Reduced ATR or Chk1 Expression Leads to Chromosome Instability and Chemosensitization of Mismatch Repair–deficient Colorectal Cancer Cells

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Genomic instability in colorectal cancer is categorized into two distinct classes: chromosome instability (CIN) and microsatellite instability (MSI). MSI is the result of mutations in the mismatch repair (MMR) machinery, whereas CIN is often thought to be associated with a disruption in the APC gene. Clinical data has recently shown the presence of heterozygous mutations in ATR and Chk1 in human cancers that exhibit MSI, suggesting that those mutations may contribute to tumorigenesis. To determine whether reduced activity in the DNA damage checkpoint pathway would cooperate with MMR deficiency to induce CIN, we used siRNA strategies to partially decrease the expression of ATR or Chk1 in MMR-deficient colorectal cancer cells. The resultant cancer cells display a typical CIN phenotype, as characterized by an increase in the number of chromosomal abnormalities. Importantly, restoration of MMR proficiency completely inhibited induction of the CIN phenotype, indicating that the combination of partial checkpoint blockage and MMR deficiency is necessary to trigger CIN. Moreover, disruption of ATR and Chk1 in MMR-deficient cells enhanced the sensitivity to treatment with the commonly used colorectal chemotherapeutic compound, 5-fluorouracil. These results provide a basis for the development of a combination therapy for those cancer patients.

INTRODUCTION

Eukaryotic cells are continuously exposed to endogenous and exogenous insults capable of damaging DNA. To maintain the integrity of their genomes, cells have evolved a series of sophisticated and complex signaling networks that allow cells to respond to genotoxic injury. Such responses include recognition of DNA lesions, activation of cell cycle checkpoints and DNA repair mechanisms, and, in the event of irreparable damage, initiation of apoptosis. Defects in many of these surveillance mechanisms result in the inability of cells to properly process genomic stresses, often leading to genomic instability and subsequently tumorigenesis. Genomic instability can be divided into two clinically distinct classes that have been extensively studied in colorectal cancers: chromosome instability (CIN) and microsatellite instability (MSI) (Kinzler and Vogelstein, 1996; Harfe and Jinks-Robertson, 2000; Rajagopalan *et al.*, 2003; Rustgi, 2007). Tumors with CIN comprise ~85% of all colorectal cancers and are characterized by gross karyotypic changes exhibiting abnormalities in chromosome structure and number (Rajagopalan *et al.*, 2003). The pathway leading to the development of CIN is not currently clear but it has been correlated with truncations in the adenomatous polyposis coli (APC) protein (Kinzler and Vogelstein, 1996; Rajagopalan *et al.*, 2003). Tumors with MSI account for the remaining 15%

of colorectal cancers. In contrast to CIN tumors, MSI tumors have a relatively stable karyotype and harbor anomalies at the nucleotide level, resulting in frameshift and missense mutations that disrupt the normal function of proto-oncogenes and tumor suppressors. This type of instability has been directly linked to silencing of mismatch repair (MMR) genes (Kinzler and Vogelstein, 1996; Raschle *et al.*, 2002; Rajagopalan *et al.*, 2003; Jiricny, 2006). It is generally accepted that both CIN and MSI are early events that drive cancer development by allowing cells to rapidly accumulate genetic changes required for the multistep tumorigenic process (Nowak *et al.*, 2002). Although APC truncations were always thought to be the driving force behind CIN initiation, new evidence indicates that ~55% of aneuploid colorectal adenomas are found to be APC wild type and only 47% of adenomas with APC mutation are aneuploid (Giaretti *et al.*, 2004). These data point to alternative mechanisms for CIN development in colorectal tumors, independent of APC mutations. In this regard, although MMR silencing alone appears to lead to a MSI phenotype (Prolla *et al.*, 1998; Rustgi, 2007), it is possible that the combination of different mutations in a given cell can contribute to the initiation of a CIN phenotype. Indeed, it has been recently shown that a small subset of colorectal tumors displays a phenotype with both microsatellite and chromosome instability (Matsuzaki *et al.*, 2005), suggesting that in certain genetic backgrounds, MMR disruptions can be affiliated with CIN development.

MMR machinery plays an integral role in maintaining genetic stability by repairing mismatched bases and insertion-deletion loops that arise during DNA replication. Activation of the MMR response inhibits recombination between nonidentical DNA sequences and provokes both checkpoint

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and apoptotic responses after certain types of DNA damage (Jiricny, 2006; O'Brien and Brown, 2006). In humans, the core MMR machinery consists of two heterodimeric protein complexes: hMutS and hMutL. The repair reaction requires mismatch recognition mediated by the hMutS complex, consisting of hMSH2 paired with hMSH3 or hMSH6. On mismatch recognition by hMutS, the hMutL complex of hMLH1 paired with hPMS2 or hPMS1 is recruited to the lesion where its enzymatic ATPase (Raschle *et al.*, 2002) and endonuclease (Jiricny, 2006; Kadyrov *et al.*, 2006) activities are required for completion of the repair process. Loss-of-function MMR defects in humans are associated with hereditary nonpolyposis colon carcinoma (HNPCC) and also with sporadic tumors. These tumors are characterized as harboring a high degree of MSI (MSI-H) and, notably, tolerance to the cytotoxic effects of commonly used chemotherapeutic agents that act by inducing DNA damage, such as 5-fluorouracil (5-FU; Meyers *et al.*, 2001; Arnold *et al.*, 2003).

