Role of c-Abl Kinase in DNA Mismatch Repair-dependent G$_2$ Cell Cycle Checkpoint Arrest Responses*□S

Mark W. Wagner[‡§1], Long Shan Li[‡1], Julio C. Morales[‡], Cristi L. Galindo[¶], Harold R. Garner[¶], William G. Bornmann[‖], and David A. Boothman[‡2]

From the ‡Laboratory of Molecular Stress Responses, Program in Cell Stress and Cancer Nanomedicine, Simmons Comprehensive Cancer Center, and the ¶McDermott Center for Human Growth and Development, Division of Translational Research, Departments of Biochemistry and Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390-8807, the §Department of Environmental Health Sciences, Case Western Reserve University, Cleveland, Ohio 44106, and the ‖Department of Experimental Therapeutics, M. D. Anderson Cancer Center, Houston, Texas 77030

Current published data suggest that DNA mismatch repair (MMR) triggers prolonged G$_2$ cell cycle checkpoint arrest after alkylation damage from N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) by activating ATR (ataxia telangiectasia-Rad3-related kinase). However, analyses of isogenic MMR-proficient and MMR-deficient human RKO colon cancer cells revealed that although ATR/Chk1 signaling controlled G$_2$ arrest in MMR-deficient cells, ATR/Chk1 activation was not involved in MMR-dependent G$_2$ arrest. Instead, we discovered that disrupting c-Abl activity using STI571 (Gleevec^{TM}, a c-Abl inhibitor) or stable c-Abl knockdown abolished MMR-dependent p73α stabilization, induction of GADD45α protein expression, and G$_2$ arrest. In addition, inhibition of c-Abl also increased the survival of MNNG-exposed MMR-proficient cells to a level comparable with MMR-deficient cells. Furthermore, knocking down GADD45α (but not p73α) protein levels affected MMR-dependent G$_2$ arrest responses. Thus, MMR-dependent G$_2$ arrest responses triggered by MNNG are dependent on a human MLH1/c-Abl/GADD45α signaling pathway and activity. Furthermore, our data suggest that caution should be taken with therapies targeting c-Abl kinase because increased survival of mutator phenotypes may be an unwanted consequence.

DNA mismatch repair (MMR)[3] proteins detect and repair mismatched bases or unpaired loops in DNA. Defects in MMR, primarily due to acquisition of mutations in both copies of the human (h) *MLH1* (*mutL* *homolog-1*) or *MSH2* (*mutS* *homolog-2*) genes (1–3), are directly linked to hereditary nonpolyposis colon cancer. A subset of sporadic colorectal cancers also lack MMR due to loss of hMLH1 protein expression caused by promoter hypermethylation (4). Cells deficient in MMR present work defects in G$_2$ cell cycle checkpoint arrest responses and display increased resistance to the lethal effects of specific DNA-damaging agents, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 6-thioguanine (6-TG), 5-fluoro-2′-deoxyuridine (FdUrd), cisplatin, and temozolomide (5). Theoretically, MMR-dependent G$_2$ arrest responses allow time for cells to repair mutagenic lesions created by these agents prior to cell entry and transit through mitosis. On several levels, MMR functions as a potent mutational avoidance system. Conversely, cells lacking MMR have a mutator phenotype as a result of failure to detect DNA lesions and subsequent absence of G$_2$ arrest and apoptotic responses (6, 7), resulting in a greatly increased colon cancer risk (8).

MNNG causes a spectrum of specific DNA lesions, including methylation of dG at the O^6-position in dG:dC base pairs. These DNA lesions are mutagenic because replication over O^6-methylguanine base pairs causes preferential pairing with Thy. Fortunately, mutagenic O^6-methylguanine:Thy mispairs are excellent MMR substrates (9); however, the exact signal transduction processes that regulate G$_2$ arrest responses remain ill defined. It is not clear if MMR signals arrest and cell death directly or if DNA replication is required in the context of newly synthesized DNA for signaling and cell death via DNA double-strand breaks (DSBs).

Two main theories have been proposed to explain MMR-dependent G$_2$ arrest and lethality responses to specific DNA damage in human cancer cells (6). In the futile cycle theory, MMR detects DNA lesions and creates DSBs due to futile cycles of repair or repair-replication fork collision. However, blocking signaling events emitted from MMR-lesion complexes may affect cell cycle and apoptotic "cleanup processes," but not lethality resulting from unrepaired DSBs. In the signaling theory, MMR acts as a DNA damage sensor, directly signaling G$_2$

arrest and cell death. Preventing these signaling pathways would ablate G$_2$ arrest and apoptosis and cause damage tolerance.

Redundant pathways promote G$_2$ arrest after DNA damage in human cells. G$_2$ arrest can be mediated by DNA damage activation of ATM (*Ataxia telangiectasia mutated*) or ATR (*Ataxia telangiectasia-Rad3-related kinase*) phosphatidylinositol 3-like kinases. These kinases phosphorylate and activate p53 and downstream Chk1 and Chk2 checkpoint kinases. Activated Chk1 or Chk2 can phosphorylate the Cdc25C phosphatase at Ser[216], thereby inhibiting Cdc25C activity by 14-3-3-mediated cytoplasmic sequestration. This prevents Cdc25C from dephosphorylating Cdc2 at Tyr[15], a main factor preventing the initiation of mitosis in mammalian cells. The phosphorylation-mediated functional activation of the p53 tumor suppressor by the ATM/Chk2 and/or ATR/Chk1 pathway leads to its transcriptional regulation of several downstream genes involved in cell cycle checkpoint arrest, including p21, *GADD45α* (growth arrest- and DNA damage-inducible-45α), and 14-3-3σ (10). MMR-dependent GADD45α protein increases, in particular, were shown after FdUrd exposure (5), and GADD45α can mediate G$_2$ arrest by direct binding to and inhibiting Cdc2 (10).

Despite numerous indirect studies suggesting activation of specific pathways by MMR that might control G$_2$ arrest, a detailed analysis of MMR-dependent *versus* MMR-independent G$_2$ arrest responses has not been completed. MMR was reported to preferentially stimulate the ATR/Chk1 or ATM/Chk2 pathway in cells after exposure to methylating agents such as 6-TG, MNNG, and temozolomide, the clinical equivalent to MNNG (11). p38α MAPK was also linked to MMR-mediated G$_2$ arrest after temozolomide treatment. Inhibition of the proposed hMLH1/p38α pathway, by small interfering RNA (siRNA) or p38α inhibitors, abrogates MMR-dependent G$_2$ arrest (12). However, activation of this pathway appears to be independent of either Chk1 or Chk2 phosphorylation/signaling. Exposure of human cells to cisplatin can also stimulate MMR-dependent activation of the c-Abl/p73α pathway, leading to apoptosis (13). However, a link between MMR-dependent c-Abl kinase activation and G$_2$ arrest was not examined.

Using MMR-deficient human RKO colon cancer cells (lacking hMLH1 protein expression) corrected by single replacement and expression of hMLH1 protein at endogenous wild-type (WT) levels, we found that the ATR/Chk1 pathway was activated after MNNG treatment; however, its downstream phosphorylation of Chk1 was not MMR-dependent because equivalent consensus site Chk1 phosphorylation was noted regardless of MMR status. Furthermore, expression of dominant-negative ATR in WT MMR cells did not affect G$_2$ arrest or cell death responses, indicating that MMR does not signal through ATR.

Instead, we provide data in this and our accompanying article (41) showing that MMR-dependent G$_2$ arrest is controlled by hMLH1/c-Abl/GADD45α signaling. Although MNNG stimulated both ATR/Chk1 and hMLH1/c-Abl/GADD45α signaling pathways that can mediate G$_2$ arrest, only c-Abl-mediated signaling was activated and regulated the more prolonged MMR-dependent G$_2$ arrest response. These data support the theory of a direct role for MMR signaling to provide time or induce cell

death (41) at the G$_2$ checkpoint to correct or eliminate cells containing mutagenic DNA lesions induced by MNNG exposure.

MATERIALS AND METHODS

Chemicals, Reagents, and Cell Treatments—MNNG (Sigma) was dissolved in Me$_2$SO as a 100 mM stock solution and stored at −20 °C. 6-TG (Sigma) was dissolved in 0.1 N NaOH, and 1 mM stock solutions were stored at −20 °C. STI571 (Gleevec™, Novartis, East Hanover, NJ) was dissolved in water at 5 mM, and stock solutions were stored at −20 °C. PD166326, a c-Abl inhibitor, was synthesized by us and dissolved at 10 mM in Me$_2$SO. Exposure of cells to IR was performed using a ^{137}Cs irradiator (6). Cells were treated with ultraviolet (UVB) radiation at 50 J/m^2.

