers and PCR kits. The promoter region of TP53 was amplified from −1000 and +288 with the following primers: promoter forward3, 5′-TGGCATCAGTTCAGAGTCCG-3′, and exon 1 reverse, 5′-AGCGATTTTCCCGAGCTGA-3′. The PCR was carried out using the PCR master mix kit (ThermoFisher) as follows: 95 °C; 5 min, 40 cycles of denaturation 95 °C; 30 s, annealing at 57 °C; 45 s, extension at 72 °C; 1.5 min, followed by a final extension at 72 °C; 10 min. PCR product was verified by running 5 μl of reaction on 2% agarose gel at 100 V for 1 h. Once the product was verified, the PCR product was purified using GeneJet PCR purification kit (ThermoFisher) and sent off for sequencing. The primers used for sequencing are as follows: forward, F3 5′-TGGCATCAGTTCAGAGTCCG-3′ and F4 5′-AGAAGGTTTCCCGTTCCCATC-3′; reverse exon 1 reverse 5′-AGCGATTTTCCCGAGCTGA-3′ and R3 5′-AGCCTGGAGAATGAGATGCAG-3′. The following two reactions were performed using the Qiagen® LongRange PCR kit. Intron 1 of the *TP53* gene was amplified using the following primers: forward 5′-CTTTCCACGACGGTGACACGCTT-3′ and reverse 5′-AGACAAGAGCAGAAAGTCAGTCCCA-3′. The PCR was as follows: 93 °C; 3 min, 10 cycles of denaturation 93 °C; 15 s, annealing at 60 °C; 30 s, extension at 68 °C; 11 min; followed by 28 cycles of denaturation 93 °C; 15 s, annealing at 60 °C; 30 s, and extension at 68 °C; 11 min plus an additional 20 s after each cycle. PCR product were run on 0.7% agarose gel overnight at 20 V. Amplification of exon 2 to intron 8 of the *TP53* gene was performed with the following primers: forward 5′-TCTCATGCTGGATCCCCACT-3′ and reverse 5′-CTCGCTTAGTGCTCCCTGGG-3′. The PCR was as follows: 93 °C; 3 min, 35 cycles of denaturation 93 °C; 15 s, annealing at 58 °C; 30 s, extension at 68 °C; 4 min. PCR product was verified by running 5 μl of reaction on 0.7% agarose gel at 40 V for 4 h. Once product was verified, PCR product was purified using

TABLE 1

p53 Mutants selected in Nutlin

HCT116 p53$^{+/+}$ and HCT116 p53$^{+/-}$ cells were cultured in the continuous presence of 2.5, 5, or 10 μM Nutlin for 2 weeks. In the presence of 10 μM Nutlin, HCT116 p53$^{+/+}$ cells formed 1–5 colonies per 10,000 cells plated, and HCT116 p53$^{+/-}$ cells formed ~1 colony per 1000 cells plated. Individual colonies were picked and expanded in the absence of Nutlin. mRNA from the expanded cells was reverse-transcribed into cDNA, which was then PCR-amplified with *TP53* specific primers. The amplified *TP53* cDNA was then sequenced. Listed in the table are the individual *TP53* mutations identified in each clone. Note the Nutlin-resistant clones isolated from HCT116 p53$^{+/+}$ cells had acquired a heterozygous mutation in one *TP53* allele, while leaving the remaining wild-type allele intact. This was evidenced by cDNA sequencing results that showed overlap of both wild-type and mutated *TP53* cDNA (Fig. 4). Note also that the majority of *TP53* mutations identified in the Nutlin-selected clones were transitions.

	HCT116 p53$^{+/+}$ clones				HCT116 p53$^{+/-}$ clones (all selected in 10 μM Nutlin)			
Selected in Nutlin	Clone	p53 status	Mutation	Type	Clone	p53 status	Mutation	Type
μM								
2.5	HN3	WT/V203E	G*T*G-G*A*G	Transversion	HCT+/− NR1	Y220C	T*A*T-T*G*T	Transition
5	HN5	WT/R248Q	C*G*G-C*A*G	Transition	HCT+/− NR2	Frameshift mutation K372		
5	HN5C10	WT/R267W	C*G*G-*T*GG	Transition	HCT+/− NR3	R72C	C*G*C-*T*GC	Transition
5	HN5C8	WT/R273H	C*G*T-C*A*T	Transition	HCT+/− NR5	V272A	G*T*G-G*C*G	Transition
5	HN5C1	WT/R175C	C*G*C-*T*GC	Transition	HCT+/− NR6	T256A	*A*CA-*G*CA	Transition
		WT/S240G	*A*GT-*G*GT	Transition	HCT+/− NR7	V217G	G*T*G-G*G*G	Transversion
10	HN9	WT/T125M	AC*G*-A*T*G	Transition	HCT+/− NR8	A138V	G*C*C-G*T*C	Transition
10	HN4	WT/P278H	C*C*T-C*A*T	Transversion	HCT+/− NR10	Y220C	T*A*T-T*G*T	Transition
10	HN11	WT/R273C	C*G*T-*T*GT	Transition	HCT+/− NR12	S260F	T*C*C-T*T*C	Transition
					HCT+/− NR14	T256A	*A*CA-*G*CA	Transition
					HCT+/− NR15	Y220C	T*A*T-T*G*T	Transition
					HCT+/− NR16	R282Q	C*G*G-C*A*G	Transition
					HCT+/− NR18	A161V	G*C*C-G*T*C	Transition
					HCT+/− NR19	Y220C	T*A*T-T*G*T	Transition
					HCT+/− NR20	Frameshift mutation P177		

GeneJet PCR purification kit (ThermoFisher) and sent off for sequencing. To verify whether SNPs known to be present in intron 7 were present, the following primers were used: forward 5′-CAAGGCGCACTGGCCTCATC-3′ and reverse 5′-CTCGCTTAGTGCTCCCTGGG-3′.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed using the MAGnify ChIP System (Invitrogen) according to the manufacturer's instructions. Briefly, cells fixed at room temperature for 10 min in the culture medium containing 1% formaldehyde and H3K27me3 (07-449) and H3Ac (06–599) antibodies (EMD Millipore, Billerica, MA) were coupled to Dynabeads, and the antibody-coupled Dynabeads were incubated with the sheared chromatin. Chromatin·antibody·Dynabeads complexes were then washed with washing buffers, and the cross-links were reversed in the presence of proteinase K. The uncross-linked DNA was purified using the DNA purification magnetic beads. The purified DNA fragments were amplified using real time quantitative PCR. Primer sets that target the p53 promoter regions can be found elsewhere (29).

Results

Nutlin Selects HCT116 Cells That Have Acquired Point Mutations in One TP53 Allele—TP53 mutations are found in ~50% of all human cancers. However, little is known about how *TP53* mutations are acquired. Nutlin-3a (Nutlin) causes growth arrest or apoptosis in p53 wild-type cells (36, 37). Thus, only p53-null or p53-mutant cells can grow and form colonies in the presence of Nutlin. We speculated colony formation in the continued presence of Nutlin would select clones from a p53 wild-type background that had acquired inactivating mutations in *TP53*. HCT116 is an MSI colon cancer cell line that expresses wild-type p53. HCT116 are MMR-deficient due to lack of *hMLH1* and *hMSH3* (39, 41). HCT116 p53$^{+/+}$ cells have both *TP53* alleles intact (38). We cultured HCT116 p53$^{+/+}$ cells in increasing doses of Nutlin (2.5, 5, or 10 μM) and isolated surviving clones. When HCT116 p53$^{+/+}$ cells were cultured in continuous Nutlin (10 μM), they formed Nutlin-resistant (Nut^r) colonies at a rate of ~1 in 10,000 cells plated. In HCT116 p53$^{+/-}$ cells, one *TP53* allele has been deleted by recombination (38). When HCT116 p53$^{+/-}$ cells were cultured in the same Nutlin dose (10 μM), they formed Nutlin-resistant (Nut^r) colonies at a rate of ~1 in 1000 cells plated. We used cDNA sequencing to address whether the selected Nutlin-resistant clones had *TP53* mutations. A total of 23 Nutlin-resistant clones from the p53$^{+/+}$ and p53$^{+/-}$ cells were examined. As shown in Table 1, all 23 clones had acquired missense mutations in *TP53*, mostly transitions (18/23 cases) followed by transversions (3/23 cases) and frameshifts (2/23 cases). The vast majority of *TP53* mutations in human cancers occur within the DNA binding domain of p53 (amino acids 100–300). In our study, 22 of the 23 mutations we identified were in the DNA binding domain. The only mutation lying outside the DNA binding domain was a frameshift mutation at lysine 372 (Lys-372). At least some of the p53 mutations we identified occurred at "hot spot" residues commonly mutated in cancer. These included mutations at hot spot residues Tyr-220, Arg-248, Arg-273, Pro-278, and Arg-282 (42). Other mutations (*e.g.* T125M and R267W) have also been observed in different cancers (43, 44). All other Nutlin-resistant clones isolated from HCT116 p53$^{+/+}$ cells had acquired a single heterozygous mutation in one *TP53* allele, while leaving the remaining wild-type alleles intact. This was evidenced by cDNA sequencing results that showed that in these clones both wild-type and mutated *TP53* mRNAs were expressed (see Fig. 4*A* as example).