Checkpoint pathways exist to halt the cell cycle in response to DNA strand breaks and DNA replication errors, allowing for the proper repair of DNA when exposed to such insults. Two members of the phosphoinositide 3-kinase-related kinase family, ataxia-telangiectasia (ATM)-mutated and ataxia-telangiectasia rad3-related (ATR), have been found to be rapidly recruited to sites of DNA damage where they convey signals to the damage response network. ATM and ATR have been found to directly regulate two major effector kinases, Chk2 and Chk1, respectively, in response to different forms of DNA damage. ATM primarily responds to DNA double-stranded breaks (DSBs). In contrast, ATR acts to monitor replication fork progression and is responsible for maintaining replication fidelity. Checkpoint activation is triggered when replication forks are stalled by abnormally structured or damaged DNA or by exposure to agents such as hydroxyurea (HU) and aphidicolin (APH). As the main substrate for ATR, Chk1 acts as a central regulator of cell cycle checkpoint delays in S- and G2-phases of the cell cycle (Abraham, 2001; Shiloh, 2003). In the complete absence of either ATR or Chk1, mammalian cells rapidly lose viability, most likely because of a profound breakdown in DNA replication fidelity, ultimately resulting in chromosome fragmentation and mitotic catastrophe (Brown and Baltimore, 2000; de Klein *et al.*, 2000; Liu *et al.*, 2000; Takai *et al.*, 2000).

Several lines of evidence suggest that MMR machinery can communicate with ATM- and ATR-dependent checkpoint signaling pathways to elicit a cellular response; however, the nature of this molecular mechanism has not yet been fully elucidated. In response to ionizing radiation during S-phase, an acute suppression of replicative DNA synthesis is triggered (Painter and Young, 1980). This requires hMutS and hMutL complexes and may involve direct interactions between hMLH1 and ATM, as one signaling complex, and hMSH2 and Chk2 as another (Brown *et al.*, 2003). In response to certain DNA damaging adducts, such as O⁶-methylguanine, hMSH2 has been found to engage in a direct signaling complex with ATR, indicating that MMR can act as damage sensors for ATR-dependent checkpoint signaling (Wang and Qin, 2003; Yamane *et al.*, 2004; Adamson *et al.*, 2005; Yoshioka *et al.*, 2006). Additionally, it has been shown that hMLH1 is necessary for p53-mediated induction of apoptosis in response to alkylating and oxygen radical-inducing agents (Luo *et al.*, 2004). Moreover, proteomic studies have revealed that hMLH1 interacts with many classes of proteins, including DNA metabolism, helicase, and cell cycle proteins (Cannavo *et al.*, 2007). Taken together, these new lines of evidence suggest that the MMR pathway may be

involved in regulating other cell processes closely linked with DNA repair, such as cell cycle progression, apoptosis, and DNA replication.

We have previously reported that the essential checkpoint signaling kinase, ATR, acts as a haploinsufficient tumor suppressor in combination with MMR-deficiency. Our studies showed that disrupting a single allele of ATR in an MLH1-deficient background increased fragile site expression and resulted in enhanced sensitivity to hydroxyurea. Additionally, ATR+/-MLH1-/- mice were more prone to early tumor development when compared with their ATR+/- or MLH1-/- littermate counterparts (Fang *et al.*, 2004). These data raise the possibility that MMR and checkpoint signaling pathways may function together to regulate genomic stability. However, it has been difficult to fully establish the presence of cross-talk between these two pathways as the basis for a novel mechanism in CIN during tumorigenesis. This challenge results from the inherent difficulty in creating cell-based model systems, whereby a single allele of checkpoint protein genes becomes silenced, and is further complicated by the cell-lethality phenotype caused by homozygous null mutations of genes as ATR and Chk1.

In this study, we use a new experimental strategy to demonstrate that reduction in ATR or Chk1 expression via partial knockdown in a MMR-deficient background results in chromosome instability. The findings support the broad presence of a mechanism by which chromosome instability is developed upon combined MMR-deficiency and checkpoint haploinsufficiency, leading to progression of tumorigenesis by permitting cells to accumulate genetic lesions without causing cell lethality. To determine the clinical significance of these findings, we show that the combination of ATR or Chk1 reduction and MMR-deficiency correlates with a sensitization of colon cancer cells to the commonly used chemotherapeutic agent 5-FU, suggesting that combination therapy should be explored for patients bearing MMR-deficient colorectal cancers.

MATERIALS AND METHODS

Cell Culture

HCT116 and RKO cells were obtained from American Type Culture Center (ATCC, Manassas, VA). HCT116.chr3 cells were kindly provided by Thomas Kunkle (National Institute Environmental Health Sciences, Research Triangle Park, NC). HCT116 and HCT116.chr3 cells were cultured in McCoy's 5A Medium (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum and 5% Pen/Strep. RKO cells were cultured in MEM supplemented with 10% FBS and 5% Pen/Strep.