Cell Culture—MMR-deficient human HCT116 (parental) colon cancer cells and an isogenic MMR-corrected HCT116 3-6 derivative (corrected for hMLH1 expression by microcell transfer of an extra chromosome 3) were provided by Dr. C. R. Boland (Baylor College, Dallas, TX). BRCA1 (breast cancer-associated gene 1) and WT HCC1937 corrected cells were obtained from Dr. K. Yamane (Case Western Reserve University, Cleveland, OH). hMLH1-deficient RKO cells were transfected with cytomegalovirus-driven full-length hMLH1 cDNA (obtained from Drs. A. Buermeyer and R. M. Liskay (Oregon Health Sciences University, Portland, OR), and clones were isolated in G418 (400 μg/ml) by limiting dilution. Clone RKO7 expressed hMLH1 protein levels comparable with WT cells, whereas clone RKO6 contained the hMLH1 expression vector, expressed *neo*[R] without stable hMLH1 or hPMS2 protein expression, and served as a negative control. U2OS-derived stable cells that conditionally regulate FLAG-tagged ATR-WT or kinase-dead ATR (ATR-KD) levels by doxycycline were provided by Drs. P. Nghiem and S. L. Schreiber (Harvard University) (14). Normal human fibroblasts (N2936B) and fibroblasts (AT2052) from ataxia telangiectasia (AT) patients were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Human ATM-deficient fibroblast YZ5 and pEBS7 cells (provided by Dr. Yosef Shilo) were stably transfected with ATM-WT and an empty vector, respectively. Mouse MEF1-1 (*Gadd45α*^{+/+}) and MEF11-1 (*Gadd45α*^{−/−}) cells were provided by Dr. Al Fornace (Georgetown University, Washington, D. C). and grown as described (15). All cells were maintained in Dulbecco's modified Eagle's medium (Cambrex Bio Science, Walkersville, MD) with 10% fetal bovine serum (HyClone, Logan, UT) supplemented with penicillin (10 units/ml) and streptomycin (10 units/ml) in a humidified 95% air and 5% CO$_2$ atmosphere. All cells used were mycoplasma-free.

MMR Status—Comparative genomics was used to evaluate the microsatellite instability (MSI) status in RKO6 *versus* RKO7 cell lines. Arrays manufactured by NimbleGen (Madison, WI) were hybridized with RNA-free DNA extracts of RKO6 and RKO7 cell lines in duplicate, along with a Promega total human standard. Each array contained seven copies of 53,735 unique probes, including WT, single-mismatch, double-mismatch, and deletion probes for all possible 1-mer through 6-mer repeats and their complements. This array also contained seven copies of poly(A) and poly(T) probes ranging from 29-mer to

47-mer in length to specifically measure MSI. Total poly(A)/poly(T) amplification of the genome at ~3900 loci, dramatically more than the five poly(A) mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27), was used to measure MSI (16). The data were robust multiarray analysis (RMA)-normalized; specificity was confirmed using mismatch probes; and two replicates for each of a total of 126 poly(A) and 126 poly(T) probes were averaged. The relative total poly(A)/poly(T) content was measured as a ratio of averages for RKO6 and RKO7 cell line genomes.

Stable Short Hairpin RNA (shRNA) Knockdown—Human p73α shRNA (shp73; RHS3979-95754010), GADD45α shRNA (shGADD45α; RHS3979-9629535), and c-Abl shRNA (shABL; RHS1764-9493323) lentiviral and retroviral vectors were purchased from Open Biosystems (Huntsville, AL). Stable shRNA knockdown clones were generated by infecting RKO6 or RKO7 cells with Polybrene-supplemented medium obtained from Phoenix packaging cells transfected with viral shRNA vectors targeting p73α, GADD45α, and c-Abl. Stable scrambled shRNA (shSCR) controls were also generated. Individual clones were isolated in medium containing both puromycin (1 μg/ml) and G418 (400 μg/ml) by limiting dilution and screened for targeting protein levels. All experiments were performed without antibiotics.

Transient siRNA Knockdown—Transient SMARTpool siRNA targeting ATR in MMR$^+$ RKO7 cells or a scrambled control (SrcII) was purchased from and used as directed by Dharmacon (Lafayette, CO). ATR expression was monitored by Western blotting (supplemental Fig. 1*B*). After transfection (48 h) when ATR levels were significantly knocked down and scrambled shRNA-transfected cells expressed basal ATR levels, cells were treated with MNNG and analyzed for G$_2$ arrest and apoptosis.

Colony-forming Ability Assays—Survival was assessed by colony-forming ability using standard techniques, wherein colonies of >50 normal-appearing cells were counted (6). Isogenic cells were treated for 1 h with MNNG or continuously with 6-TG for 6–7 days. Pretreatment with STI571 or other c-Abl inhibitors consisted of a 2-h pretreatment prior to MNNG (1 h) exposure.

Cell Cycle Analyses—Human cells were synchronized by growth to >95% confluence and maintained for 48 h in Dulbecco's modified Eagle's medium containing low serum (0.1% fetal bovine serum). To initiate synchrony, confluent cells were trypsinized and reseeded at 1:15 in 10% fetal bovine serum-containing Dulbecco's modified Eagle's medium. MNNG was added 16 h after release, corresponding to a time prior to S phase initiation, and beyond the p53-mediated G$_1$ checkpoint (5). Pretreatment with the STI571 inhibitor occurred 2 h prior to MNNG exposure (10 μM, 1 h). At various times post-treatment, cells were fixed and treated with RNase A (100 μg/ml) and propidium iodide (50 μg/ml in phosphate-buffered saline) overnight at 4 °C. Cells were then analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). MPM-2 staining was performed to distinguish G$_2$ and M phases of the cell cycle as described (5). Quantification of cell cycle populations was assessed using ModFit LT Version 3.0 software (Verity Software House, Topsham, ME). The results presented were from three or more independent experiments.

Immunoblot and c-Abl Kinase Activity Analyses—Human cells were synchronized and treated with MNNG, and whole cell extracts and Western blots were prepared (5). Antibodies to hMLH1 (Ab-2), hMSH2 (Ab-1), hPMS2 (Ab-1), ATR (Ab-2), and c-Abl (Ab-1) were obtained from Oncogene Research Products (Boston, MA). Anti-cyclin B$_1$ (H-20), anti-GADD45α (H-165), anti-Cdc2 (C-19), anti-Chk1 (FL-476), and anti-Chk2 (A-12) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All phospho-specific antibodies were purchased from Cell Signaling (Beverly, MA): phospho-Chk1 (Ser^{296}, Ser^{317}, and Ser^{345}), phospho-Chk2 (Thr^{68}), and phospho-Cdc2 (Tyr^{15}). Antibodies for p73β (259A) and p73α (5B429), β-actin (AC-15), and glyceraldehyde-3-phosphate dehydrogenase (6C5) were from Imgenex Corp. (San Diego, CA), Sigma, and Calbiochem, respectively. Appropriate horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology) and Super-Signal chemiluminescence substrate (Pierce) were used for detection. Signals were detected using Fuji RX medical x-ray film.

Nonradioactive c-Abl kinase assays were performed as described (SignaTECT, Promega) with modifications. Cell extracts were prepared as described (5) with addition of two phosphatase inhibitor mixtures (sc-45044 and sc-45045, Santa Cruz Biotechnology). GST-Abltide peptide (~28 kDa; Upstate Biotechnology, Lake Placid, NY) was used as a high affinity c-Abl substrate; GST-Abltide contains the sequence EAIYAAPFAKKK, identical to GST-CRK. Kinase reactions were initiated with cell extracts (5 μg) and Abltide substrate (1 μg) for 30 min. Separate c-Abl kinase reactions using purified protein were performed as controls. Extracts were resolved and visualized by Western blotting, and membranes were probed using an anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology), normalized by Ponceau S staining.

Statistics—All experiments described were performed in triplicate with representative images shown. Quantification of protein levels or c-Abl kinase activities was performed by scanning x-ray films and analyzing scans using NIH Image J software. Statistical significance was determined using paired Student's *t* tests.

RESULTS

Restoration of hMLH1 Expression and MMR Function in MMR-deficient RKO Cells—RKO cells lack hMLH1 expression due to promoter hypermethylation (4). hMLH1 expression was restored by stably transfecting RKO cells with cytomegalovirus-driven hMLH1 cDNA. Two separate clones designated RKO6 (MMR$^-$), which remained deficient for hMLH1 expression, and RKO7 (MMR$^+$), which expressed hMLH1 protein levels equivalent to MMR-proficient HCT116 3-6 cells (Fig. 1*A*) corrected by chromosome 3 microcell fusion (6, 7), were isolated and chosen for further investigation. RKO7 (MMR$^+$) cells showed stable hPMS2 protein levels; hMLH1 and hPMS2 are binding partners (MutLα) that require each other for stability. Notably, hMLH1 protein was not expressed in MMR-deficient RKO6 cells, as found in parental RKO cells. Using a compara-
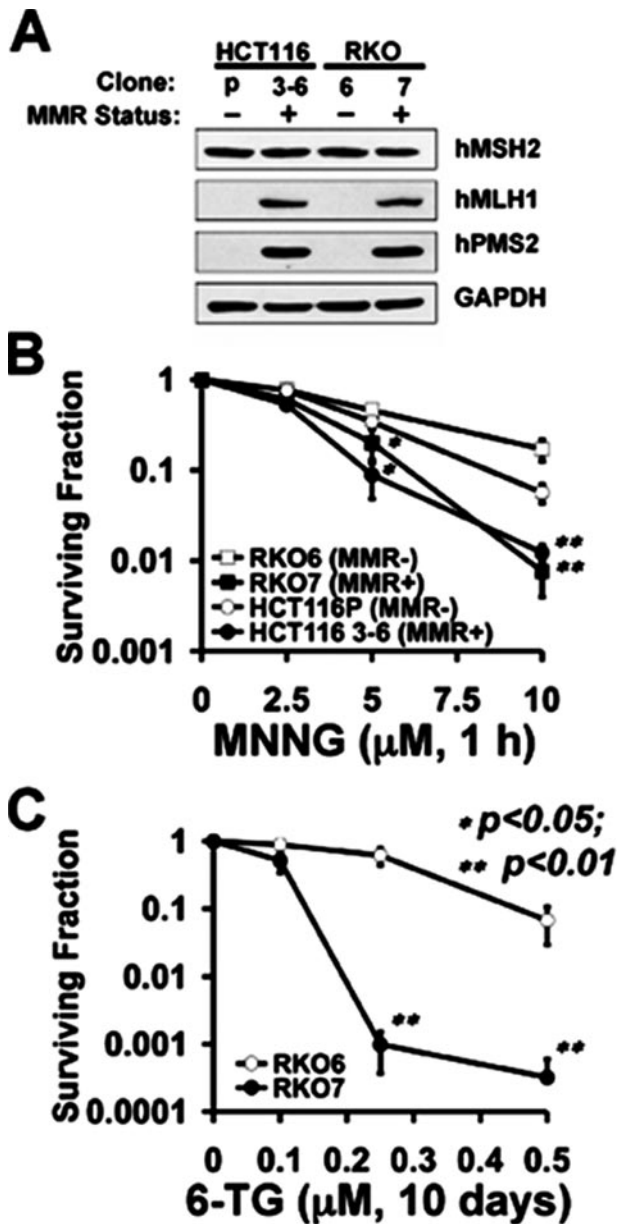
FIGURE 1. **Re-expression of hMLH1 and MMR restoration increase the sensitivity of MMR-deficient RKO cells to MNNG or 6-TG exposure.** Parental RKO cells were transfected with cytomegalovirus-driven hMLH1 cDNA, and clones were isolated by limiting dilution. *A*, Western blots show re-expression of hMLH1 and hPMS2 stabilization in RKO7 cells to a level similar to that in HCT116 3-6 cells (5). hMSH2 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) levels served as a loading controls. MMR$^-$ HCT116 and MMR$^+$ HCT116 3-6 cells served as negative and positive controls, respectively. Shown are survival assays of MMR$^-$ RKO6 and HCT116 cells and matched hMLH1-expressing MMR$^+$ RKO7 and HCT116 3-6 cells exposed to MNNG (μM, 1 h) (*B*) or after continuous 6-TG (μM) exposure (*C*). Data are the means $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$.