MMR-deficient Cells Acquire TP53 Mutations at a Higher Rate than Proficient Counterparts—Because HCT116 are MMR-deficient, we speculated the *TP53* mutations we identified may have arisen from defects in MMR. To examine this, we obtained HCT116 cells in which the MMR defect has been corrected by transfer of individual chromosomes. Transfer of chromosome 3 into HCT116 cells restores MLH1 expression and completely restores MMR at mono- and dinucleotide

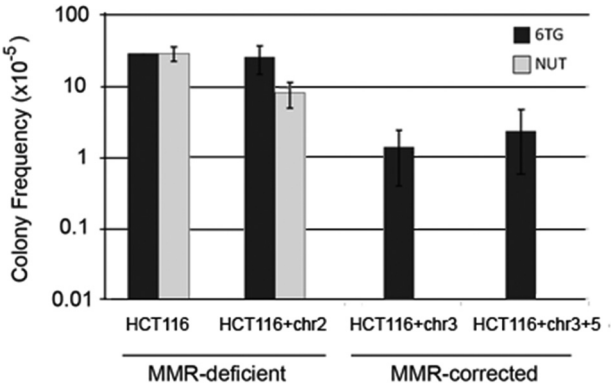


FIGURE 1. **MMR-corrected HCT116 cells do not form Nutlin-resistant colonies.** Colony-forming ability in the presence of 6TG or Nutlin (*Nut*) (10 μM) was determined in MMR-deficient (HCT116, HCT116 + chromosome 2) and MMR-corrected (HCT116 + chromosome 3, HCT116 + chromosomes 3 + 5) cells. MMR-corrected cells formed 6TG-resistant colonies at ~10-fold lower rate than MMR-deficient cells. Note, only MMR-deficient cells formed Nutlin-resistant, *TP53*-mutated colonies (mutations are listed in Table 1).

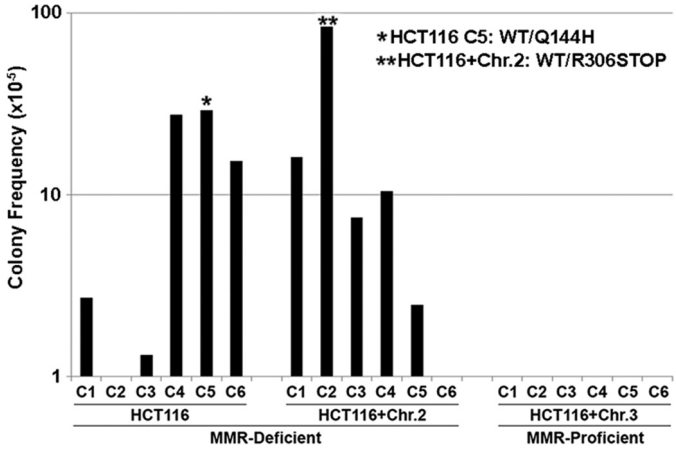


FIGURE 2. **MMR deficiency increases *TP53* mutation acquisition.** MMR-deficient (HCT116, HCT116 + chromosome 2) and MMR-corrected (HCT116 + chromosome 3) cells were plated at single cell density in the absence of Nutlin and allowed to form colonies. Individual colonies were picked and expanded to 2×10^6 cells. At this time, 1×10^6 cells were plated in the continuous presence of 10 μM Nutlin, and colony-forming ability was assessed and plotted. Only MMR-deficient HCT116 and HCT116 + chromosome 2 colonies contained cells capable of colony formation in Nutlin. cDNA sequencing of selected Nutlin-resistant colonies confirmed they were *TP53* mutated (*, **).

repeats (15, 41, 45). Transfer of chromosome 5 into HCT116 cells restores MSH3 expression and corrects a form of MSI called EMAST (Elevated Microsatellite Instability at Selected Tetranucleotide repeats) (46). Transfer of chromosome 2 into HCT116 cells does not restore MMR activity, and these cells served as a control. MMR-deficient (HCT116 and HCT116+Chr.2) and MMR-corrected (HCT116+Chr.3 and HCT116+Chr.3+5) cells were plated in continuous Nutlin (10 μM), and colony formation was assessed. As shown in Fig. 1, MMR-deficient HCT116 and HCT116+Chr.2 cells formed Nutlin-resistant colonies, but MMR-corrected cells did not. We used colony formation in the presence of 6-thioguanine (6TG) to confirm transfer of chromosomes 3 and 5 restored MMR. Mutations at the *HPRT* locus allow growth in 6-thioguanine (15). Glaab *et al.* (15) found MMR corrected HCT116+Chr.3 cells acquired HPRT mutations at a decreased rate and therefore formed ~10-fold fewer 6TG-resistant colonies than MMR-deficient counterparts. Consistent with this, MMR-corrected HCT116+Chr.3 and HCT116+Chr.3+5 cells in our study formed ~10-fold fewer 6TG^r colonies than MMR-deficient HCT116 and HCT116+Chr.2 cells (Fig. 1).

The results from Fig. 1 suggest MMR-deficient cells may acquire *TP53* mutations at a higher rate than MMR-proficient cells. However, the ability to form colonies in Nutlin may reflect the number of *TP53*-mutated cells already present in the population, but not necessarily the rate at which the cells acquire mutations in *TP53.* Therefore, we sought further evidence that MMR-deficient cells acquire *TP53* mutations at a faster rate than MMR proficient/corrected counterparts. HCT116 and HCT116+Chr.2 cells formed Nutlin-resistant *TP53*-mutated cells at a rate of 1–5 cells per 10,000 cells plated (Fig. 1). This suggests that, at most, 1 in 2000 cells within the HCT116 and HCT116+Chr.2 populations may already be *TP53*-mutated and Nutlin-resistant, whereas the remaining cells (nearly 100%) would be *TP53* wild-type and Nutlin-sensitive. MMR-deficient (HCT116 and HCT116+Chr.2) and MMR-corrected (HCT116+Chr.3 and HCT116+Chr.3+5) cells were plated at single cell density in the absence of Nutlin and allowed to form colonies. Individual colonies were then picked and expanded to 2×10^6 cells. At this time, 1×10^6 cells were plated in the continuous presence of Nutlin (10 μM), and Nutlin-resistant colony-forming ability was assessed. As shown in Fig. 2, individual clones from MMR-deficient HCT116 and HCT116+Chr.2 formed Nutlin-resistant colonies with varying ability. cDNA sequencing of selected clones confirmed they were *TP53*-mutated (marked by *asterisk* in Fig. 2). In contrast, individual clones from the MMR-corrected HCT116+Chr.3 and HCT116+Chr.3+5 cells did not form Nutlin-resistant colonies. Based on these results, we conclude that the MMR-deficient cells had most likely acquired *TP53* mutations during the expansion from a single cell to 2×10^6 cells that allowed them to form Nutlin-resistant colonies, and this did not happen in MMR-corrected cells. An alternative possibility is that MMR-deficient cells acquired *TP53* mutations shortly after the 1×10^6 cells were plated in the presence of Nutlin, and this did not occur in the MMR-corrected cells. The results support the notion that MMR deficiency increases the rate at which cells acquire *TP53* mutations.

p21 Levels Are Increased in p53 WT/mut Heterozygotes Exposed to Increasing Nutlin Doses—Nutlin-resistant clones selected from HCT116 p53$^{+/+}$ cells were heterozygote (p53 WT/mut), expressing both wild-type and mutant p53. We reasoned that increasing doses of Nutlin might increase the levels and activity of wild-type p53 in these cells. In this case, we predicted that culturing the p53 (WT/mut) cells in increasing Nutlin doses would apply selective pressure for loss of the wild-type *TP53* allele and that only cells that had lost or silenced the remaining wild-type allele would survive. As a first test, five different p53 (WT/mut) clones were treated with the Nutlin dose in which they were selected (2.5 μM Nutlin for V203E, 5 μM for R248Q, R267W, and R273H, and 10 μM Nutlin for T125M) and also with increasing Nutlin doses (10–30 μM). Levels of p53 and its downstream target protein p21 were assessed. p21 is a potent inhibitor of cyclin·cyclin-dependent kinase complexes, and p53 arrests the cell cycle in G_1-phase by

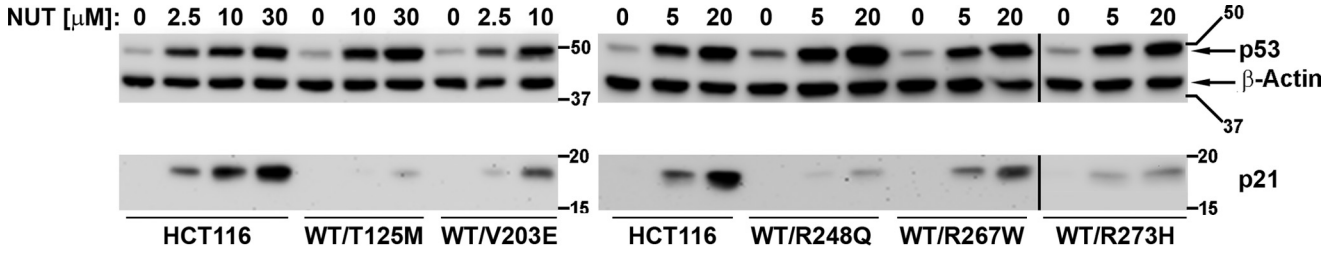


FIGURE 3. **High dose Nutlin induces p53 and p21 expression in HCT116 p53 wild-type/mutant heterozygotes.** Parental HCT116 and the HCT116 *TP53* heterozygotes were cultured in the indicated dose of Nutlin (*NUT*) for 24 h. p53 and p21 protein levels were then determined by immunoblotting. 30 μg of protein was loaded in each lane. Actin was used as a loading control.

inducing p21 expression. As shown in Fig. 3, p53 and p21 were induced in HCT116 $p53^{+/+}$ cells at all Nutlin doses used, and the induction occurred in a dose-dependent manner. In contrast, p21 was not induced or was less induced in the p53 (WT/mut) cells than the $p53^{+/+}$ cells at lower Nutlin doses (2.5–10 μM), although it was induced by the higher Nutlin doses (10 μM for WT/V203E and 20 and 30 μM for the other four clones). These data support the idea that higher doses of Nutlin may activate wild-type p53- and p21-dependent cell cycle arrest in the heterozygote cells and therefore may select for cells in which the remaining wild-type allele is lost or inhibited.