Small Interfering RNA Strategies

To stably knockdown Chk1 levels in Hct116 cells, gene-specific inserts were cloned into the pSuper.gfp.neo according to manufacturer's protocol (Oligo-Engine, Seattle, WA). Vector control and knockdown constructs were transfected using the Fugene6 reagent (Roche Applied Science, Indianapolis, IN). After 48 h, cells were transferred and grown in selection medium containing G418. To obtain single cell clones, cells were plated at a density of 500 cells per plate. Once colonies developed, they were isolated and grown in a 24-well plate. Clonal populations were then tested for Chk1 knockdown using immunoblot analysis. To stably knockdown Chk1 levels in HCT116.chr3 and RKO cells, gene-specific inserts were cloned into the pSuper.retro plasmid according to manufacturer's instructions (OligoEngine). Control and small interfering RNA (siRNA)-carrying plasmids were transfected into 293T cells using Fugene6 reagent. At 48 h after transfection, virus-containing media was harvested for infection. The targeting sequence for stable knockdown of Chk1 was 5'-caggagagaaggcaatc-3' and for ATR was 5'-cagcgcatctctatgcctcttgac-3'.

Immunoblot Analysis

Antibodies used for immunoblotting were as follows: α -ATR (Santa Cruz Biotechnology, Santa Cruz, CA), α -Chk1 (Santa Cruz), α -tubulin (Sigma, St. Louis, MO), α -MLH1 (BD Bioscience, San Jose, CA), α -p-ATMS1981 (Cell Signaling Technology, Beverly, MA) and α -p-H2AX (Cell Signaling Technology). Cells were grown to 80% confluency and were harvested by trypsiniza-

tion. Cell pellets were then lysed in lysis buffer (50 mM HEPES, pH 7.4, 30 mM sodium chloride, 0.5% NP-40, 1 mM magnesium chloride, 1.5 mM EGTA, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 ng/ml microcystin, 5 μ g/ml aprotinin, and 5 μ g/ml leupeptin). Total cellular extracts were prepared, resolved by SDS-PAGE, and transferred to PVDF membranes. Protein blots were probed with the indicated antibodies, and detection was performed with the Supersignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL). Relative band size was measured, and percentages compared with control were obtained using ImageJ (<http://rsb.info.nih.gov/ij/>).

Chromosome Preparation

Cells were treated for 30 min with colcemid (50 ng/ml). Metaphase chromosome spreads were prepared according to standard cytogenetic procedures. Cells were dropped onto slides and baked for 45 min at 95°C before G banding. Cells were imaged and karyotypic analyses were carried out using Cytovision (ver. 2.7) computer-assisted karyotyping system (Applied Imaging, Santa Clara, CA). Abnormalities were described according to the International System for Human Cytogenetic Nomenclature (Mitelman, 1995).

Fragile Site Analysis

Cells were treated with 0.1 μ M APH for 18 h, and chromosome spreads were prepared and imaged as described above. The numbers of breaks and gaps per metaphase were counted for 20 metaphase cells in each case. A 100 $\times$ oil magnification lens was used to visualize metaphase spreads.

Immunofluorescence Analysis

Monolayer cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.5% Triton X-100. After three phosphate-buffered saline (PBS) washes, cells were stained with DAPI. For centrosome analysis, cells were first probed with a γ -tubulin antibody (Sigma), followed by DAPI. Samples were visualized using a Zeiss Axio Imager (Thornwood, NY); 40 $\times$ and 63 $\times$ objective lenses used.

Clonogenic Survival Assay

Log-phase cells were plated overnight and treated with indicated concentrations of 5-FU (Duke Pharmacy) and oxaliplatin (Duke Pharmacy) for 48 h. After 48 h, cells were harvested, and 500 cells/well were plated in triplicate into 60-mm dishes. Cells were then cultured for 12 d before fixation and staining with crystal violet. The number of colonies per plate was assessed and then expressed as percent survival relative to untreated cells.

MTS Assay

Cells were treated with varying concentrations of 5-FU for 72 h. After 72 h, cells were plated in 96-well plates at a density of 2500 cells/well. Cells were allowed to recover for 96 h before adding MTS assay reagent (Promega) for 90 min. Absorbance was recorded at 490 nm with a 96-well plate reader.

G2/M Assay

Cells were treated with 0.1 μ M APH for indicated times. Cells were then harvested by trypsinization and fixed in 70% ethanol. Cells were subsequently spun and washed several times in PBS and resuspended in a 0.25% Triton/PBS mixture. Cells were then incubated with phospho-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY) for 3 h at room temperature. DNA was stained with propidium iodide (PI). Mitotic cells were then analyzed using FACS.