tive genomic hybridization array-based method, the MSI status of the RKO6 *versus* RKO7 genome was quantitatively measured at ~3900 poly(A)/poly(T) sites within the human genome. A >2.3 $\pm$ 0.1-fold reduction of poly(A)/poly(T) content in the RKO7 genome relative to the RKO6 genome was observed, thus confirming its restoration to a more MSI-stable genome.

To determine whether re-expression of hMLH1 in RKO7 and RKO6 cells was functional, survival was assessed after MNNG or 6-TG exposure. RKO7 (MMR$^+$) cells were signifi-

cantly more sensitive than RKO6 cells to each agent ($p < 0.001$) (Fig. 1, *B* and *C*). RKO7 cells were as sensitive as HCT116 3-6 (MMR$^+$) cells after MNNG exposure, whereas RKO6 cells were as resistant as hMLH1-deficient HCT116 P cells (Fig. 1*B*). RKO7 cells were also more sensitive than RKO6 (MMR$^-$) cells to 6-TG ($p < 0.001$) (Fig. 1*C*). These results demonstrated that restoration of MMR by exogenous hMLH1 expression in RKO7 cells enhanced their sensitivity to MNNG or 6-TG. In contrast, MMR-deficient RKO6 and HCT116 P cells were resistant and damage-tolerant.

Expression of hMLH1 Restores MMR-dependent G$_2$ Arrest Responses—To determine whether the RKO clones established above (Fig. 1*A*) were concomitantly corrected for MMR-dependent G$_2$ arrest responses (6, 7), we synchronized cells as described under "Materials and Methods," treated them with MNNG (5 μM for HCT116 and 10 μM for RKO, 1 h) at the indicated times (16 h after synchronous release) (Fig. 2*A*), and examined their cell cycle profiles as described (6, 7). In response to MNNG, both MMR$^+$ and MMR$^-$ cells showed an initial G$_2$ arrest response 20 h after MNNG exposure. Thereafter, MMR-deficient RKO6 cells rapidly progressed through the cell cycle. In contrast, MMR-corrected RKO7 cells exhibited a prolonged G$_2$ arrest response (6, 7). However, because these cells were synchronized, we noted that, contrary to other groups (17), MNNG-dependent G$_2$ arrest occurred within the first round of replication after treatment. Differential cell cycle G$_2$ arrest was also observed in matched RKO clones following continuous exposure to 2.5 μM 6-TG (data not shown).

Changes in expression of cyclin B$_1$ and Cdc2 protein levels were examined to confirm differential G$_2$ arrest responses in MMR-proficient *versus* MMR-deficient cells. Elevated levels of cyclin B$_1$, corresponding to the relative number of cells in G$_2$, were observed in MNNG-treated MMR-competent cells (Fig. 2*B*). The activity of Cdc2, a G$_2$ cyclin-dependent kinase, depends on its association with cyclin B$_1$ and its phosphorylation of specific amino acid residues (18, 19). In particular, the specific phosphorylation of Tyr[15] inhibits its activity, preventing cells from migrating through G$_2$ to M phase of the cell cycle. Using an antibody that specifically recognizes phosphorylated Tyr[15] of Cdc2, we noted enhanced levels of Cdc2 Tyr[15] phosphorylation in MMR-proficient cells, corresponding directly to higher levels of G$_2$-arrested cells (Fig. 2*B*). In contrast, total cellular Cdc2 levels were constant throughout the cell cycle. β-Actin served as a loading control, with constant levels noted.

We originally noted that p53 was preferentially stabilized in MNNG-, 6-TG-, or IR-treated MMR-proficient cells, a response later confirmed by others (6, 20). Using an antibody specific for p53 phosphorylated at Ser[15], we showed that MMR-proficient HCT116 3-6 and RKO7 cells displayed elevated p53 Ser[15] phosphorylation and stabilized p53 levels compared with isogenic MMR$^-$ cells (41). Interestingly, p21 (an inhibitor of cyclin-dependent kinase and a well characterized p53-dependent downstream gene) displayed similar induction levels irrespective of MMR status after MNNG treatment (41). Nevertheless, selective MMR-dependent p53 phosphorylation (and stabilization) was theorized to be a result of upstream signaling by phosphatidylinositol 3-like kinases such as ATM and its related kinase ATR, as well as their downstream checkpoint
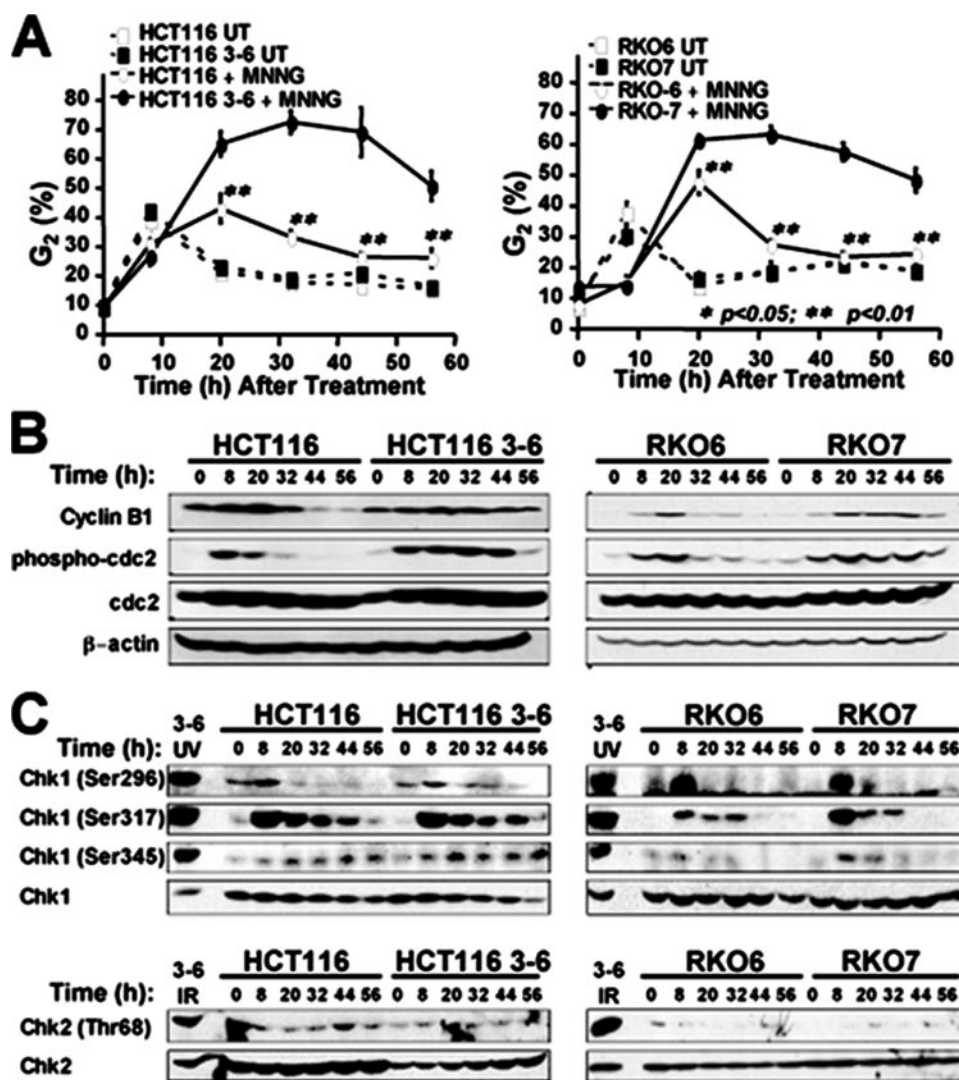
FIGURE 2. **Enhanced G_2 arrest in synchronized MMR-proficient cells after MNNG exposure.** MMR^- and MMR^+ HCT116 and RKO cells were synchronized and exposed to MNNG (5 or 10 μM, respectively; 1 h) at 16 h post-release, and samples were prepared for flow cytometry and Western analyses at the indicated times. *A*, percentage of G_2/M cells by fluorescence-activated cell sorter analyses. *B*, Western analyses of cyclin B_1, Cdc2, phospho-Cdc2 (Tyr[15]), and β-actin expression. *C*, Western analyses of phosphorylated and total Chk1 and Chk2 levels. Data are the means $\pm$ S.E. of three or more independent experiments. *, $p < 0.05$; **, $p < 0.01$. *UT*, untreated.

shown). UV radiation (50 J/m²) and ionizing radiation (10 grays (Gy)) were used as positive controls for ATR and ATM activation and associated Chk1 and Chk2 damage-induced phosphorylation, respectively (Fig. 2C). These data suggest that ATR/Chk1, but not ATM/Chk2, is activated after MNNG exposure in synchronized cells and, notably, that activation of the ATR/Chk1 pathway is independent of MMR function.