Increasing Nutlin Doses Apply Selective Pressure to Lose/Silence the Remaining Wild-type TP53 Allele—Next, the p53 (WT/mut) heterozygotes were cultured in continuous high dose Nutlin (10–30 μM), and surviving colonies were isolated. mRNA was isolated from the surviving clones, which was then used to generate and sequence p53 cDNA. The p53 cDNA sequencing revealed that the surviving clones expressed only the mutated p53 mRNA (Fig. 4*B*), apparently having lost or silenced the wild-type allele. To show that p53 activity was lost in these surviving clones, the clones were treated with increasing Nutlin doses, and p21 expression was monitored as an indicator of p53 activity. As shown in Fig. 5, p21 was induced to a lesser extent after Nutlin treatment in the cells that expressed mutant p53 only compared with either HCT116 $p53^{+/+}$ cells or the WT/mut heterozygotes. These data support the idea that silencing of the wild-type *TP53* allele caused loss of p53 activity in these clones. Thus, high dose Nutlin can select p53-mutated cells that have silenced the remaining wild-type allele. The clones selected in high dose Nutlin that express only mutant p53 are hereafter referred to as Mutant p53/WT*silenced*.

Wild-type TP53 Allele Is Present and Intact but Not Expressed in the Mutant p53/WTsilenced Clones—The *TP53* locus is located on the chromosome 17 short arm. To address whether the selected clones had lost/deleted chromosome 17 (Chr.17) or the *TP53* allele, we carried out FISH analysis with *TP53* and Chr.17 probes (Fig. 6). These studies showed ~100% of cells from the HCT116 heterozygotes (WT/mut) had two copies of *TP53* and Chr.17. Importantly, ~100% of cells from the Mutant p53/WT*silenced* clones also showed two copies of *TP53* and Chr.17. This result suggested the wild-type *TP53* gene is present in the clones but not expressed due perhaps to epigenetic silencing. To test this possibility further, we isolated mRNA and genomic DNA from the p53 heterozygote (WT/mut) and Mutant p53/WT*silenced* clones. We sequenced the p53 cDNA to determine which alleles were expressed and also sequenced genomic DNA to determine which *TP53* alleles were present (Fig. 4, *A* and *B*). p53 heterozygotes (WT/mut) expressed both wild-type and mutant p53 mRNA, evidenced by overlap of wild-type and mutant p53 in cDNA sequencing. Genomic DNA sequencing confirmed the presence of both wild-type *TP53* and mutated *TP53* genes in the heterozygotes, evidenced by overlap of wild-type and mutant *TP53* in genomic DNA sequencing. cDNA sequencing in the Mutant p53/WT*silenced* clones showed only mutant p53 was expressed (Fig. 4*B*). However, genomic DNA sequencing revealed that both wild-type *TP53* and mutated *TP53* sequences were present in these clones. This was evidenced by overlap of wild-type and mutant *TP53* sequences in the genomic DNA. These results support the idea that the wild-type *TP53* gene is present but not expressed in the Mutant p53/WT*silenced* clones.

Next, we considered the possibility that portions of the *TP53* locus or promoter may be deleted in the Mutant p53/WT*silenced* clones and that this could result in silencing of the wild-type allele. We used a PCR approach to address this question. First, we PCR-amplified the genomic DNA fragment encompassing exons 2–8 in the heterozygote and Mutant p53/WT*silenced* clones. In each case we obtained a single ~3-kb PCR product that appeared identical in size (data not shown). This suggested there were no large deletions between exons 2 and 8 in the Mutant p53/WT*silenced* clones. We then carried out DNA sequencing of the mutated region as well as a T/G polymorphic SNP located at position +13,511 in intron 7 to determine whether both alleles had been PCR-amplified. In each case we detected both the wild-type and mutated *TP53* sequences in this ~3-kb amplicon as well as the T/G SNP at position +13,511 (results for R267W are shown in Fig. 7*C* as an example). This result indicates the genomic region between exons 2 and 8 is intact in each of the Mutant p53/WT*silenced* clones. We next examined the *TP53* promoter region. Sequencing of the promoter demonstrated the presence of a G/A polymorphic SNP located 171 bp upstream of the p53 transcription start site (*position −171,* Fig. 7). We took advantage of this SNP to ask whether the *TP53* promoter was intact or deleted in the Mutant p53/WT*silenced* cells. We PCR-amplified the *TP53* promoter fragment spanning ~1000 bp upstream of the transcription start site to ~500 bp downstream of exon 1 in the heterozygote and Mutant p53/WT*silenced* clones. In each case, we obtained a single ~1.5-kb PCR product that appeared identical in size. We then carried out DNA sequencing of the entire promoter fragment, including the −171 SNP to determine whether both alleles had been PCR-amplified. For each Mutant

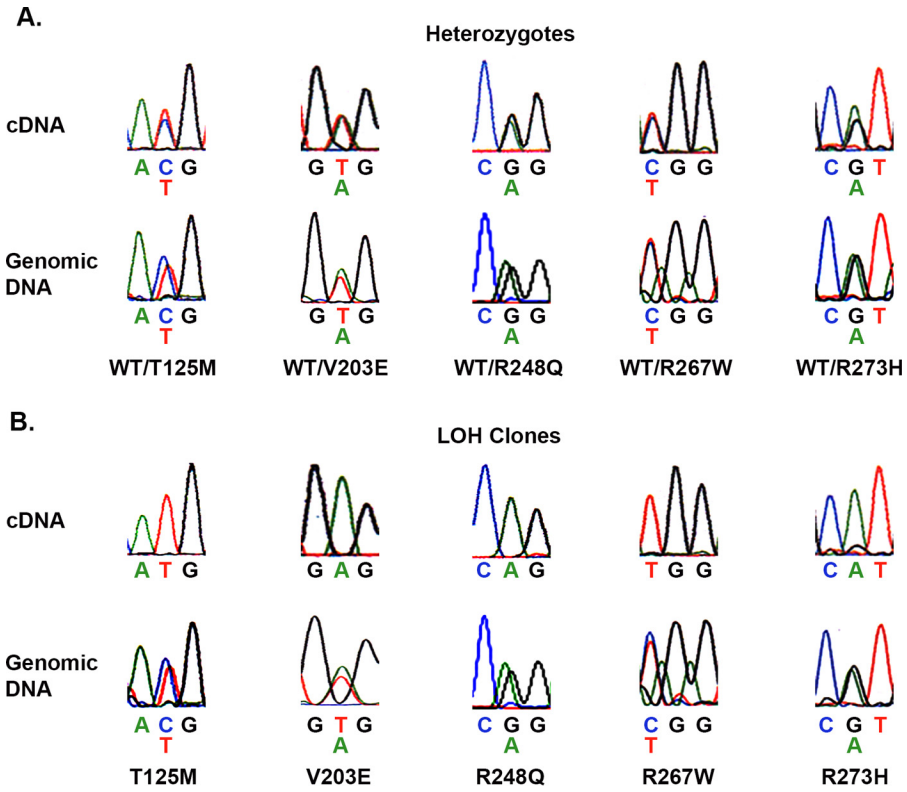


FIGURE 4. **Wild-type *TP53* allele is present but not expressed in the Mutant p53/WT*silenced* clones.** *TP53* cDNA and *TP53* genomic DNA from both heterozygote (*A*) and LOH clones (*B*) were sequenced. Sequencing results for the mutated region of each clone are shown. Note cDNA sequencing in the WT*silenced*/Mutant p53 clones shows only the mutated *TP53* allele is expressed, although genomic DNA sequencing reveals that both mutated and wild-type copies of *TP53* are present, as evidenced by detection of both wild-type and mutated *TP53* genomic sequence.

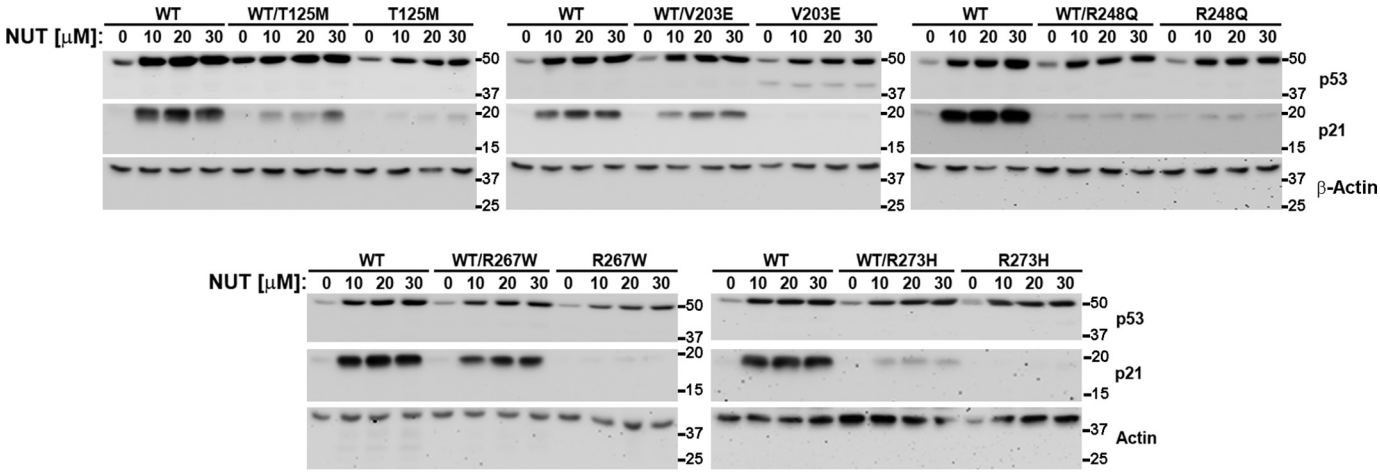


FIGURE 5. **p53 activity is lost in the WT*silenced*/Mutant p53 clones.** HCT116, heterozygote, and WT*silenced*/Mutant p53 clones were treated with increased doses of Nutlin (*NUT*) (10, 20, and 30 μM) for 24 h. p53 and p21 protein levels were determined by immunoblotting. 30 μg of protein was loaded in each lane.