RESULTS

ATR or Chk1 Partial Knockdown in a MMR-deficient Background Leads to the Formation of Chromosomal Breaks and Gaps

We have previously reported that the essential checkpoint signaling kinase, ATR, acts as a haploinsufficient tumor suppressor in a MMR-deficient background. MLH1-deficient HCT116 cells in which a single allele of ATR had been inactivated by somatic recombination were found to exhibit signs of chromosome instability (Fang *et al.*, 2004). To further investigate the relationship between ATR haploinsufficiency and MMR machinery in a cell-based system, we took a new approach to partially inhibit the function of this protein by using RNA interference (RNAi) to lower ATR protein levels by ~50%, consequently creating a genetic background that mimics ATR haploinsufficiency. In addition, we selected the RKO colorectal cancer cell line for this experiment to ensure that the original observations with the HCT116 colon cancer

Figure 1. ATR or Chk1 knockdown in MMR-deficient cells leads to an increase in chromosome breaks and gaps. (A) Left, expression of ATR protein in RKO colorectal cancer cells. Right, average number of chromosome breaks and gaps per metaphase spread. Results are representative of 20–25 total spreads scored for each vector control and ATR knockdown RKO cell lines. Spreads were prepared as described in *Materials and Methods*. (B) Left, expression of Chk1 protein in HCT116-derived clones compared with vector control. (B) Right, average number of chromosome breaks and gaps per metaphase spread. Results are representative of 20 total spreads scored for each vector control and Chk1 knockdown HCT116 cells. (C) Left, expression of Chk1 protein levels in RKO-derived cell line. (C) Right, average number of chromosome breaks and gaps per metaphase spread. Results are representative of data derived from counting 20 metaphase spreads. * $p < 0.05$.

cells were not cell line-specific. The RKO cells carry an MLH1 silencing mutation and display a high degree of MSI without gross chromosomal abnormalities. As shown in Figure 1A, left, ATR protein expression is reduced by 50% with the stable transfection of sequences encoding small hairpin RNAs (shRNAs) directed against ATR.

One commonly used indicator of CIN phenotype is an increase in fragile site instability. Fragile sites are defined as being genomic “hot spots” for chromosome breakage and rearrangements (Arlt *et al.*, 2006). Treatment with very low levels of APH triggers replicative stress, upon which, cells experiencing chromosome instability are prone to the generation of breaks and/or gaps in mitotic chromosomes, whereas chromosome-stable cells are expected to fully recover from the stress (Casper *et al.*, 2002). Consistent with our previous results from HCT116 cells, the RKO-ATR3 partial knockdown cells exhibited a substantial increase in the number of chromosome breaks and gaps when compared with vector control RKO cells after 18 h of 0.1 μ M APH treatment (Figure 1A, right). This result suggests that the CIN phenotype induced by a reduction in ATR level upon the MMR-deficient background is not limited to a specific cell line and also demonstrates the validity of utilizing a RNAi-mediated knockdown strategy as an effective alternative to single-allelic somatic targeting.

To determine whether a combination of reduced activity of the checkpoint signaling pathway and MMR deficiency represents a novel mechanism leading to chromosome instability, we also studied the Chk1 checkpoint kinase, a direct downstream effector of ATR. To do so, we used the same RNAi-based system to partially knockdown Chk1 expression in both HCT116 and RKO cell lines, respectively. For HCT116 cells, two clones HCT116-Chk1B.B6 and HCT116-Chk1B.B7 were selected for further analysis based on their levels of Chk1 expression that are ~50% of that found in HCT116-vec control cells (Figure 1B, left). Similarly, we reduced Chk1 expression levels by ~50% in the RKO cells to generate the RKO-Chk1B stable cell line (Figure 1C, left). We next determined whether Chk1 knockdown cells were more sensitive to APH-induced replicative stress. As shown in Figure 1, B and C (right), both types of cells exhibited a marked increase in chromosome gaps and breaks, suggesting that concurrent Chk1 protein reduction and MMR deficiency triggers the development of chromosome instability.

ATR or Chk1 Partial Knockdown Leads to Abnormal Centrosome Number

It is well known that successful centrosome duplication is important in regulating proper chromosome segregation. After completing a successful round of mitosis, each daughter cell is left with one centrosome, a microtubule-organizing center (MTOC) that will become essential for proper chromosomal segregation. In early S-phase, centrosomes are duplicated and by M-phase will be on opposite ends of the cell, thus establishing cell polarity for spindle formation. Chromosomal-instable cancers have been found to exhibit multiple centrosomes, a phenotype that is thought to both contribute to CIN via mis-segregation of chromosomes and to be a consequence of CIN (Fukasawa, 2005). To determine whether ATR and Chk1 knockdown cells were more likely to exhibit supernumerary centrosomes, cells were stained with anti- γ -tubulin antibody and DAPI to visualize centrosomes and chromatin, respectively. γ -Tubulin is known to be concentrated at the centrosome, making it easy to score the number of centrosomes per cell (Fukasawa, 2005). RKO-ATR3 cells exhibited a significant increase in the number of cells containing more than two centrosomes when compared with RKO-vec control cells (Figure 2A). In addition, HCT116-Chk1B.B6, HCT116-Chk1B.B7, and RKO-Chk1B knockdown cells exhibited a higher frequency of multiple centrosomes when compared with control cells (Figure 2, B and C). The presence of multiple centrosomes in these Chk1 knockdown cells further suggests that these cells exhibit signs of aneuploidy. As such, it is possible that the supernumerary centrosomes observed in these cells are contributing to the process of CIN or are the result of existing chromosome instability.