Neither ATM nor ATR Mediates MMR-dependent G_2 Arrest—To explore the role of ATM in MMR-dependent G_2 arrest responses, we compared synchronized AT (pEBS7) or ATM-corrected (YZ5) AT fibroblast cells for their responses to IR (0.75 Gy) *versus* MNNG (10 μM, 1 h). YZ5 (ATM^+) cells showed significantly greater ($p < 0.05$) G_2 arrest compared with pEBS7 cells with IR treatment (0.75 Gy), consistent with prior reports of a role for ATM in G_2 arrest after IR (22). In contrast, when treated with MNNG (10 μM, 1 h), pEBS7 ($ATM^{-/-}$) cells showed prolonged G_2 arrest responses compared with YZ5 (ATM^+) cells (Fig. 3A), similar to results found using synchronized AT (AT2052) fibroblast *versus* normal human fibroblast (N2639B) cells after MNNG exposure (10 μM, 1 h) (supplemental Fig. 1A). We also explored the role of ATR in G_2 arrest responses using U2OS cells containing doxycycline-inducible ATR-WT or ATR-KD (Fig. 3B). As

kinases Chk1 and Chk2, respectively. We therefore examined the potential involvement of the ATM/Chk2 and ATR/Chk1 signaling pathways in MMR-dependent G_2 arrest responses after MNNG exposure.

ATR can activate Chk1 and ATM can activate Chk2 by phosphorylating specific amino acid residues (21). Using antibodies that specifically recognize these phosphorylated forms of Chk1 and Chk2, we found that both RKO6 and RKO7 cells displayed equivalent Chk1 phosphorylation levels at Ser[296], Ser[317], and Ser[345] in response to MNNG (Fig. 2C). Notably, no phosphorylation of Chk2 at Thr[68] was observed in either cell line after MNNG exposure. Interestingly, parental HCT116 cells appeared to have much greater overall levels of Chk2 compared with MMR-corrected HCT116 3-6 cells. The overall protein levels of Chk1 and Chk2 remained constant throughout the cell cycle, with no increases in phosphorylation of either Chk1 or Chk2 in synchronized untreated control cells (data not

with ATM deficiency (Fig. 3A), loss of functional ATR due to ATR-KD expression did not influence G_2 arrest responses compared with U2OS cells expressing ATR-WT after various MNNG doses (Fig. 3, C–E). These data are consistent with the lack of differential MMR-dependent downstream Chk1 and Chk2 phosphorylation responses (Fig. 2) and strongly suggest that neither ATM nor ATR influences G_2 arrest after MNNG exposure. In contrast, loss of functional ATR greatly affected IR-induced G_2 arrest responses, as well as G_2 arrest induced by UVB light (14). Results with ATR-KD were confirmed using siRNA oligomer knockdown of ATR, wherein no significant change in MNNG-induced G_2 arrest was noted in MMR^+ RKO7 ATR siRNA cells (where ATR levels were knocked down by ~85%) compared with MMR^+ RKO7 cells exposed to scrambled siRNA oligomers (supplemental Fig. 1, B and C).

c-Abl Kinase Is Required for MMR-dependent G_2 Arrest—Because MMR-dependent activation of c-Abl kinase after cispla-
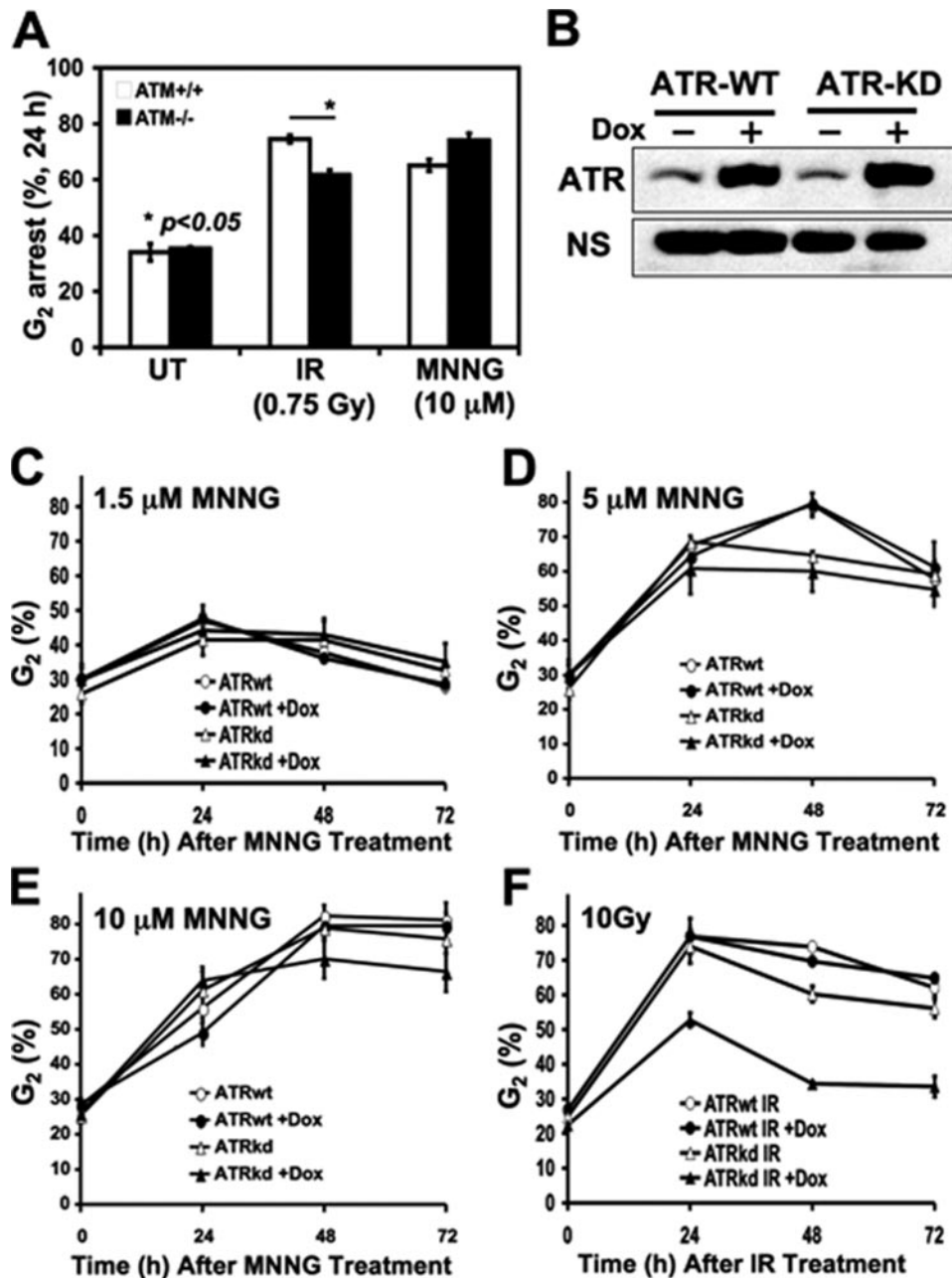
A

B

We also performed these experiments by treating cells with 5 or 10 μM STI571 for 16–24 h using previously published procedures (supplemental Fig. 2, *A* and *B*) (25). Consistent with data in Fig. 4 (*A* and *B*), we found that, at these lower concentrations of STI571, MNNG-induced MMR-dependent G_2/M checkpoint responses were abrogated. These data further support a role for MMR-activated c-Abl activity in these checkpoint responses. Because STI571 can inhibit other tyrosine kinases in addition to c-Abl, we tested another c-Abl/Src kinase inhibitor, PD166326, which was developed to overcome STI571 resistance (24). Similar to STI571, pretreatment of MMR$^+$ cells with PD166326 (5 μM, 2 h) preferentially abrogated MMR-dependent G_2 arrest after MNNG exposure. Treatment with either STI571 or PD166326 alone had no effect on the cell cycle. These findings showed that pretreatment with either STI571 or PD166326 abrogated MMR-dependent G_2 arrest and strongly suggest that c-Abl kinase is critical for MMR-dependent G_2 arrest signaling.