p53/WT*silenced* clone, we detected the heterozygous SNP at position −171, indicating both alleles had been PCR-amplified (see *R267W* in Fig. 7*A*). These results indicate the genomic region from ~1000 bp upstream of the transcription start site to ~500 bp downstream of exon 1 is intact in each of the Mutant p53/WT*silenced* clones. Finally, we focused on the ~10.7-kb intron 1 in *TP53*. We used primers in exon 1 and exon 2 to carry out long range PCR of intron 1 in each of the heterozygote and Mutant p53/WT*silenced* clones. We reasoned that if there is a deletion in intron 1 of the WT *TP53* allele, then we would detect two PCR products in the Mutant p53/WT*silenced* genomic DNA (one product from the expressed Mutant *TP53* allele and a second shorter product from the silenced WT allele). However, in each of the heterozygote (WT/mut) and Mutant p53/WT*silenced* clones, we detected a single ~10.7-kb intron 1 fragment and no shorter fragments in the Mutant p53/WT*silenced* clones (see *R267W* in Fig. 7*B*). This result suggests intron 1 is intact in the Mutant p53/WT*silenced* clones. The FISH analysis (Fig. 6), genomic DNA sequencing (Fig. 4*B*), and PCR anal-

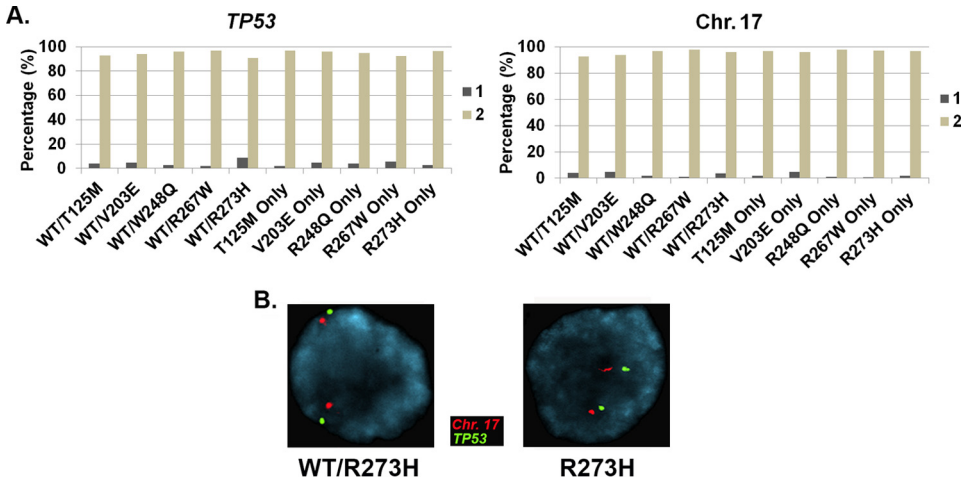


FIGURE 6. **Mutant p53/WT*silenced* clones retain two alleles for *TP53*.** FISH analysis was performed with chromosome 17 (*Chr.17*) and *TP53*-specific probes in the *TP53* heterozygotes (*WT/mutant*) and WT*silenced*/mutant p53 clones. *A,* nearly 100% of the heterozygote cells and Mutant p53/WT*silenced* cells displayed two signals for chromosome 17 and *TP53*. *B,* images of WT/R273H and WTp53*silenced*/R273H cells stained for chromosome 17 (*red*) and *TP53* (*green*).

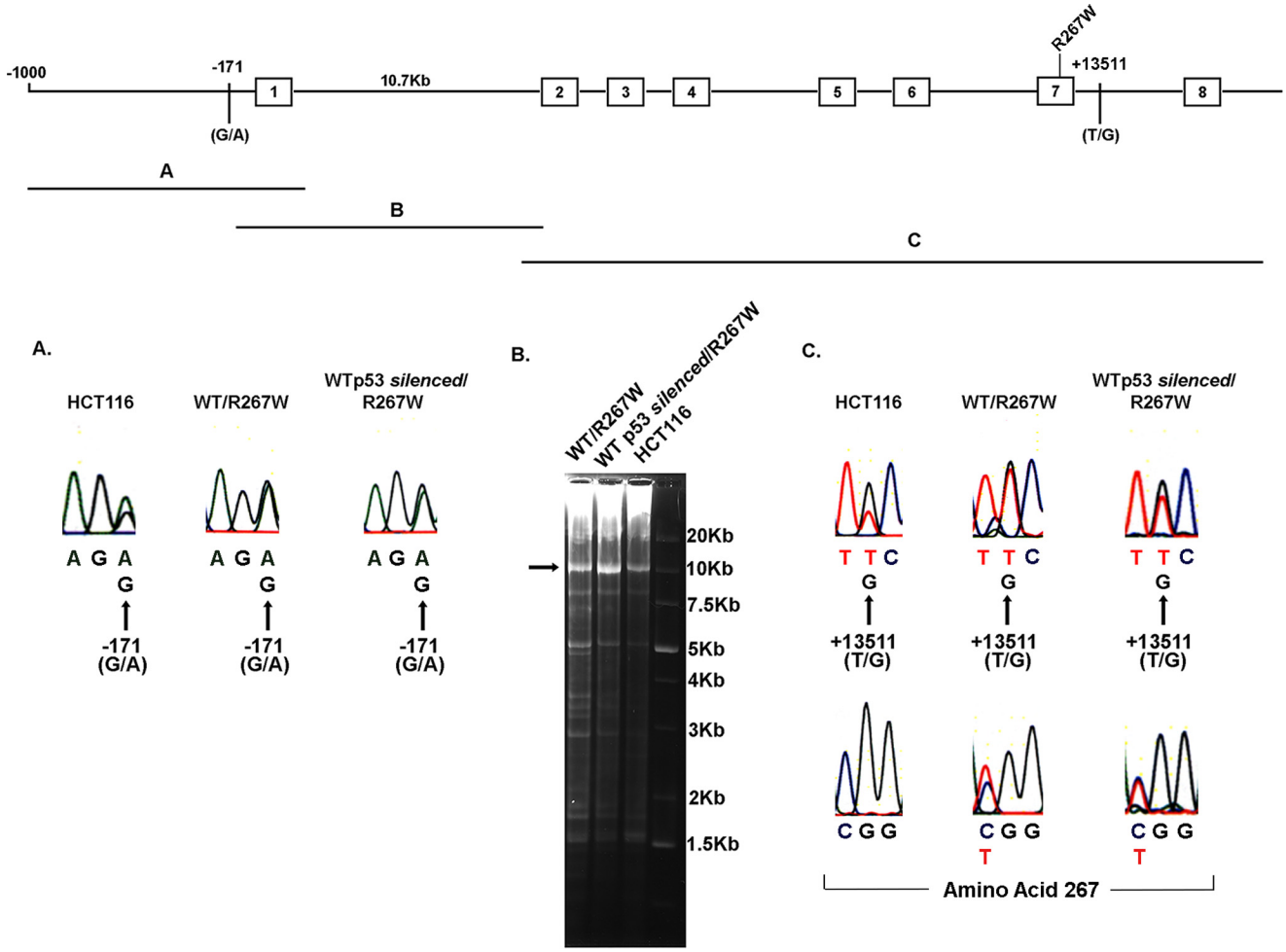


FIGURE 7. ***TP53* gene is intact in the Mutant p53/WT*silenced* clones.** *Upper,* schematic of the *TP53* allele from ~1000 bp upstream of the transcription start site. *Numbered boxes* indicate exons 1–8. The position of polymorphic SNPs at position −171 upstream of exon 1 and position +13,511 in intron 7 are indicated. *Lines A–C* indicate PCR amplification products used to verify both *TP53* alleles are intact. *A,* ~1500-bp fragment spanning exon 1 was amplified from each p53 WT/mut heterozygote and Mutant p53/WT*silenced* clone and sequenced. The −171 SNP was detected in all Mutant p53/WTsilenced clones, indicating both alleles *TP53* alleles were detected (sequence results for the R267W cell pair is shown). *B,* ~10.7-kb intron 1 fragment was amplified from each p53 WT/mut heterozygote and Mutant p53/WT*silenced* clone and resolved on an agarose gel. A single ~10.7-kb fragment was detected in all WT/mut heterozygotes and Mutant p53/WTsilenced clones, suggesting intron 1 is intact in each Mutant p53/WT*silenced* clone (results for the R267W cell pair is shown). *C,* ~3-kb fragment spanning exon 2 to exon 8 was amplified from each p53 WT/mut heterozygote and Mutant p53/WT*silenced* clone and sequenced. The +13,511 SNP and *TP53* mutation was detected in all Mutant p53/WTsilenced clones, indicating both alleles *TP53* alleles were detected (sequence results for the R267W cell pair is shown).

yses (Fig. 7) in combination demonstrate the wild-type *TP53* gene is present and intact but not expressed in the Mutant p53/WT*silenced* clones.

Wild-type TP53 Silencing Does Not Occur in Promoter DNA Methylation—Multiple gene silencing mechanisms have been described, including DNA promoter methylation, histone methylation, and histone deacetylation. Gene silencing by promoter methylation results from methylation of CpG sites within a gene promoter. Sequencing of bisulfite-treated DNA allows one to distinguish methylated and non-methylated cytosines. There are 16 CpG sites within the p53 gene promoter (see Fig. 8, *A* and *B*). To ask whether silencing of the wild-type *TP53* allele resulted from DNA promoter methylation, we bisulfite-treated genomic DNA from HCT116 and the Mutant p53/WT*silenced* clones and then PCR-amplified and cloned the *TP53* promoter region. We sequenced the *TP53* promoter from 9 to 10 individual clones to determine the CpG methylation status. Unmethylated CpG islands are represented by *open circles* in Fig. 8. The results showed that all CpG sites were unmethylated in HCT116 $p53^{+/+}$ cells and WT/mut clones (Fig. 8*A*). All or most of the CpG sites were also unmethylated in the Mutant p53/WT*silenced* clones, although methylation at single CpG sites was detected in individual clones from the R267W/WT*silenced* cells (CpG methylation represented by *closed circles* in Fig. 8*B*).

