DNMT1 deficiency triggers mismatch repair defects in human cells through depletion of repair protein levels in a process involving the DNA damage response

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DNA methyltransferase 1 (DNMT1) maintains methylation at CpG dinucleotides, important for transcriptional silencing at many loci. It is also implicated in stabilizing repeat sequences: DNMT1 deficiency causes microsatellite instability in mouse embryonic stem cells, but it is unclear how this occurs, how repeats lacking CpG become unstable and whether the effect is confined to stem cells. To address these questions, we transfected hTERT-immortalized normal human fibroblasts (hTERT-1604) with a short hairpin RNA construct targeting DNMT1 and isolated stable integrants with different levels of protein. DNMT1 expression levels agreed well with methylation levels at imprinted genes. Knockdown cells showed two key characteristics of mismatch repair (MMR) deficiency, namely resistance to the drug 6-thioguanine and up to 10-fold elevated mutation rates at a CA17 microsatellite reporter, but had limited viability. The likely cause of MMR defects is a matching drop in steady-state protein levels for key repair components in DNMT1 knockdown cells, affecting both the MutLα and MutSα complexes. This indirect effect on MMR proteins was also seen using a different targeting method in HT29 colon cancer cells and did not involve transcriptional silencing of the respective genes. Decreased levels of MMR components follow activation of the DNA damage response and blocking this response, and in particular poly(ADP-ribose) polymerase (PARP) overactivation, rescues cell viability in DNMT1-depleted cells. These results offer an explanation for how and why unmethylated microsatellite repeats can be destabilized in cells with decreased DNMT1 levels and uncover a novel and important role for PARP in this process.

INTRODUCTION

Faithful maintenance of DNA methylation at mammalian CpG sites is carried out by the DNA methyltransferase 1 (DNMT1) enzyme. Methylation plays a clearly established role in transcriptional silencing of a number of sequence classes, including imprinted genes (1), genes on the inactive X chromosome (2) and some types of selfish DNA repeats such as IAP elements (3). However, methylation also appears to play an important, but less well-understood role in genomic stability. A number of mouse models with targeted deletions of either Dnmt1, or the genes for the de novo methyltransferases Dnmt3a and Dnmt3b, have shown that DNA methylation is required to stabilize some forms of repeats, not all of which are likely to be transcribed (reviewed in 4). Expansion and

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Microsatellites consist of short runs of nucleotides and are widely dispersed in the genome, including in the coding regions of some genes. They are frequently the cause of DNA polymerase slippage during replication and the increase or decrease in repeat number can result in a frameshift and inactivation of the gene concerned (12). The mismatch repair (MMR) system counteracts this by detecting any mutations at microsatellite repeats and recruiting enzymes for their repair (13). It comprises the MutS complex, which detects and binds to the misincorporated base, and the MutL complex, which binds to MutS and recruits repair enzymes (14). There are two main MutS complexes in humans: MutSα consists of the MutS homologue 2 (MSH2) protein bound to MSH6 and can recognize single-base mismatches or small insertion/deletion loops (indels), whereas MutSβ contains MSH2 and MSH3 and repairs only indels (15). The main complex for MutL in somatic cells is MutLα, consisting of a heterodimer of MLH1 and PMS2 (13). Mutations in the MLH1 and MSH2 genes, and to a lesser extent in MSH6, are primary causes of Lynch Syndrome, also known as hereditary non-polyposis colon cancer (16,17). Loss of MLH1 through mutation or through epigenetic silencing by DNA hypermethylation is also found in many sporadic colon cancers, as well as some stomach, endometrial and ovary cancers (18).

A number of studies using mouse ES cell lines with different mutations in the Dnmt1 gene detected 4- to 10-fold increases in mutation rate at microsatellite repeats (8–10). The microsatellites affected included several (e.g. a CA\textsubscript{17} and an A\textsubscript{25} repeat) which contain no CpG site and are therefore not targets for methylation. Dnmt1 mutant cells could also grow on 6-thioguanine (6TG), a nucleotide analogue bound to MSH6 and can recognize single-base mismatches or small insertion/deletion loops (indels), whereas MutSβ contains MSH2 and MSH3 and repairs only indels (15). The main complex for MutL in somatic cells is MutLα, consisting of a heterodimer of MLH1 and PMS2 (13). Mutations in the MLH1 and MSH2 genes, and to a lesser extent in MSH6, are primary causes of Lynch Syndrome, also known as hereditary non-polyposis colon cancer (16,17). Loss of MLH1 through mutation or through epigenetic silencing by DNA hypermethylation is also found in many sporadic colon cancers, as well as some stomach, endometrial and ovary cancers (18).