Although STI571 is more specific for c-Abl than for other tyrosine kinases *in vivo*, it may inhibit related enzymes at higher doses (*e.g.* platelet-derived growth factor, Src, and c-Kit) (24). Therefore, we also employed a genetic approach using stable shRNA to specifically knock down c-Abl protein levels and enzymatic activity. We generated isogenically matched MMR-proficient and MMR-deficient RKO cell lines expressing specific shRNA targeting c-Abl (shABL) or scrambled sequence (shSCR) (Fig. 5*A*). Notably, hMLH1 protein levels were not

C

D

E

F

FIGURE 3. **MMR-dependent G₂ arrest responses are not mediated by ATM or ATR.** *A*, AT pEBS7 (ATM$^{-/-}$) or corrected YZ5 (ATM$^{+/+}$) human fibroblasts with identical doubling times were synchronized, exposed to IR (0.75 Gy) and MNNG (10 μM, 1 h) at 16 h post-synchronization, and analyzed for G_2 arrest responses. *UT*, untreated. *B*, doxycycline (*Dox*) induction of FLAG-tagged ATR-WT or dominant-negative ATR-KD was confirmed in U2OS cells. *NS*, nonspecific band for loading. *C–F*, asynchronous cells from *B* were exposed to 1.5, 5.0, or 10 μM MNNG for 1 h or to IR (10 Gy), respectively. G_2 arrest responses were monitored at various times. Changes in G_2 arrest were noted only after IR exposure (10 Gy). *, $p < 0.05$.

tin exposure was reported previously (23), we examined a role for its activation in MNNG-induced G_2 arrest. Pretreatment of isogenic MMR$^{+/-}$ RKO cells with the c-Abl inhibitor STI571 (Gleevec^{TM}) (25 μM, 2 h) prior to MNNG exposure dramatically abrogated subsequent G_2 arrest responses (Fig. 4*A*); The BCR-ABL inhibitor STI571 was designed to target the BCR-ABL fusion gene product or Philadelphia translocation that causes >90% of chronic myelogenous leukemia (24). Interestingly, STI571 did not abrogate the more transient G_2 arrest responses observed at 20 h in MMR-deficient cells (Fig. 4*A*).

affected in RKO7-shABL or RKO7-shSCR clones (Fig. 5*A*). RKO6-shABL clone 2 and RKO7-shABL clone 27 had the greatest c-Abl knockdown, with <60% and <80% reduced protein levels, respectively. The c-Abl-deficient (shABL) and shSCR-containing RKO7 cells were then examined for differences in MNNG-induced G_2 arrest (Fig. 5*B*). Further time course examination of all clones revealed that loss of c-Abl protein and activity resulted in loss of G_2 arrest responses (Fig. 5*C*). Collectively, these results suggest that c-Abl plays an important and specific role in MMR-dependent G_2 arrest responses to
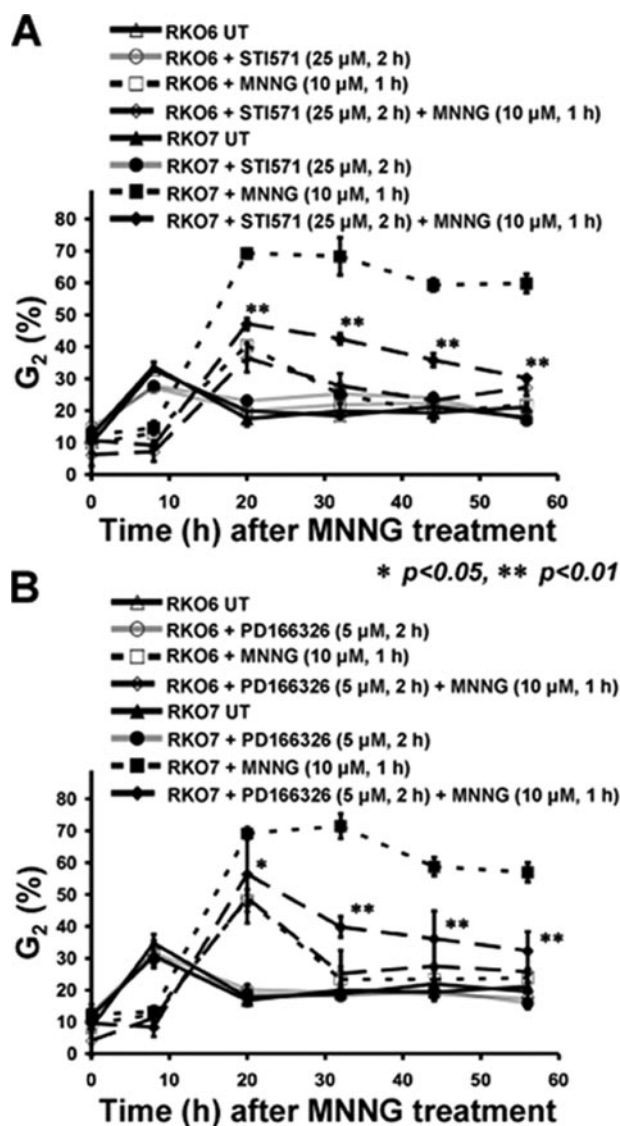
FIGURE 4. **Pharmacological inhibition of c-Abl abrogates MMR-dependent G$_2$ arrest.** Synchronized isogenic MMR$^+$ and MMR$^-$ RKO cells were pretreated for 2 h with STI571 or PD166326 at the indicated doses prior to MNNG exposure (10 μM, 1 h). *A*, time course of G$_2$ arrest in synchronized isogenic MMR$^-$ and MMR$^+$ RKO cells mock-treated or treated with STI571 (25 μM, 2 h), MNNG (10 μM, 1 h), and STI571 (25 μM, pretreatment for 2 h) + MNNG (10 μM, 1 h). *B*, time course of G$_2$ arrest in synchronized MMR$^-$ and MMR$^+$ RKO cells mock-treated or treated with PD166326 (5 μM, 2 h), MNNG (10 μM, 1 h), and PD166326 (5 μM, pretreatment for 2 h) + MNNG (10 μM, 1 h). *, $p < 0.05$; **, $p < 0.01$. *UT*, untreated.
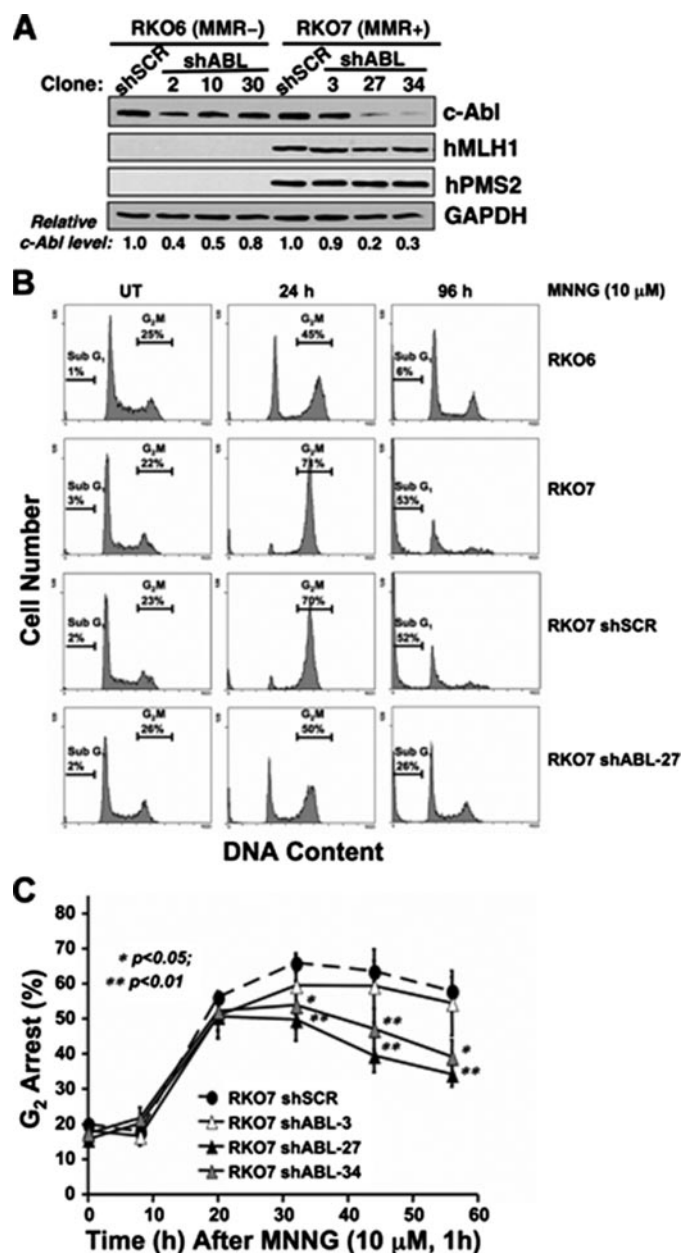
FIGURE 5. **c-Abl knockdown by shRNA abrogates MMR-dependent G$_2$ arrest after MNNG exposure.** MMR$^-$ RKO6 and MMR$^+$ RKO7 cells were knocked down for c-Abl expression using pShag2-c-Abl shRNA (shABL) or pShag2-scrambled shRNA vector containing noncoding nonsense sequences (shSCR). *A*, Western blot analyses of c-Abl, hMLH1, hPMS2, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) protein expression in isogenic MMR$^+$ and MMR$^-$ RKO cells stably expressing shSCR or shABL sequences. Glyceraldehyde-3-phosphate dehydrogenase levels were monitored for loading. *B*, G$_2$ arrest response at the 24-h time point and apoptosis at the 96-h time point in synchronized isogenic MMR$^-$ RKO6, MMR$^+$ RKO7, and MMR$^+$ RKO7-shABL-27 clones after MNNG exposure (10 μM, 1 h). *UT*, untreated. *C*, time course of G$_2$ cell cycle checkpoint arrest responses in synchronized RKO6 and RKO7-shABL knockdown clones after MNNG exposure (10 μM, 1 h). *, $p < 0.05$; **, $p < 0.01$.