The fact that most or all CpGs were not methylated in the Mutant p53/WT*silenced* clones suggests silencing of the wild-type allele in these clones does not result from DNA methylation. However, a caveat of this interpretation is that methylation at CpG sites outside the promoter region analyzed in Fig. 8 might also inhibit wild-type p53 expression. Therefore, to more directly ask whether DNA methylation contributes to wild-type p53 silencing, we monitored p53 mRNA and protein expression in HCT116 cells and the clones treated with the DNA methyltransferase inhibitor Aza. As shown in Fig. 9*B*, p53 mRNA levels were not increased in HCT116 and were slightly decreased in the Mutant p53/WT*silenced* cells treated with Aza. We used *TKTL1* and *ESR1* as positive controls for genes that can be induced by Aza, because previous studies showed that both *TKTL1* and *ESR1* gene promoters are silenced by DNA methylation in HCT116 cells and treatment with Aza can induce their expression (47, 48). As shown in Fig. 9*A*, both *TKTL1* and *ESR1* mRNA levels were induced in Aza-treated HCT116 and the Mutant p53/WT*silenced* cells, consistent with both genes being silenced by DNA methylation. Finally, we carried out p53 cDNA sequencing in the Mutant p53/WT*silenced* cells that had been treated with Aza. Although the cDNA sequencing showed mutant p53 was expressed in the mutant only clones treated with Aza, there was no evidence for increased expression from the wild-type p53 allele (Fig. 9*C*). The results suggest wild-type p53 gene silencing in the Mutant p53/WT*silenced* clones does not result from DNA promoter methylation.

Wild-type TP53 Silencing Does Not Occur through Histone Deacetylation—Next, we wished to ask whether silencing of the wild-type *TP53* allele resulted from changes in histone H3 acetylation or histone H3 methylation. To this end, we first used ChIP analysis to compare the levels of two histone modifications that have different effects on gene transcription: H3 Lys-27 trimethylation (H3K27me3), which represses gene transcription, and H3 Lys-9 acetylation (H3Ac), which activates gene transcription. H3K27me3 was mostly undetected in heterozygote and R267W/WT*silenced* and R273H/WT*silenced* cells (Fig. 10, *A* and *B*). In contrast, H3Ac was detected at all *TP53* promoter fragments, and notably, there was a trend toward decreased H3Ac in each of the WT-*silenced* clones (Fig. 10, *A* and *B*). This suggested reduced H3Ac might contribute to silencing of the WTp53 allele. If this were the case, then we predicted histone deacetylase (HDAC) inhibitors would increase H3Ac levels and restore expression of the wild-type allele. To test this, we treated the Mutant p53/WT*silenced* clones with two different HDAC inhibitors, AR42 and SAHA. As shown in Fig. 11*A*, both HDAC inhibitors caused a robust increase in H3K9Ac levels. Previous studies showed p21 mRNA increases in a p53-independent way in HDAC inhibitor-treated cells. We therefore used p21 as a positive control for a gene induced by HDAC inhibitors. The results show p21 mRNA and protein expression were induced by AR42 and SAHA treatment in the treated cells (Fig. 11, *A* and *B*). Contrary to our prediction, however, p53 mRNA levels were not increased but were instead decreased in AR42- and SAHA-treated cells. This reduction in p53 mRNA is consistent with studies suggesting HDAC inhibitors can inhibit *TP53* transcription (49). p53 protein levels were also reduced slightly in the Mutant p53/WT*silenced* clones (Fig. 7, *A* and *B*). In contrast, wild-type p53 protein levels in HCT116 cells were slightly increased upon AR42 and SAHA treatment (Fig. 7*A*). We speculate this could result from increased acetylation of lysine residues in p53 that block ubiquitination and thus stabilize the protein (50). Finally, we carried out p53 cDNA sequencing in Mutant p53/WT*silenced* cells that had been treated with SAHA. Although the cDNA sequencing showed mutant p53 was expressed in the Mutant p53/WT*silenced* clones treated with SAHA, there was no evidence for increased expression from the wild-type p53 allele (Fig. 11*C*). The fact that HDAC inhibitors increased H3Ac levels but did not restore wild-type p53 expression suggests reduced acetylation is not the mechanism for silencing the wild-type *TP53* allele.

Wild-type TP53 Silencing Does Not Occur through Histone Methylation—Gene silencing can also result from histone H3 methylation at lysine residues. Thus, histone H3 methylation inhibitors have been developed that can block methylation and activate silenced genes (51, 52). In our initial ChIP analysis (Fig. 10), H3K27me3 was not detected in *TP53* promoter fragments immediately adjacent to the transcription start site. Nonetheless, it remained possible that H3K27me3 at more distant sites could contribute to silencing of the wild-type allele. Therefore, to ask whether wild-type *TP53* allele silencing results from histone H3 methylation at lysines, we treated cells with two different H3 methylation inhibitors as follows: BIX-01294, which inhibits H3 Lys-9 dimethylation (H3K9me2), and GSK126, which inhibits H3 Lys-27 trimethylation (H3K27me3). Immunoblotting showed H3K9me2 and H3K27me3 decreased by treatment with BIX-01294 and GSK126 alone or in combination (Fig. 12*A*). This confirmed the activity of the methylation inhibitors. However, p53 mRNA levels were unchanged or slightly reduced by BIX-

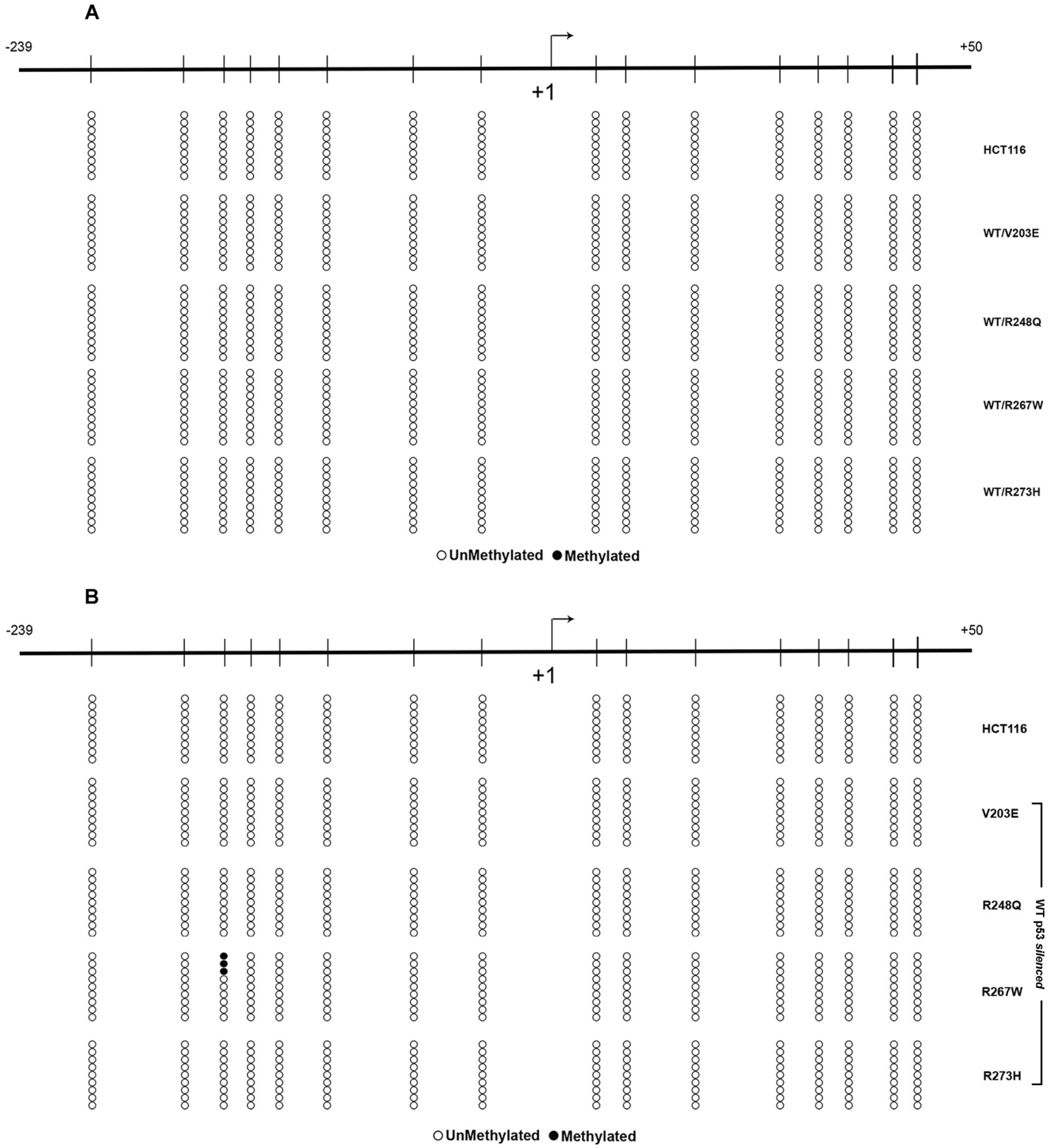


FIGURE 8. **Bisulfite sequencing of the *TP53* promoter in heterozygote Mutant p53/WT*silenced* clones.** *A,* genomic DNA was isolated from heterozygote clones and modified by bisulfite. The *TP53* promoter was amplified by nested PCR and cloned into pCR4-TOPO TA cloning vector. 9–10 colonies were picked and sequenced. *Open circles* represent non-methylated cytosine. No methylated cytosines were detected in the *TP53* promoter from heterozygote clones. *B,* genomic DNA was isolated from the Mutant p53/WT*silenced* clones and modified by bisulfite. *TP53* promoter was amplified by nested PCR and cloned as described above. 9–10 colonies were picked and sequenced. *Open circles* represent non-methylated cytosine, and *solid circles* represent methylated cytosine.