ATR or Chk1 Partial Knockdown Induces Chromosome Bridge and Micronuclei Formation

Chromosomal instable cells are known to exhibit abnormal nuclear structures, such as chromatin bridges and micronuclei (Iarmarcovai *et al.*, 2007). To further characterize the CIN phenotype of the ATR and Chk1 knockdown cells, we analyzed the formation of chromosome bridges and the presence of micronuclei. Cells were stained with DAPI to visualize chromatin using fluorescence microscopy and subsequently scored for the presence of these types of chromosomal abnormalities. When compared with cells carrying an empty vector construct, we observed a significant increase in the formation of chromatin bridges and micronu-

Figure 2. Centrosome number in ATR and Chk1 knockdown RKO and HCT116 cells. Average percentage of cells containing more than two centrosomes in ATR knockdown cells. Representative of three independent experiments for each cell line is shown. (A) Centrosome number in RKO-vec control and RKO-ATR3 knockdown cells. Left, representative images from control and Chk1 knockdown RKO cells. Centrosomes are highlighted in green (γ -tubulin) and nuclei are highlighted in blue (DAPI). Arrows point to centrosomes. Right, quantification of the percentage of cells containing more than two centrosomes. (B) Centrosome number in HCT116-vec control and knockdown HCT116-Chk1B.B6 and HCT116-Chk1B.B7 clones. Left, representative images from control and Chk1 knockdown clones. Centrosomes are highlighted in green (γ -tubulin) and nuclei are highlighted in blue (DAPI). Arrows point to centrosomes. Right, the percentage of cells containing more than two centrosomes was quantified and represented graphically. (C) Centrosome number in RKO-vec control and RKO-Chk1B knockdown cells. Left, representative images from vector control and Chk1 knockdown RKO cells. γ -Tubulin is seen in green and depicts centrosomes; DAPI stain for nucleus. Right, quantification of the percentage of cells containing more than two centrosomes from RKO-vec control and RKO-Chk1B knockdown cells. * $p < 0.05$.

clei in RKO-ATR3 (Figure 3A). As shown in Figure 3, B–D, both stable Chk1 knockdown cell lines, RKO-Chk1B and HCT116-Chk1B clones also exhibited a substantial increase in chromosome bridges and micronuclei. These results further support the notion that reduction in checkpoint-signaling activity in combination with a MMR-deficient genetic background leads to the development of chromosomal instability.

Chk1 Partial Knockdown Leads to Spontaneous Chromosome Instability Due to the Formation of DSBs

Because Chk1 knockdown cells with MMR deficiency proved to be more sensitive to low levels of genotoxic stress, we speculated that these cells could also have a higher basal level of chromosome instability. To address this question, we examined metaphase spreads from the vector control HCT116-vec cell line and the two Chk1 knockdown HCT116-Chk1B.B6 and HCT116-Chk1B.B7 cell clones. Karyotypic analysis revealed that the control HCT116-vec cells exhibited a relatively stable chromosomal content and signature chromosome alterations (Supplemental Figure S1A), consistent with

Figure 3. ATR and Chk1 knockdown of MMR-deficient cells form nuclear bridges and micronuclei. (A) Percent field of views containing chromosome bridges and micronuclei in RKO-vec and RKO-ATR3 knockdown cells. Cells were stained with DAPI as described in *Materials and Methods*. (B) Representative image of HCT116-vec control, HCT116-Chk1B.B6, and HCT116-Chk1B.B7 knockdown HCT116 clones. Nuclei are highlighted with DAPI. Arrows point to bridge and/or micronuclei. (C) Quantification of percentage of fields of view containing nuclear bridges and/or micronuclei in HCT116-vec control, HCT116-Chk1B.B6, and HCT116-Chk1B.B7 knockdown HCT116 cells. (D) Percentage of field of views containing micronuclei and/or nuclear bridges in RKO-vec control and RKO-Chk1B knockdown cells. * $p < 0.05$.

the notion that gross chromosomal alterations are quite rare in cells with MSI (Masramon *et al.*, 2000). In contrast, HCT116-Chk1B.B6 and HCT116-Chk1B.B7 knockdown clones contained substantially increased levels of chromosomal abnormalities including translocations, deletions, rearrangements, and chromosome losses and gains (Supplemental Figure S1, B and C). Because of the dramatic karyotypic difference observed between HCT116-vec control and HCT116 Chk1 knockdown clones, the total number of chromosomes was determined for each spread, in order to assess the extent of aneuploidy. Indeed, HCT116-Chk1B.B6 and HCT116-Chk1B.B7 knockdown clones exhibited a wide range of chromosome number per mitotic spread when compared with the control HCT116-vec cells (Figure 4A). These results support the postulation that partial loss of checkpoint signaling in an MMR-deficient background is sufficient to

cause a dramatic increase in chromosomal instability, even in the absence of exogenous genotoxic agents. Although it has been shown that CIN cancer cells can both gain and lose chromosomes, our results show a trend toward an overall loss of chromosomes. The number of total chromosomes in CIN cancer cells can range from 60 to 90, suggesting that cells may lose chromosomes before a doubling event occurs (Kinzler and Vogelstein, 1996; Lengauer *et al.*, 1997; Nowak *et al.*, 2002; Rajagopalan *et al.*, 2003). We speculate that the chromosome loss observed in our data are a relatively early step and that chromosome number may eventually increase at a later time during the tumorigenic process. It is also possible that those cells with very low chromosome number represent cells where chromosomes have been mis-segregated and may initiate apoptosis before the next round of cell division. If this is the case, there may likely exist cells that received too many chromosomes during the mis-segregation event that were not captured in our experiments. Either way, HCT116-vec cells did not exhibit clear signs of dramatic chromosome loss, suggesting that this observation is particular to the HCT116-Chk1B.B6 and HCT116-Chk1B.B7 cell clones.