MNNG. Furthermore, c-Abl does not play a role in the more transient MMR-independent G$_2$ arrest responses observed, which are probably mediated by ATR/Chk1 signaling.

Stabilization of p73α, Expression of GADD45α, and Enhanced c-Abl Tyrosine Kinase Activity after MNNG Treatment Are MMR-dependent—We then sought direct evidence for MMR-dependent c-Abl activation. The p53 homolog p73α is a known downstream responsive gene controlled by c-Abl (26), and c-Abl-dependent stabilization of p73α after cisplatin exposure requires functional MMR (13). Stabilization during exposure to specific DNA-damaging agents is dependent on c-Abl-mediated phosphorylation at Tyr^{99} of p73α (27). We examined p73α expression in MMR$^-$ RKO6 and MMR$^+$ RKO7

cells after MNNG treatment (10 μM, 1 h) by Western analyses (Fig. 6*A*). We noted a MMR-dependent increase in p73α expression 8–56 h post-treatment in MMR$^+$ RKO7 cells, but only a transient increase in p73α protein stabilization at 8 h in MNNG-treated RKO6 cells. Interestingly, MNNG-induced p73α protein stabilization mirrored G$_2$ arrest responses, with
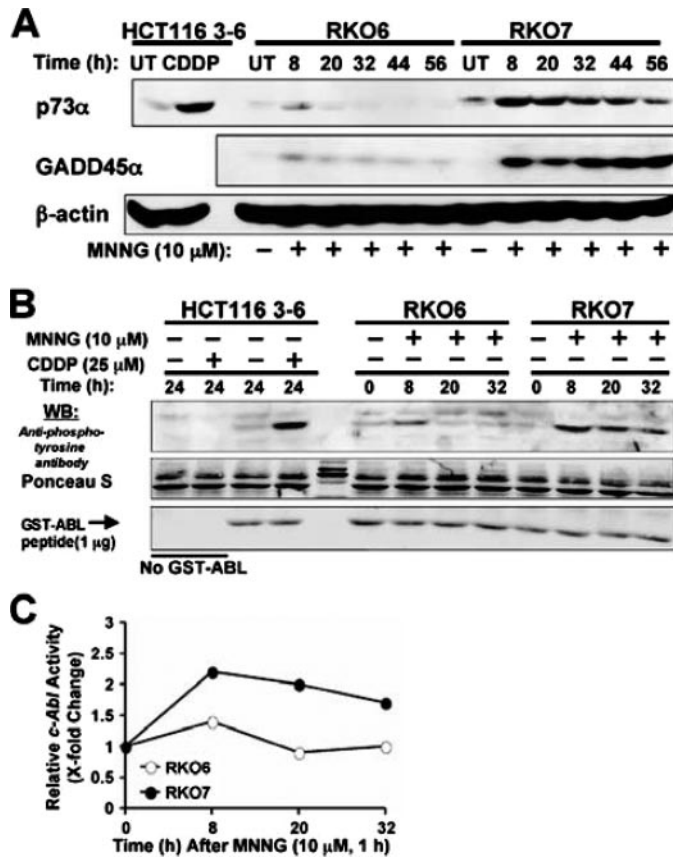
FIGURE 6. **MMR-dependent increases in p73α stabilization, GADD45α expression, and c-Abl-mediated kinase activity after MNNG treatment.** Synchronized isogenic MMR⁺ and MMR⁻ RKO cells were exposed to MNNG (10 μM, 1 h), and the time courses of proteins and c-Abl activities were monitored at the times indicated. *A*, Western blot analyses of p73α, GADD45α, and β-actin protein expression. *UT*, untreated. *B*, analyses of c-Abl activities from isogenic MMR⁺ and MMR⁻ RKO cells exposed to MNNG (10 μM, 1 h). *C*, the relative levels of phosphotyrosine expression of a GST-Abltide (*ABL*) peptide were monitored, quantified, and graphed. Ponceau S staining of membranes displayed loading of the GST-Abltide peptide substrate. MMR⁺ HCT116 3-6 cells treated with cisplatin (*CDDP*; 25 μM, 24 h) or mock-treated cells were used as positive and negative controls, respectively, for p73α stabilization and c-Abl kinase activation.

increases prior to peak G₂ levels. Thus, p73α was preferentially stabilized in an MMR-dependent manner after MNNG exposure. Cisplatin (25 μM, 24 h) and mock-exposed HCT116 3-6 cells were used as positive and negative controls, respectively, for p73α stabilization as described (13).

Prior work from our laboratory showed that GADD45α levels were elevated in MMR-proficient cells after FdUrd exposure (5). GADD45α was previously implicated in G₂ arrest regulation, making it a likely candidate in controlling MMR-dependent G₂ arrest (10). We therefore determined whether GADD45α expression was differentially expressed in isogenic MMR⁺/⁻ RKO cells after MNNG exposure. As we previously found after FdUrd exposure (5), MMR-dependent induction of GADD45α levels was noted after MNNG exposure (Fig. 6A). MMR-dependent GADD45α induction was also observed in isogenic HCT116 3-6 *versus* HCT116 cells after MNNG exposure (data not shown). Although minor transient increases in both p73α and GADD45α expression were also noted in MMR-deficient cells after MNNG treatment, these experiments

clearly demonstrate the MMR dependence of expression for each of these proteins.

Because stabilization of p73α after cisplatin treatment is attributed to MMR-dependent increases in c-Abl tyrosine kinase activity (13), we wanted to determine whether c-Abl kinase activity was directly activated in an MMR-dependent manner after MNNG exposure. We therefore assessed the relative levels of c-Abl kinase activities by measuring phosphorylation at a specific recognition protein sequence within the GST-Abltide peptide. c-Abl activities were measured in synchronized MMR⁻ RKO6 and MMR⁺ RKO7 cells before and after MNNG exposure. Enhanced GST-Abltide tyrosine phosphorylation in MMR-proficient cells treated with either cisplatin (25 μM, 24 h) or MNNG (10 μM, 1 h) was noted (Fig. 6B; quantified in *C*). As with p73α stabilization, the levels of c-Abl kinase activity, judged by tyrosine phosphorylation, correlated with G₂ arrest responses in MMR⁺ RKO7 cells (Fig. 2). In contrast, MMR⁻ RKO6 cells showed transient increases in c-Abl activity from basal levels at 8 h (Fig. 6, *B* and *C*), and these corresponded with transient increases in p73α and GADD45α protein expression (Fig. 6A) and G₂ arrest (Fig. 2).

To determine whether MMR-dependent p73α stabilization and GADD45α induction in response to MNNG were dependent on c-Abl kinase, we examined mock- or MNNG-treated RKO7-shSCR or RKO7-shABL-27 cells or RKO7-shSCR cells pretreated with STI571 (25 μM, 2 h). Decreased p73α and GADD45α protein levels concomitant with decreased c-Abl and G₂ arrest responses were noted in shABL-27 knockdown RKO7 cells (Fig. 7A). Furthermore, STI571 pretreatment of RKO7-shSCR (shown) or parental RKO7 (not shown) cells greatly suppressed p73α and GADD45α expression (Fig. 7A) and c-Abl activity (Fig. 7B) after MNNG treatment (10 μM, 1 h). As expected, STI571 also inhibited c-Abl activity in cisplatin-exposed MMR⁺ HCT116 3-6 cells (Fig. 7B). These data further support a role for MMR-dependent activation of c-Abl activity that controls GADD45α and p73α downstream gene expression to potentially regulate G₂ arrest and cell death.

GADD45α, But Not p73α, Regulates MMR-dependent G₂ Arrest—Because upstream MMR-dependent activation of c-Abl appears to regulate p73α and GADD45α levels, we explored the functional roles of p73α and GADD45α in MMR-dependent G₂ arrest responses to MNNG. We generated knockdown cells using shRNA lentivirus specific for p73α (Fig. 8A) or GADD45α (shGADD45α) (Fig. 8C). RKO6-shp73 clone 15 and RKO7-shp73 clone 4 had the greatest p73α knockdown, with 90% reduced protein levels each compared with shSCR-containing RKO cells. Surprisingly, silencing p73α failed to abrogate MMR-dependent G₂ arrest despite its pro-survival effects on apoptosis and survival (Fig. 8B) (41). Thus, knocking down p73α in RKO7 cells significantly abrogated the apoptotic and lethality responses to MNNG without affecting MMR-dependent G₂ arrest. These data are the first to demonstrate uncoupling of the MMR-dependent G₂ arrest signaling from lethality.