01294 and GSK126 in HCT116 and the Mutant p53/WT*silenced* clones (Fig. 12*B*). Notably, GSK126 caused a more pronounced reduction in p53 mRNA levels in HCT116 than in the Mutant p53/WT*silenced* clones. Finally, cDNA sequencing showed no evidence for expression/reactivation of the wild-type p53 allele in the Mutant p53/WT*silenced* clones treated with BIX-01294 and/or GSK126 (Fig. 12*C*). This result suggests wild-type p53 gene silencing in the

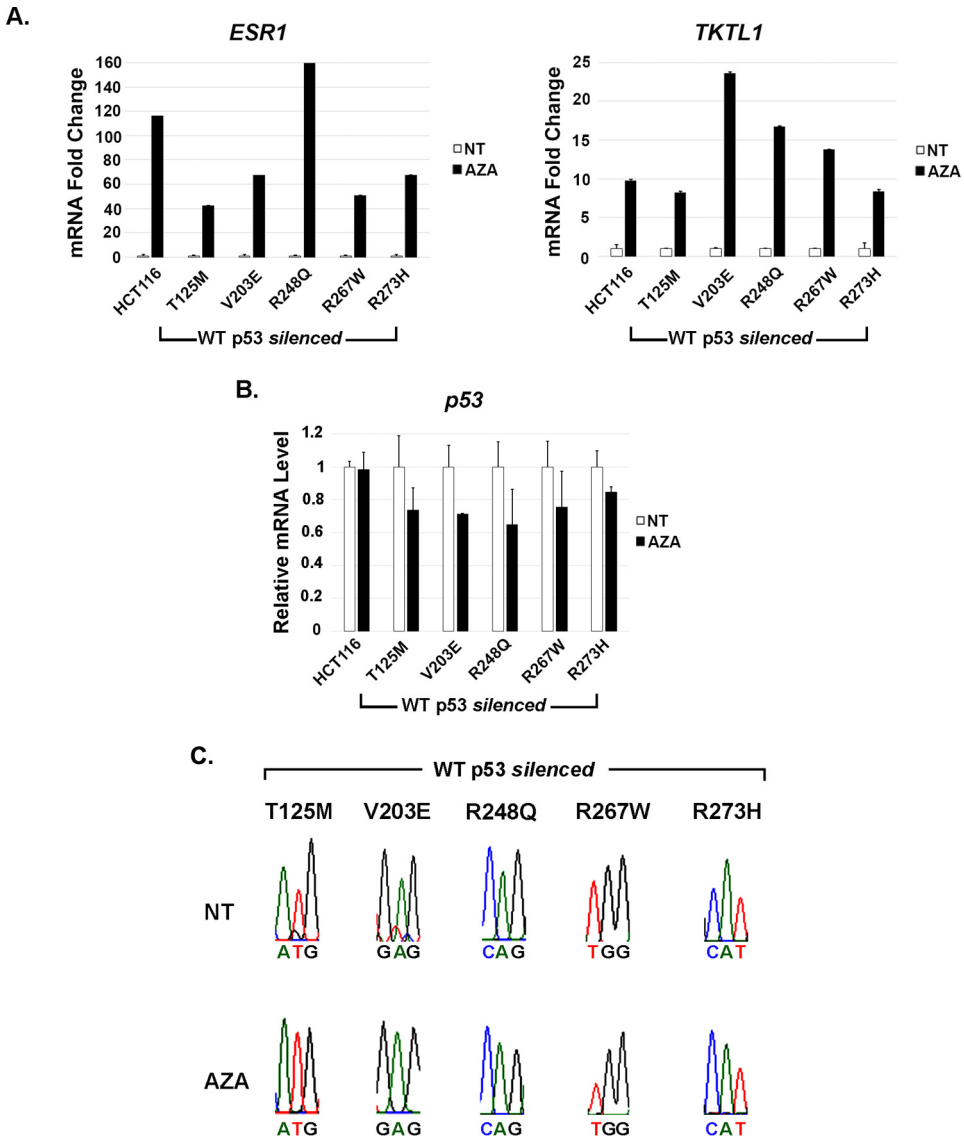


FIGURE 9. **DNA methyltransferase inhibitor does not reactivate wild-type *TP53*.** *A*, HCT116 and LOH clones were treated for 3 days with 10 μM Aza. Following treatment with Aza, both *ESR1* and *TKTL1* were induced, suggesting that DNA methylation silences expression of both genes. Shown are the average results from two separate experiments, each done in triplicate; *bars* indicate S.E. *B*, *TP53* gene expression was also measured following Aza treatment, which resulted in no induction of the *TP53* gene. Shown are the average results from two separate experiments, each done in triplicate; *bars* indicate S.E. Aza did not cause a significant decrease in p53 mRNA as determined by Student's *t* test using $p < 0.05$ as a cutoff. *C*, cDNA sequencing from LOH clones following Aza treatment shows that the wild-type allele of p53 is not expressed. *NT*, not treated.

Mutant p53/WT*silenced* clones does not result from histone H3 di- or tri-methylation at Lys-9 or Lys-27.

Finally, we considered there may be multiple redundant mechanisms in play to silence the *TP53* wild-type allele. To examine this possibility, we treated R267W/WT*silenced* and R273H/WT*silenced* cells with different combinations of Aza (DNA methyltransferase inhibitor), AR42 (HDAC inhibitor), and BIX-01294 or GSK126 (histone methylase inhibitors). Immunoblotting for H3K27me3, H3K9me2, and H3K9Ac confirmed activity of the histone methylation and HDAC inhibitors (Fig. 13*A*). We used cDNA sequencing to address whether the wild-type p53 allele was reactivated and expressed in response to these treatments (Fig. 13*B*). For each combination treatment, cDNA sequencing showed no evidence for expression/reactivation of the wild-type p53 allele. The results suggest silencing of the wild-type p53 allele does not result from a combination of DNA methylation, histone methylation, and/or histone deacetylation.

Discussion

Inactivation of the p53 tumor suppressor pathway is considered essential for the development of most or all human cancers. Over 50% of cancers harbor inactivating missense mutations in *TP53* that destroy p53 function (1). In most cases, mutation in one *TP53* allele is followed by LOH, such that the cancer expresses only a mutant inactive form of the p53 protein. Despite the importance of p53 inactivation in cancer development, little is known about how *TP53* mutations arise or how the remaining wild-type allele is lost or silenced. Approximately 15% of human CRCs are DNA MMR-deficient (6, 7). Loss of MMR is believed to drive cancer development by allowing cells to rapidly accumulate the genetic changes required for the mul-

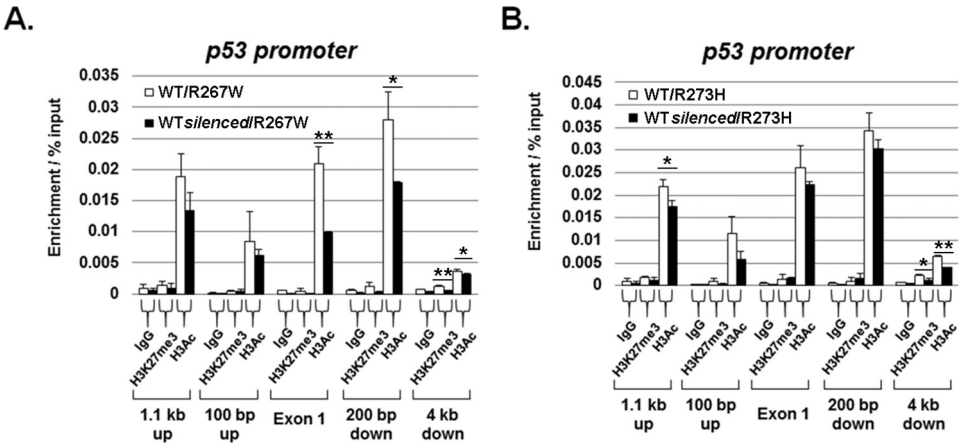


FIGURE 10. **Occupancy of H3K27me3 and H3Ac at the *TP53* promoter in the Mutant p53/WT*silenced* clones compared with their heterozygote (WT/mut) counterparts.** *A,* WT/R267W and WTsilenced/R267W cells, or *B,* WT/R273H and WTsilenced/R273H cells were subjected to ChIP analysis using anti-IgG, anti-H3K27me3, and anti-H3Ac antibodies. *TP53* promoter regions were amplified using the designated primer sets. *A* and *B,* the average results are plotted from two separate experiments, each done in triplicate; *bars* indicate S.E. Significance was tested by Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

tistep tumorigenic process (16, 25). The finding of mutations in specific MMR genes in families predisposed to CRC supports this hypothesis (53–55). Interestingly, *TP53* mutations are observed in ~50% of all CRCs, but they are relatively rare in MMR-deficient CRCs, occurring in only 10–20% of cases (22, 23). It is unknown whether MMR deficiency in CRC gives rise to *TP53* mutations, and the mechanism by which the wild-type *TP53* is inhibited or lost in MMR-deficient CRCs has also not been examined. Nutlin is a potent stabilizer and activator of wild-type p53 that is being developed as an anti-cancer therapeutic (36). HCT116 is a p53 wild-type CRC cell line that is MMR-deficient. In this study, we used Nutlin as a tool to select HCT116 cells that had acquired *TP53* mutations and lost expression of the remaining wild-type *TP53* allele. MMR-deficient HCT116 cells acquired initiating point mutations in one *TP53* allele and formed heterozygote (p53 wild-type/mutant) colonies in the continued presence of Nutlin, although MMR-corrected counterparts did not. Placing the p53 (wild-type/mutant) heterozygotes in increasing Nutlin doses selected clones that expressed only the mutant *TP53* allele. Analysis of these clones revealed the remaining wild-type allele was inactivated through a copy-neutral, gene silencing mechanism that does not appear to involve histone deacetylation, histone methylation, or *TP53* promoter DNA methylation. Taken together, our data indicate the following: 1) that MMR deficiency can accelerate the *TP53* mutation acquisition rate and give rise to initiating point mutations in one *TP53* allele; 2) that loss of the remaining wild-type *TP53* allele in these MMR-deficient cells can occur through a copy-neutral, gene silencing mechanism; and 3) that data highlight the ability to use MDM2 antagonists as tools to study *TP53* mutation acquisition and gene-silencing mechanisms in cancer.