To begin to address a possible mechanism by which CIN is initiated, we hypothesized that CIN observed in HCT116.Chk1 knockdown clones could possibly be due to the formation of DNA DSBs. Western blot analysis revealed an increase in the levels of phospho-ATMS1981 and phospho-H2AX, two markers of DSBs, in response to low levels of APH (Figure 4B). Interestingly, there appeared to also be an increase in the basal levels of phospho-ATMS1981 and phospho-H2AX, suggesting that HCT116.Chk1-B6 and HCT116.Chk1-B7 cell clones are not only more sensitive to genotoxic agents but are also likely to be undergoing spontaneous chromosomal alterations, a result that is consistent with our karyotypic data. In addition, HCT116-Chk1 partial knockdown cell clones seemed to fail to initiate G2/M checkpoint activation in response to low doses of APH (Figure 4D), further supporting our hypothesis that these cells are able to accumulate genetic lesions.

Chk1 Partial Knockdown in a MMR-proficient Background Does Not Induce Chromosomal Instability

Total abrogation of ATR or Chk1 alone induces rapid cell death due to gross chromosomal abnormalities and subsequent mitotic catastrophe (Brown and Baltimore, 2000; de Klein *et al.*, 2000; Liu *et al.*, 2000; Takai *et al.*, 2000; Durkin *et al.*, 2006). Thus, it is possible that the observations described above were solely the result of a change in gene dosage of ATR or Chk1 without the contribution from the MMR-deficient background. However, it is impossible to reverse the CIN phenotype in those cells by restoring hMLH1 activity in the ATR and Chk1 knockdown cells once the underlying process for the development of CIN is initiated. To address this question, we stably knocked down Chk1 protein levels by ~50% in HCT116.chr3 cells. These cells contain a small piece of chromosome 3 carrying the MLH1 gene (Figure 5A), rendering HCT116.chr3 cells MMR proficient (Koi *et al.*, 1994). As shown in Figure 5B, HCT116.chr3-Chk1B knockdown cells did not exhibit any significant increase in chromosome gaps and breaks compared with the HCT116.chr3-vec control cells when treated with low levels of APH. Furthermore, HCT116.chr3-Chk1B knockdown cells had similar chromosome content to that of HCT116.chr3-vec control cells (Figure 5C) and displayed no significance difference in the percentage of cells containing supernumerary centrosomes (Figure 5D). Taken together, these results illustrate that there is no evidence of chromosome instability

Figure 4. Spontaneous chromosomal instability in Chk1 partial knockdown HCT116-derived cell clones. (A) Forty spreads for each of the HCT116-vec control, HCT116-Chk1B.B6, and HCT116-Chk1B.B7 cell clones were analyzed for total chromosome content. Each dot represents one spread. (B) HCT116-Chk1B.B6 and HCT116-Chk1B.B7 cell clones harbor a higher degree of p-H2AX staining (green); nuclei are highlighted in red (DAPI). (C) Western blot analysis shows a higher expression level of p-ATM S1981 and p-H2AX, two markers for DNA DSBs, in HCT116-Chk1B.B6 and HCT116-Chk1B.B7 cell clones both basally and in the presence of APh. (D) FACS analysis for PI versus p-histone H3 shows that HCT116-Chk1B.B6 and HCT116-Chk1B.B7 cell clones do not activate G2/M checkpoint in response to low doses of APh.

induction when only Chk1 expression is partially reduced in MLH1-reconstituted cells. This finding strongly supports the notion that the development of CIN phenotype is the result of combined defects in checkpoint signaling and MMR machinery.

ATR or Chk1 Partial Knockdown in MMR-deficient Background Leads to Enhanced Chemosensitivity to 5-FU

Treatment of patients with MMR-deficient colorectal tumors has proven to be difficult using conventional chemotherapeutic regimens as those tumors are reported to be insensitive to a variety of drug classes including antimetabolites, topoisomerase inhibitors, and most platinum-based agents (Meyers *et al.*, 2001; Ragnhammar *et al.*, 2001; Claij and Te Riele, 2002; Arnold *et al.*, 2003; Ju *et al.*, 2007; Wang *et al.*, 2007).