In contrast, reduction of GADD45α levels affected both G₂ arrest and lethality. RKO6-shGADD45α clone 1 and RKO7-shGADD45α clone 2 had the greatest GADD45α knockdown, with <70% and <80% reduced protein levels, respectively.
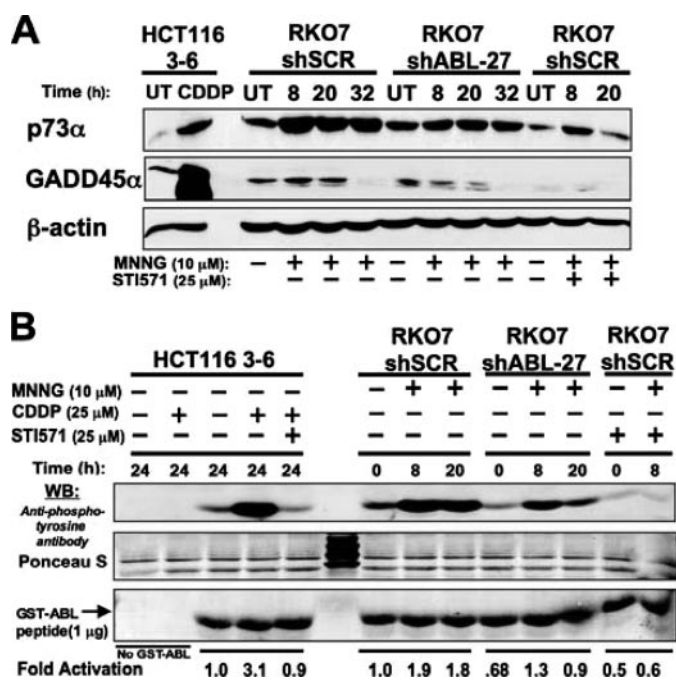
FIGURE 7. **c-Abl inhibition, by STI571 or genetic silencing, suppresses MMR-dependent p73α stabilization, GADD45α expression, and c-Abl-mediated kinase activity after MNNG treatment.** At various times, cell lysates were simultaneously prepared for Western blot and c-Abl kinase activity analyses from synchronized isogenic MMR$^+$ RKO7-shSCR and MMR$^+$ RKO7-shABL-27 stable knockdown cells after MNNG treatment (10 μM, 1 h). A portion of the cells were pretreated with STI571 (Gleevec^{TM}, 25 μM, 2 h) prior to MNNG exposure. *A*, Western analyses of p73α, GADD45α, and β-actin protein expression. *UT*, untreated. *B*, Western blot (*WB*) analyses of the relative phosphotyrosine levels of GST-Abltide (*ABL*). Ponceau S staining was used for GST-Abltide peptide loading. HCT116 3-6 cells were treated with cisplatin (*CDDP*; 25 μM, 24 h) or left untreated as positive and negative controls, respectively.

Silencing GADD45α significantly abrogated MMR-dependent G$_2$ arrest responses to MNNG ($p < 0.05$) (Fig. 8*D* and supplemental Fig. 3), as well as apoptosis and survival (41). These data strongly suggest that MMR-dependent G$_2$ arrest responses to MNNG are regulated by GADD45α and not by p73α.

Inhibition of c-Abl Kinase Activity Reverses MMR-dependent Sensitivity to MNNG—The findings above indicate that c-Abl plays a role in MMR-dependent G$_2$ arrest and p73α stabilization. To determine whether c-Abl also plays a role in MMR-dependent cell survival after MNNG treatment, MMR$^+$ RKO7-shABL cells were pretreated with 25 μM STI571 prior to MNNG exposure (10 μM, 1 h). STI571 decreased sensitivity to MNNG preferentially in MMR-proficient cells (Fig. 9*A*). To confirm these results, we examined whether isogenic c-Abl shRNA knockdown cells were more resistant to MNNG treatment (Fig. 9*B*) than RKO7-shSCR or parental MMR$^+$ RKO7 cells. We found that MMR$^+$ RKO7-shABL-27 and RKO7-shABL-34 cells were significantly more resistant to MNNG, similar to STI571. In contrast, the sensitivities of MMR$^-$ RKO6-shABL-2 and RKO6-shABL-30 cells were not affected compared with RKO6-shSCR or parental cells after MNNG exposure. Finally, we treated RKO7-shABL-27 and RKO7-shABL-34 cells with low doses of STI571 ($\leq$5 μM). Silencing c-Abl protein expression by shRNA was predicted to make these cells more responsive (*i.e.* survive better) after STI571 doses (Fig. 9*C*). Indeed, c-Abl kinase knockdown cells were
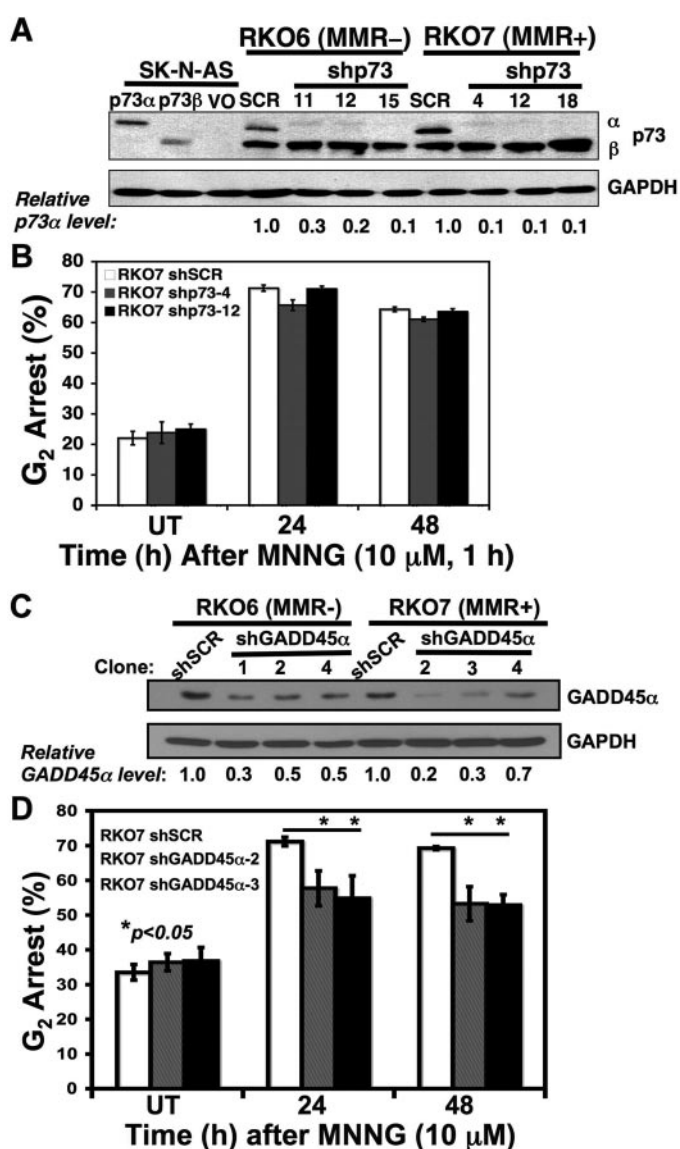

FIGURE 8. **c-Abl-mediated stabilization of GADD45α, but not p73α, regulates G$_2$ arrest responses in MMR$^+$ cells after MNNG treatment.** *A*, generation of p73α knockdown RKO6 and RKO7 clones. RKO6 (clones 11, 12, and 15) and RKO7 (clones 4, 12, and 18) clones expressing stable shp73 or a control shSCR vector were generated. Note that p73β levels were not affected in knockdown clones. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *B*, specific knockdown of p73α does not abrogate G$_2$ arrest responses to MNNG (10 μM, 1 h). *UT*, untreated. *C*, generation of GADD45α knockdown RKO6 and RKO7 clones. RKO6 (clones 1, 2, and 4) and RKO7 (clones 2, 3, and 4) clones expressing stable shGADD45α or a control shSCR vector were generated. *D*, RKO7-shSCR, RKO7-shGADD45α-2, and RKO7-shGADD45α-3 clones were exposed to MNNG (10 μM, 1 h) and assessed for G$_2$ arrest. Specific knockdown of GADD45α abrogated G$_2$ arrest responses to MNNG. *, $p < 0.05$.

spared by 1–5 μM STI571, whereas control RKO7-shSCR cells required 5–15-fold greater STI571 levels (*i.e.* 15–25 μM). Thus, STI571 was more efficacious at lower doses in c-Abl shRNA knockdown cells than in RKO7-shSCR cells (Fig. 9*C*).