The MMR machinery helps maintain genetic stability by repairing mismatched bases or insertion-deletion loops that arise during DNA replication. The core MMR machinery in humans consists of three heterodimeric protein complexes involved in either mismatch recognition or repair. Most current models suggest hMUTSα (composed of hMSH2 and hMSH6) preferentially recognizes DNA single base mispairs, although hMUTSB (composed of hMSH2 and hMSH3) preferentially recognizes insertion-deletion loops (18–20). The hMUTL complex (composed of hMLH1 paired with hPMS2 or hPMS1) is recruited after mismatch recognition by either hMUTSα or hMUTSB and functions in the repair of both single base mispairs and insertion-deletion loops (11, 12, 21). The enzymatic ATPase and endonuclease activities of hMUTL are required to complete repair. HCT116 cells lacking *hMSH3* and *hMLH1* (39, 41) and are therefore deficient in the recognition and repair of DNA insertion-deletion loops. Deficient repair of insertion-deletion loops is expected to yield mostly frameshift mutations. Thus, it was somewhat unexpected in this study that the vast majority (21 of 23) of *TP53* mutations identified in Nutlin-selected cells were single-base point mutations, although only two *TP53* frameshift mutations were identified. However, it is important to note studies from Kunkel and coworkers (56) that demonstrated functional overlap between hMSH3 and hMSH6. In these studies, the MMR-deficient endometrial cancer cell line HHUA was used that is a mutant in both *hMSH3* and *hMSH6*. Transfer of chromosome 5 into HHUA cells restored wild-type *hMSH3* expression and partially restored the ability of the cells to repair base-base mismatches. One possibility is that the single-base point mutations in *TP53* we identified resulted from lack of *hMSH3* and subsequent inability of the hMSH3 protein to promote repair of single-base mismatches.

Knudsen's two-hit hypothesis states that inactivation/loss of both alleles of a tumor suppressor gene are required for loss of tumor suppressor function and cancer development. An interesting finding in this study was that HCT116 cells with only one intact *TP53* allele (p53+/−) formed *TP53*-mutated, Nutlin-resistant colonies at a rate ~10-fold greater than HCT116 $p53^{+/+}$ cells with both *TP53* alleles intact. The HCT116 p53+/− cells need only acquire a mutation in one *TP53* allele to completely lose p53 function and become Nutlin-resistant. However, assuming the overall mutation rates are comparable in HCT116 $p53^{+/+}$ and HCT116 $p53^{+/-}$ cells, one would expect that *TP53* mutations would be acquired at comparable rates in the $p53^{+/+}$ and $p53^{+/-}$ cells. The fact that *TP53*-mutated Nutlin-resistant

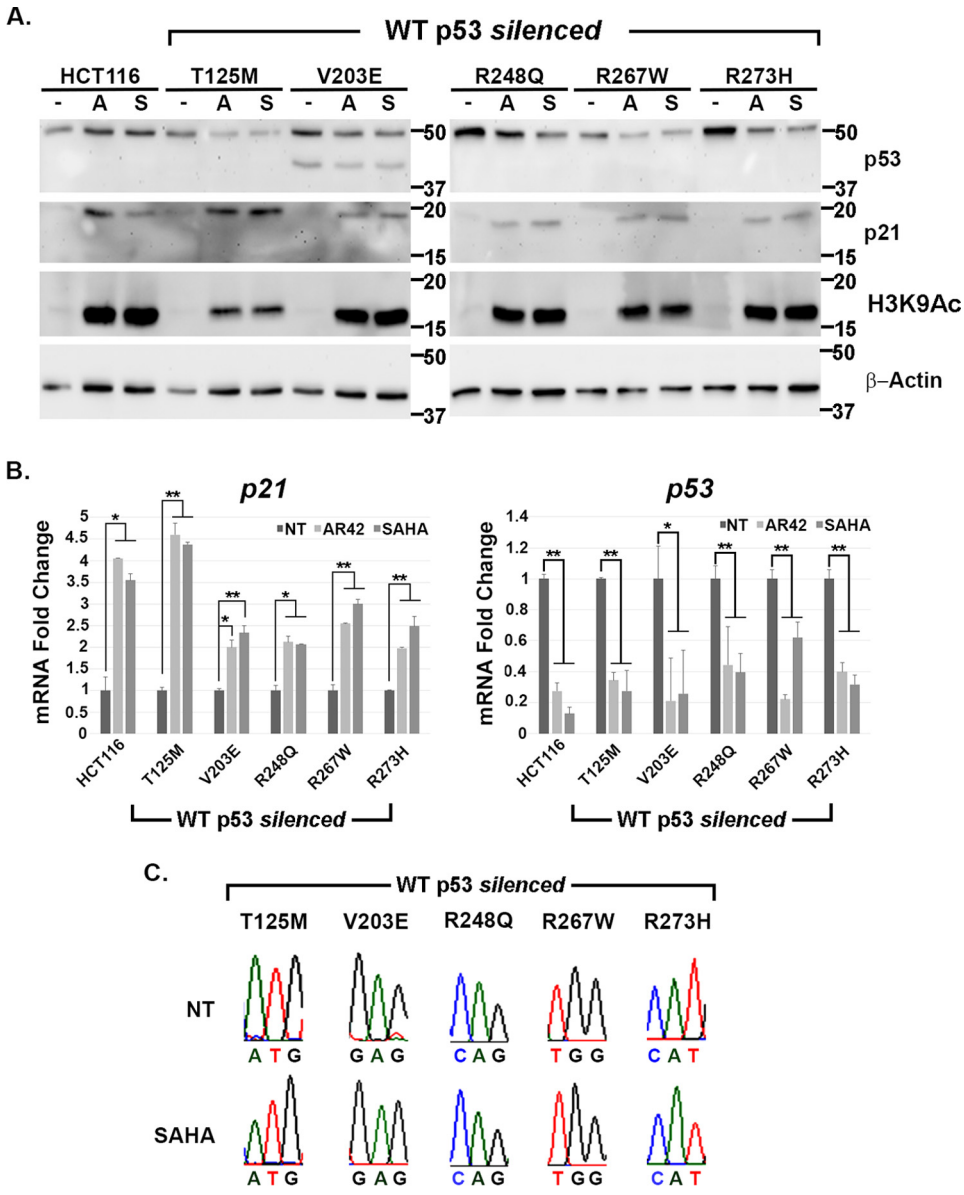
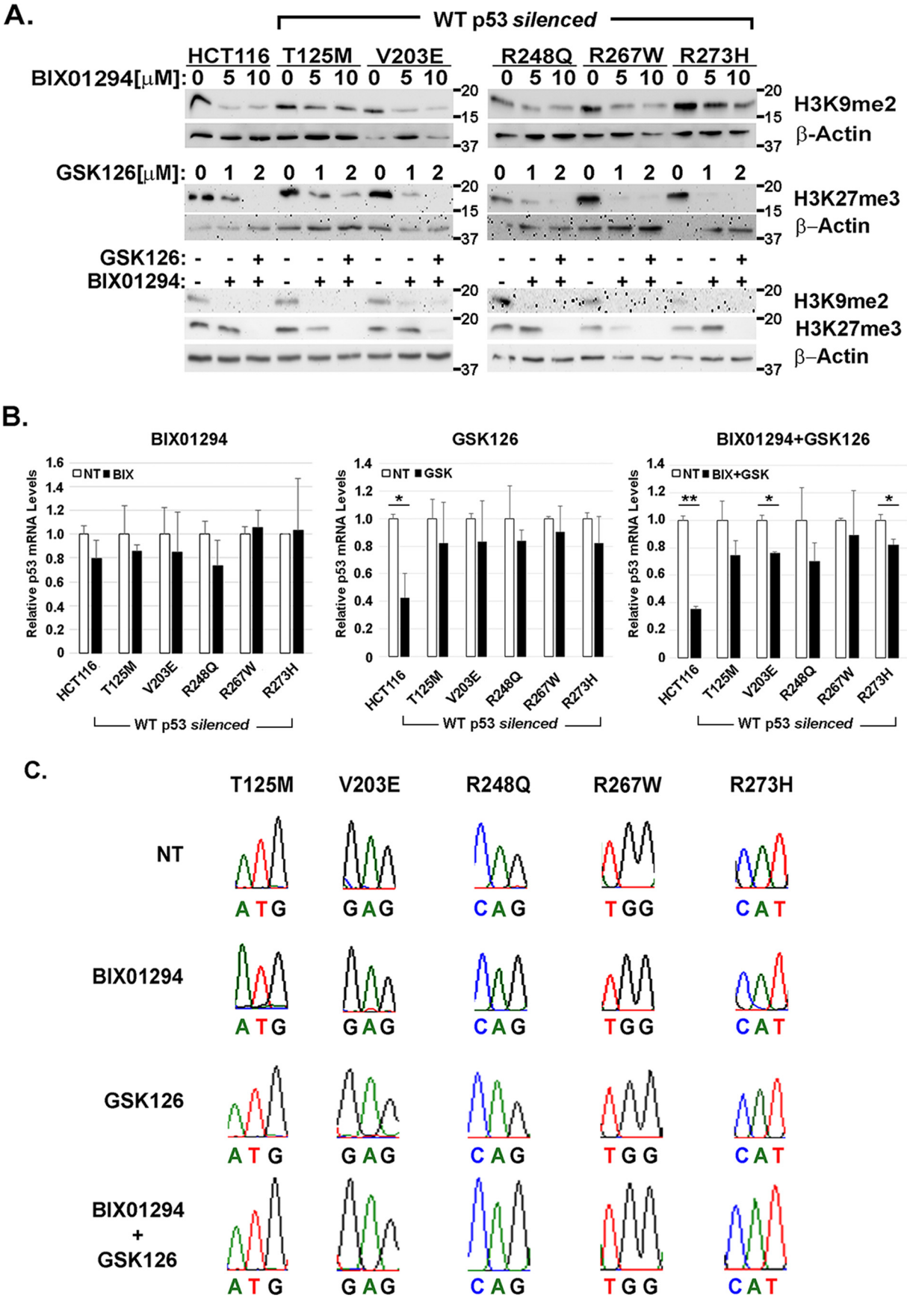