Because we have shown that a partial reduction of ATR or Chk1 protein levels renders MMR-deficient colorectal cancer cells more sensitive to genotoxic insults, we tested if these cells were also more sensitive to the most commonly used, first-line chemotherapeutic drug for colorectal cancer, 5-FU. RKO-vec control and RKO-ATR3 partial knockdown cells were exposed to varying concentrations of 5-FU for 72 h. After a 96-h recovery period, MTS assay reagent was added to each well and survival curves were obtained. As seen in Figure 6A, RKO-ATR3 knockdown cells showed an increased sensitivity to treatment with 5-FU when compared with RKO-vec control cells. To determine if Chk1 partial knockdown HCT116-Chk1B.B6 and HCT116-Chk1B.B7 cells were more susceptible to 5-FU, control and Chk1 knockdown clones were exposed to varying concentrations of

Figure 5. MLH1-reconstituted HCT116.chr3 cells do not exhibit signs of chromosomal instability when Chk1 is partially knocked down. (A) Expression of MLH1 protein in the reconstituted HCT116.chr3 cell line (top). The HCT116.chr3 cells were then used to generate stable cell lines expressing an approximate 50% reduction in Chk1 protein levels (bottom). Whole blot image is shown in Supplementary Figure S5. (B) A total of 20 metaphase spreads were examined for each of the HCT116.chr3-vec control and HCT116.chr3-Chk1B knockdown cells. The number of chromosome breaks and gaps observed is shown as an average per five metaphase spreads. (C) Total number of chromosomes observed in 15 metaphase spreads for HCT116.chr3-vec control and HCT116.chr3-Chk1B knockdown cells. Chromosome number remained stable in both vector and Chk1 knockdown HCT116.chr3 cells. Representative images (D) and quantification of cells containing supernumerary centrosomes in HCT116.chr3-vec and HCT116.chr3-Chk1B knockdown cells. γ-Tubulin (green) was used as a marker for centrosomes; DAPI (blue) was used to highlight the nucleus; arrows point to centrosomes. The quantification was expressed as the average number of cells containing more than two centrosomes for each HCT116.chr3-vec and HCT116.chr3-Chk1B knockdown cell lines.

Figure 6. RKO ATR and HCT116 Chk1 knockdown cells are more sensitive to treatment with 5-FU. (A) MTS assay for cell survival in RKO-vec control and RKO-ATR3 knockdown cells. Representative of four independent experiments conducted in quintuplicate and as described in *Materials and Methods*. Absorbance was read at 490 nm, and cells were normalized to untreated cells. (B–D) Clonogenic survival assay. Surviving colonies were counted after drug treatment at stated concentrations and as described in *Materials and Methods*. Representative image (B) and survival curves (C and D) for HCT116-vec control, HCT116-Chk1.B6, and HCT116-Chk1.B7 knockdown clones and for HCT116.chr3 cells in response to treatment with 5-FU. Samples were normalized to untreated cells.

5-FU for 48 h. Cells were then plated sparsely to assay for clonogenic survival. After a 10-d period cell colonies were fixed and stained, and survival curves were obtained. As shown in Figure 6, B and C, HCT116-Chk1.B6 and HCT116-Chk1.B7 knockdown cells exhibited an enhanced sensitivity to 5-FU when compared with control HCT116-vec cells. Interestingly, the sensitivity of HCT116-Chk1 partial knockdown clones exposed to 5-FU was similar to that of the MMR proficient cell line, HCT116.chr3 (Figure 6D). To rule out the possibility that HCT116-Chk1 partial knockdown clones have a heightened sensitivity to all stress inducers, cell viability was measured after serum starvation. HCT116-Chk1 partial knockdown clones did not exhibit a lower level of cell viability in response to serum starvation when compared with HCT116-vec control cells (Supplemental Figure S2). These results demonstrate that the combined effect of reduction in Chk1 protein levels and MMR-deficiency renders colorectal cells highly susceptible to treatment with the most commonly used first-line chemotherapeutic agent for this disease.

To evaluate if Chk1 partial inhibition combined with 5-FU treatment could be a possible therapeutic course in the clinic, we tested whether inhibition of Chk1 using the small-molecule inhibitor UCN-01 would give similar results to those observed in genetically modified cell lines with Chk1 partial knockdown. Western blot analysis of phospho-CDC25C levels indicated that in HCT116 cells, treatment with 0.1 μ M UCN-01 caused a partial reduction in Chk1 activity (Figure 7A). Indeed, simultaneous treatment with this concentration of UCN-01 and 5-FU led to a significantly lower clonogenic survival rate when compared with cells treated with 5-FU alone (Figure 7B).

Figure 7. Combinatorial treatment of HCT116 cells with 5-FU and UCN-01 leads to reduced cellular survival. (A) Western blot analysis showing dose response for Chk1 activity levels by examining p-CDC25C levels in response to treatment with UCN-01. (B) HCT116 cells treated with both 0.1 μ M UCN-01 and indicated concentrations of 5-FU. HCT116 cells treated with UCN-01 and 5-FU show a decreased survival when compared with control HCT116 cells and survival similar to HCT116 Chk1 knockdown clones.