DISCUSSION

MMR senses and repairs damaged lesions, but also appears to directly activate cell cycle arrest signaling pathways. The long-held theory that cells arrest at specific checkpoints, such as in G$_2$, to allow time for repair of mutations prior to cell cycle
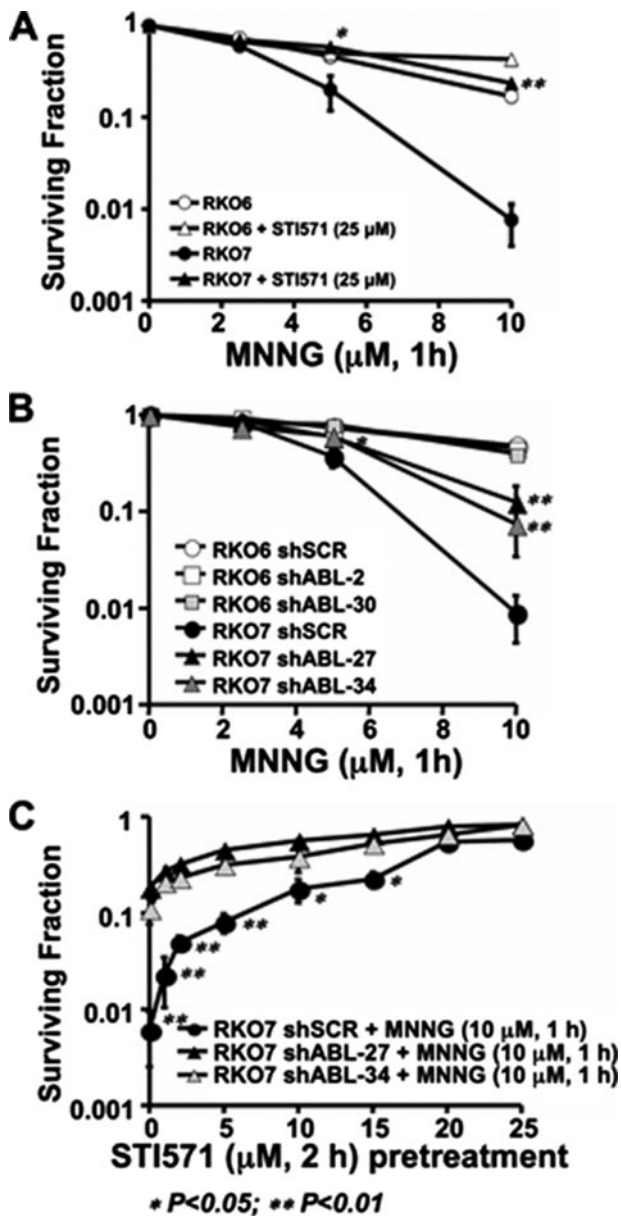
FIGURE 9. **c-Abl inhibition, by STI571 or genetic silencing, suppresses MMR-dependent lethality after MNNG treatment.** *A*, survival assays of isogenic MMR$^+$ and MMR$^-$ RKO cells pretreated with STI571 (25 μM, 2 h) and exposed to varying doses of MNNG (μM, 1 h). *B*, survival of isogenic MMR$^+$ and MMR$^-$ RKO-shSCR clones compared with c-Abl knockdown shABL clones exposed to various MNNG doses (μM, 1 h). *C*, survival of MMR$^+$ RKO7 and MMR$^+$ RKO7-shABL knockdown clones pretreated with various STI571 doses (μM, 2 h) prior to MNNG exposure (10 μM, 1 h).

progression appears to be very applicable to MMR processes. MMR-dependent G$_2$ arrest and elimination of severely damaged cells by apoptotic responses clearly result in mutational avoidance in human MMR$^+$ cells that is several orders of magnitude lower than that in matched MMR$^-$ cells (28). To understand the signal transduction processes involved in these mutational avoidance processes, we functionally reconstituted MMR in hMLH1-deficient RKO cells. Stable hMLH1 re-expression in synchronized RKO cells restored MMR-dependent G$_2$ arrest and sensitivity to MNNG. For the first time, using both genetic and pharmacological approaches, we elucidated a role for c-Abl kinase in G$_2$ arrest responses in MMR$^+$ cells after

MNNG treatment. Disrupting c-Abl kinase (*a*) abrogated MMR-dependent G$_2$ arrest, (*b*) decreased expression of MMR-dependent GADD45α protein and p73α stabilization, and (*c*) abrogated MMR-dependent lethality (*i.e.* damage tolerance) in response to MNNG. Our data provide the first evidence that MMR-dependent G$_2$ arrest responses are dependent on c-Abl activation. Our results suggest that futile cycling may not be a means to MMR-dependent cell death because DNA breaks created by processing DNA lesions by MMR would be upstream from c-Abl/p73α activation. Abrogating c-Abl activity or p73α levels should not have affected MMR-mediated DNA single-strand breaks or DSBs and therefore lethality, as predicted (5, 6). Abrogating c-Abl, GADD45α, and, even more so, p73α leads to damage tolerance and increased survival, presumably without affecting MMR activity. These data suggest that if DNA single-strand breaks or DSBs are being created by MMR processing of damage, they do not mediate G$_2$ arrest and/or lethality. Thus, MMR appears to detect specific, potentially mutagenic MNNG-induced DNA lesions and selectively signals to c-Abl kinase to regulate G$_2$ arrest, apoptosis (41), and lethality. c-Abl activation causes GADD45α up-regulation, which we propose is a major determinant in MMR-dependent G$_2$ arrest, suggesting that MMR directly signals this checkpoint response, leading to cell death (Fig. 8) (41). Notably, inhibition of c-Abl by STI571 exposure or stable c-Abl shRNA knockdown resulted in increased MNNG resistance, raising the question of whether DSBs are formed because MMR processes are not affected. Preliminary assays using comet assays suggest that DSBs are formed via MMR-dependent processes, but are not affected by STI571 or c-Abl knockdown.

Although a link between MMR and c-Abl activation has been made for MMR-dependent apoptosis mediated by p73α after cisplatin exposure (13), the link to G$_2$ arrest has not. c-Abl kinase can phosphorylate Tyr^{99} of p73α protein, stabilizing it in a manner similar to p53. Cisplatin also activates ATM, which, in turn, stimulates c-Abl, leading to p73α accumulation (13). p73α accumulation then directly stimulates apoptosis.

The mechanism by which MMR-dependent c-Abl mediates increases in GADD45α expression and how GADD45α controls both G$_2$ arrest and apoptosis remain unknown. MMR-dependent GADD45α protein expression was originally shown after FdUrd or 5-fluorouracil exposure (5). These responses occurred in the first cell division after drug exposure, wherein MMR directly detected fluoro-Ura:Gua mispairing (5). Overexpression of GADD45α can mediate G$_2$ arrest by direct binding and inhibiting Cdc2 (10). Loss of GADD45α leads to defective G$_2$ checkpoint signaling after DNA damage (10). Here, we established a link between GADD45α and MMR/c-Abl signaling. GADD45α can directly signal to p38α kinase (MAPK) after DNA damage and oncogenic transformation by Ras (29). We believe that this puts p38α stress kinase downstream from MMR/c-Abl/GADD45α signaling. p38α activity was previously linked to MMR-mediated G$_2$ arrest in response to temozolomide (an MNNG analog) (12). Thus, p38α may well be part of the c-Abl-mediated G$_2$ arrest pathway. Indeed, blocking G$_2$ arrest by p38α inhibition has no effect on Chk1 phosphorylation after temozolomide treatment (12), similar to our results after MNNG exposure (Fig. 2C). Indeed, both p38α and Chk1

signal transduction pathways contribute separately, but equally, to MMR-dependent temozolomide-induced G_2 arrest (30). Thus, we theorize that p38α kinase is downstream not only from c-Abl, but also from GADD45α. More direct evidence for involvement of GADD45α in MMR-dependent lethality is shown in the accompanying article (41).

Early onset BRCA1 is a potential candidate protein that could bridge the gap between MMR and c-Abl signaling to control GADD45α induction. BRCA1 has been proposed to play a dual role in G_2 arrest and homologous DSB repair. BRCA1 can be phosphorylated and activated after genotoxic stress, allowing it to act as a transcription factor for several downstream genes, including *GADD45α*. Thus, MMR-dependent GADD45α induction (Fig. 6*A*) may be a result of BRCA1 activation, linked to c-Abl activity in response to MMR signaling. Evidence of a role for BRCA1 in MMR-dependent signaling, independent of ATR or Chk1 for lethality, was reported recently (31). BRCA1 can be activated by ATM kinase after a DSB, disrupting its interaction from a stable inactive complex with c-Abl (32). This allows both proteins to separate and become active. A direct interaction between MMR and BRCA1 was noted (33), and BRCA1 can physically interact with hMSH2 and two of its binding partners, hMSH3 and hMSH6 (34). BRCA1 and hMSH2 are part of a proposed multiprotein complex involved in DNA damage recognition and repair, known as BASC (<u>B</u>RCA1-<u>a</u>ssociated genome <u>s</u>urveillance <u>c</u>omplex) (35). Recently, Yamane *et al.* (31) noted a minor role for BRCA1 in MMR-dependent G_2 arrest signaling, similar to our own findings (supplemental Fig. 4); however, caution should still be taken in attributing a role for BRCA1 in MMR-specific processes without more detailed investigations.

Recently, Kim *et al.* (25) found that c-Abl may physically interact with hMLH1; however, they failed to evaluate how this interaction might affect cell cycle and apoptotic responses. They also determined that MMR activates the JNK/MAPK signaling cascade after MNNG exposure; however, these results are not surprising because MMR-dependent activation of the c-Abl/JNK pathway has previously been observed after cisplatin exposure (36). A direct interaction between c-Abl and hMSH5 has also been shown (37). In fact, a single point mutation (Y823H) in hMSH5, acting similarly to loss of hMSH2, results in damage tolerance to MNNG or temozolomide (38). Because the hMSH4-hMSH5 heterodimer is involved in Holliday junction recognition and resolution (39), hMSH4-hMSH5 may stabilize and preserve a meiotic bimolecular DSB repair intermediate. How MMR-specific c-Abl kinase activation participates in these processes will require further research.

The dramatic pro-survival effects achieved by inhibiting c-Abl kinase activity using STI571 or c-Abl kinase silencing on MNNG-dependent cytotoxicity have major clinical implications. Others have demonstrated that c-Abl inhibition by Gleevec™ can protect cells from DNA-damaging agents, including H_2O_2, Ara-C, and curcumin (40). Collectively, these results suggest that caution should be taken in clinical protocols that combine STI571 (or other c-Abl/Src inhibitors) with chemotherapeutic agents that are modulated by MMR (*e.g.* cisplatin, 5-fluorouracil, and temozolomide).

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