FIGURE 11. **HDAC inhibitors do not reactivate the wild-type allele in the Mutant p53/WT*silenced* clones.** *A,* HCT116 and Mutant p53/WT*silenced* clones were treated with either 1 μM AR-42 (*A*) or 5 μM SAHA (*S*) for 24 h. p53 and p21 levels were determined by immunoblotting. 30 μg of protein was loaded in each lane. p53 protein levels were reduced, and p21 protein levels were induced following treatment with HDAC inhibitors. Acetylated histone H3 was used as a treatment control, and actin was used as a loading control. *B,* mRNA levels for p53 and p21 were determined following treatment with AR-42 or SAHA. Shown are the average results from two separate experiments, each done in triplicate; *bars* indicate S.E. Significance was tested by Student's *t* test; *, $p < 0.05$; **, $p < 0.01$. *NT*, not treated. *C,* p53 cDNA from Nutlin-resistant Mutant p53/WT*silenced* clones was sequenced following treatment with 5 μM SAHA.

colonies formed with ~10-fold lower frequency in the $p53^{+/+}$ cells suggests that only certain *TP53* mutations are capable of promoting Nutlin-resistant growth in these cells. Some but not all p53 mutants can function as dominant-negative inhibitors of the wild-type protein. We speculate that in the HCT116 $p53^{+/+}$ background, only those p53 mutants that can function as dominant-negative inhibitors of the wild-type protein may allow Nutlin-resistant growth and therefore be selected.

p21 is a potent inhibitor of cyclin·cyclin-dependent kinase complexes, and wild-type p53 arrests the cell cycle by inducing p21 expression (57, 58). p21 was highly induced when HCT116 p53 (wild-type/mutant) heterozygotes were treated with increasing doses of Nutlin (Fig. 3). This suggests that increasing Nutlin doses can increase and activate wild-type p53 to high enough levels to activate the *p21* gene. Based on this, we reasoned that increasing doses of Nutlin would apply selective pressure for loss or silencing of the remaining wild-type allele. Indeed, when p53 heterozygote (wild-type/mutant) cells were placed in increasing Nutlin doses, the surviving clones expressed only the mutant *TP53* allele. FISH analysis with p53 and chromosome 17-specific probes suggested loss of wild-type p53 expression did not result from *TP53* allele or chromosome loss (Fig. 6). Genomic DNA sequencing and PCR analyses revealed that the LOH clones maintained a copy of both the wild-type and mutant *TP53* alleles, despite the fact that only the mutant *TP53* allele was expressed (Figs. 4*B* and 7). Based on these results, we conclude wild-type p53 was lost through a copy-neutral, gene silencing mechanism.


A.
WT p53 silenced
HCT116 T125M V203E R248Q R267W R273H
BIX01294[μM]: 0 5 10 0 5 10 0 5 10 0 5 10 0 5 10 0 5 10
H3K9me2
β-Actin
GSK126[μM]: 0 1 2 0 1 2 0 1 2 0 1 2 0 1 2 0 1 2
H3K27me3
β–Actin
GSK126:
BIX01294:
H3K9me2
H3K27me3
β–Actin
B.
BIX01294
GSK126
BIX01294+GSK126
Relative p53 mRNA Levels
NT BIX
NT GSK
NT BIX+GSK
HCT116 T125M V203E R248Q R267W R273H
WT p53 silenced
C.
T125M V203E R248Q R267W R273H
NT
BIX01294
GSK126
BIX01294 + GSK126
A T G G A G C A G T G G C A T

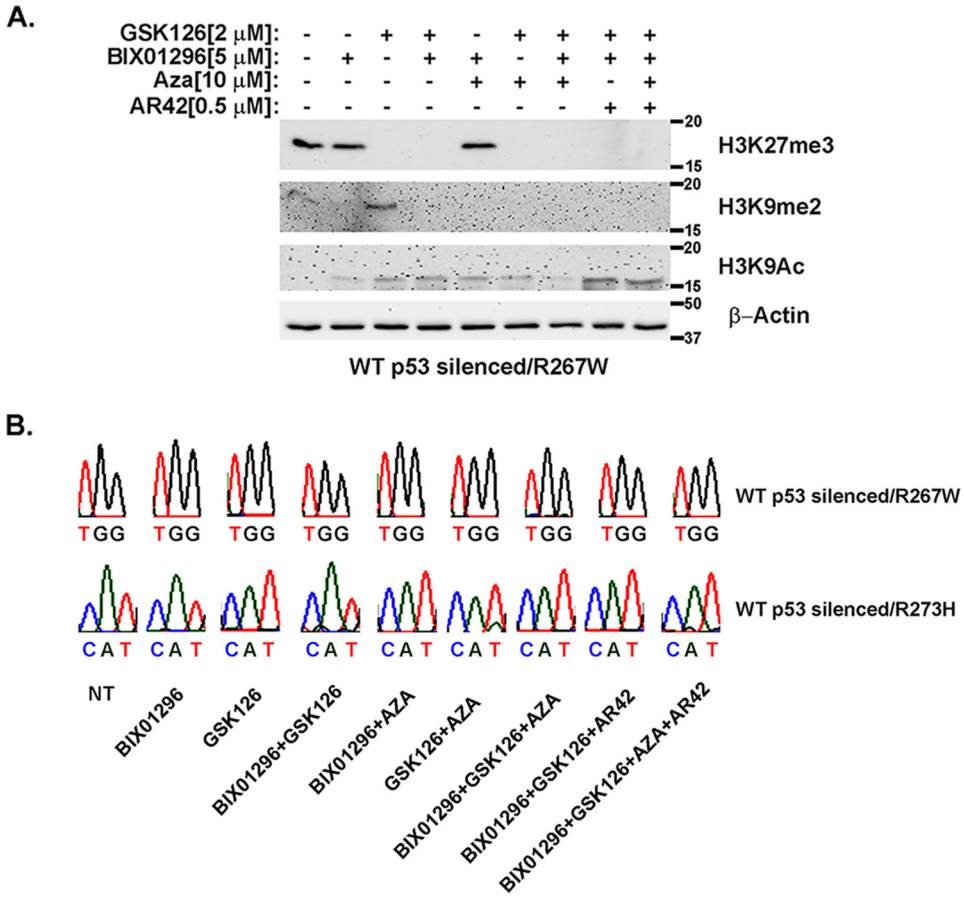


FIGURE 13. **Combined treatment with HDAC inhibitors and inhibitors of DNA methylation and histone methylation does not reactivate the wild-type allele in the Mutant p53/WT*silenced* clones.** R273H/WT*silenced* and R267W/WT*silenced* cells were treated with DNA methylation inhibitor (Aza), histone methylation inhibitors (BIX-01294 and GSK126 alone), and HDAC inhibitor (AR42) in different combinations as indicated. *A,* H3K9me2, H3K27me3, and H3K9Ac levels were determined by immunoblotting. 30 μg of protein was loaded in each lane. *B,* p53 cDNA from the treated cells showed no evidence of wild-type *TP53* gene reactivation by histone methylation inhibition. *NT*, not treated.

Three common mechanisms for gene silencing include histone deacetylation, histone methylation, and DNA promoter methylation. We used bisulfite sequencing as well as histone deacetylase, histone methylase, and DNA methyltransferase inhibitors to ask whether silencing of the wild-type *TP53* locus resulted from histone deacetylation, histone methylation, or promoter DNA methylation. We found no evidence of *TP53* promoter methylation in the clones in which wild-type p53 was silenced and, furthermore, no evidence that treatment with histone deacetylase (Figs. 11 and 13), DNA methyltransferase (Figs. 9 and 13), or histone methylase inhibitors (Figs. 12 and 13) could reactivate the silenced *TP53* locus. The results suggest silencing of the wild-type *TP53* locus in the LOH clones does not result from histone deacetylation, histone methylation, or *TP53* promoter DNA methylation. We should note that we have also examined expression of genes surrounding *TP53* on chromosome 17 and saw no evidence that the surrounding genes were silenced or less expressed in the Mutantp53/WT*silenced* clones compared with the p53 WT/mut heterozygotes. This leads us to believe that silencing of the *TP53* wild-type allele does not result from a global shutdown of the chromosome 17 region in which *TP53* resides. At present, the mechanism of wild-type *TP53* silencing in Mutantp 53/WT*silenced* cells remains unknown.

In summary, we used Nutlin as a tool to select cells and examine the mechanism by which initiating point mutations in one *TP53* allele are acquired and how expression of the remaining WT *TP53* allele is lost. Our results are the first to conclusively demonstrate MMR deficiency in CRC gives rise to initiating point mutations in one *TP53* allele. Furthermore, our results indicate expression of the second *TP53* allele is lost through a novel gene silencing mechanism that appears independent of DNA or histone methylation or histone deacetylation. We anticipate these findings may also be true in other cancers asso-

FIGURE 12. **Histone methylation inhibitors do not reactivate the wild-type allele in the Mutant p53/WT*silenced* clones.** HCT116 and Mutant p53/WT*silenced* clones were treated with histone methylation inhibitors BIX-01294 or GSK126 alone or in combination for 24 h. *A,* H3K9me2 and H3K27me3 levels were determined by immunoblotting. 30 μg of protein was loaded in each lane. Decreased H3K9me2 levels were used to demonstrate BIX-01294 activity, and decreased H3K27me3 levels were used to demonstrate GSK126 activity. *B,* p53 mRNA levels were determined in HCT116 and the Mutant p53/WT*silenced* clones following treatment with BIX-01294 or GSK126, alone or in combination. Relative p53 mRNA levels are plotted in comparison with the no treat level, which is considered "1.0." Shown are the average results from an experiment done in duplicate; *bars* indicate S.E. Significance was tested by Student's *t* test; *, $p < 0.05$; **, $p < 0.01$. *C,* p53 cDNA from Mutant p53/WT*silenced* clones showed no evidence of wild-type *TP53* gene reactivation by histone methylation inhibition. *NT*, not treated.

ciated with MMR deficiency in which p53 is mutated, including endometrial, gastric, ovarian, and skin cancer (59). Finally, our results highlight the use of MDM2 antagonists to identify *TP53* mutation and gene silencing mechanisms in cancer cells, information which could ultimately reveal targets for cancer prevention or treatment.

Author Contributions—R. E. P., H. S., and L. D. performed the experiments. R. H. K. and N.-H. P. conceived and interpreted the ChIP experiments. T. K. performed the ChIP experiments. R. E. P. wrote an initial draft of the manuscript. C. G. M. and H. S. conceived the studies. C. G. M. wrote the final version of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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