DISCUSSION

In this study, we have provided strong evidence for the broad presence of a mechanism through which genomic instability is greatly accelerated via the induction of CIN in MMR-deficient tumors, a process that renders these cells more sensitive to the chemotherapeutic agent, 5-FU. Given that biallelic mutations in ATR or Chk1 lead to cell lethality (Brown and Baltimore, 2000; de Klein *et al.*, 2000; Liu *et al.*, 2000; Takai *et al.*, 2000), it is unlikely that homozygous null mutations exist in human disease, even in late-stage malignant cells. Several clinical reports have recently demonstrated that checkpoint signaling proteins are indeed found to be heterozygously mutated in multiple types of tumors with mismatch repair deficiencies, including malignancies of the stomach, the endometrium, and the colon (Menoyo *et al.*, 2001; Lewis *et al.*, 2005; Bertholon *et al.*, 2006; Kim *et al.*, 2007; Lewis *et al.*, 2007). However, it remains unclear how these ATR and Chk1 mutations contribute to the tumorigenic process. Through siRNA-mediated knock-down and reconstitution experiments, our findings demonstrate that defects in both checkpoint signaling, with a partial loss of ATR or Chk1 activity, and MMR machinery are required to drive the rapid development of CIN, consequently contributing to a higher level of genomic instability and the multistep tumorigenic process.

The functional cooperation between the checkpoint and MMR pathways in the induction of CIN demonstrated here is strongly supported by existing experimental evidence at the molecular level. For example, MSH2 has been reported to interact with ATR upon treatment with MNNG, a DNA-methylating agent, suggesting that the hMutS/hMutL complexes of the MMR machinery can serve as DNA damage sensors in response to specific types of genotoxic insults (Wang and Qin, 2003). A functioning MMR system is also necessary for full activation of an ATM-dependent S-phase checkpoint, triggered by DSBs during replication (Brown *et al.*, 2003). Loss of hMLH1 or hMSH2 would render the cells completely reliant on ATR for the suppression of DNA replication when DSBs are encountered. Another major role where MMR and checkpoint proteins may play coopera-

tively is to maintain recombination fidelity, as MMR-deficient cells are known to exhibit high levels of illegitimate recombination events (Harfe and Jinks-Robertson, 2000). It is plausible that partial loss of ATR or Chk1 exacerbates the intensity and frequency of promiscuous recombination, resulting in gene amplification, chromosome translocation, and chromosome breaks, all of which are hallmarks of the CIN phenotype. This opens the possibility that the ATR-Chk1 checkpoint signaling pathway represents a barrier between MSI and CIN in MMR-deficient tumors. On losing MMR proficiency, cells develop what has been termed the "mutator" phenotype, making it more likely that genes containing microsatellite sequences, such as ATR and Chk1, become mutated. These circumstances would inevitably lead to CIN in a subset of cells that would further provide leverage toward unregulated growth and accelerated tumor development.

Understanding the cell biology behind genetic alterations in cancer cells can be of great importance in the clinic as it can significantly influence treatment options. In this regard, it has recently become evident that MMR-deficient tumors are often resistant to treatment with 5-FU, the first-line standard therapeutic for colorectal cancer (Ragnhammar *et al.*, 2001), a clinical feature that is consistent with the phenotype displayed by the same type of cancer cells in culture (Meyers *et al.*, 2001; Arnold *et al.*, 2003; Ju *et al.*, 2007; Wang *et al.*, 2007). Indeed, it has been shown that the MMR system plays a prominent role in the detection and removal of fluoropyrimidine-induced lesions (Meyers *et al.*, 2001; Meyers *et al.*, 2003, 2005). The exact mechanism between loss of MMR and chemoresistance is not completely understood, although it is known that MMR-deficient cells can tolerate a higher level of DNA damage induced by 5-FU, an observation confirmed by our studies.

In this study, we clearly demonstrate that a reduction in ATR or Chk1 protein levels in a MMR-deficient background renders these colorectal cancer cells more susceptible to treatment with 5-FU. The mechanism by which this occurs is not yet fully understood; however, the data presented here suggest that cells with MMR deficiency combined with partially reduced checkpoint activity are more prone to the formation of DNA DSBs. This finding provides the basis for further exploration of potentially novel therapies for patients bearing MMR-deficient tumors. In support of this postulation, a recent report indicated that patients with high-MSI colorectal tumors harboring ATR mutations exhibit an enhanced disease-free survival time when compared with patients with high-MSI tumors without ATR mutations (Lewis *et al.*, 2005). Therefore, our results suggest that treatment of MMR-deficient tumors with a combination of checkpoint inhibitors and other chemotherapeutic agents may represent a more effective regiment to increase disease-free survival. In support of this theory, we further show that the use of a pharmacological inhibitor of Chk1, UCN-01, in combination with 5-FU vastly reduces survival of MMR-deficient cells. Although UCN-01 has shown significant anti-neoplastic promise in both animal models and human trials, it has also displayed a high level of toxicity and adverse side effects. Because our data suggest that a partial inhibition of checkpoint activity was sufficient to sensitize MMR-deficient cancer cells to the effects of the chemotherapeutic agent 5-FU, it is possible that effective therapy may be achieved with a lower dosage of pharmacological checkpoint inhibitors in combination with other chemotherapeutics. Ultimately, our observations show that this could serve as a means to widen the therapeutic window for the treatment of

cancers harboring mismatch repair deficiencies and to increase disease-free survival.

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