In these latter studies, Dnmt1 transient knockdown to 34% of WT levels gave an ~ 2-fold increase in contraction rate at a CAG triplet repeat, and the effects of knockdown of MLH1 and Dnmt1 were additive (11,19). The mechanism by which loss of Dnmt1 enzyme results in MMR defects has not yet been uncovered; however, no changes in transcription levels of MMR genes were found in the previous studies and the regions around the microsatellites showed no changes in methylation.

Loss of MLH1 and Dnmt1 both also affect the DNA damage response (DDR) in the cell. The DDR is an integrated system for detecting DNA lesions, signalling their presence, recruiting repair enzymes, regulating the rate of cell-cycle progression and activating cell-death pathways (21). As well as its role in post-replicative repair of microsatellites, MMR has a second function in signalling DNA damage resulting from a number of exogenous agents, such as the base analogue 6TG in the case of MLH1 (22). MLH1 can sense the presence of 6TG in the DNA and trigger phosphorylation of the histone variant H2A.X at the damage site by the ATM and ATR protein kinases (23,24). These kinases are central mediators of the DDR and can cause activation of p53 as well as slow down or halt the cell cycle to allow repair (21). The DDR can also activate several independent pathways to cause programmed cell death in the presence of excessive damage (25,26). One pathway leads through p53 activation to the caspase cascade and classical apoptosis (27). Interestingly, among the preferred targets for caspases are DNA repair proteins such as ATM, PARP, BLM1, BRCA1 and MLH1 (28–33), thus speeding the loss of DNA integrity.

Even in the absence of exogenous damaging agents, loss of Dnmt1 in differentiated cells has been shown to cause activation of ATM/ATR, phosphorylation of H2A.X and p53, loss of cell viability and subsequent apoptosis (34,35) which can be partly rescued by either blocking p53 or caspase activity (36,37). We have recently shown that a second, and largely independent pathway to cell death, can be triggered by MLH1 involving the base excision repair (BER) protein poly(ADP-ribose) polymerase (38,39). PARP is a nuclear enzyme which responds to DNA damage by adding PAR groups to many nuclear targets, including histones (40). PARP activation causes a sharp drop in nicotinamide adenine dinucleotide (NAD+) levels, resulting in mitochondrial membrane depolarization and translocation of apoptosis inducing factor (AIF) to the nucleus (41), which is sufficient in itself to cause chromatin condensation and DNA fragmentation and also causes a late activation of caspases (39). There have been no previous reports of the activation of this second pathway in response to the loss of Dnmt1, although recent evidence suggests PARP may be involved in DNA demethylation in 1-cell embryos (42,43).

A number of possible mechanisms for cross-talk between Dnmt1 and MLH1 have been suggested based on protein interactions. Dnmt1 is present at the replication fork, it can interact with both the PCNA and 9-1-1 sliding clamp complexes (35,44) and in irradiated human cells can be recruited to the site of damage (35,45). MLH1 also has a PCNA-interacting region and is recruited to the same irradiated sites, although with slower kinetics (45,46). Another possible interaction is through the BER protein MBD4 (discussed in 47). MBD4 is crucial for the correction of G/T mismatches formed at methylated CpG sites through spontaneous deamination of the methylated cytosine (48,49). MBD4 and thymidine deglycosylase (TDG) have functional overlap and have been shown to interact with the de novo methyltransferases (50,51). MBD4 was isolated as a methyl-CpG binding protein (52) and then independently in a screen for proteins which bind to MLH1 (53). In one study, loss of MBD4 caused destabilization of MMR proteins and conferred drug resistance (54), but these effects were not seen in mice with mutations in the gene (48,49). In another study in Xenopus, it was shown to bind both MLH1 and Dnmt1 and to be crucial in the apoptotic response to the loss of Dnmt1 (45).
However, these previous studies have not uncovered the sequence of events which lead from the initial loss of DNMT1 to the observed MMR defects.

We were therefore interested to see if decreases in DNMT1 levels in normal differentiated human cells could indeed cause MMR defects and what relationship there was in these cells, if any, between DDR signalling, MMR repair and cell death. Previously generated human models for DNMT1 deficiency were not suitable for this study as they employed cancer cell lines, most often the HCT116 colon cancer cell line, which lacks both MLH1 and MSH3 and is MMR-deficient (34,55). For these reasons, we employed the hTERT-1604 fibroblast cell line, a normal diploid cell line immortalized by telomerase over-expression, in which we have carried out previous studies of MMR (38). This line has intact cell-cycle checkpoints and p53 responses, lacks MSI and is non-tumorigenic (56–58). From this parental line, we generated novel isogenic derivatives with a range of DNMT1 expression levels. This was also important, since previous studies have indicated that the effects of DNMT1 loss on DDR and methylation are dependent on protein level (34,59). Using hTERT-1604 cells, we show that depleting DNMT1 alone is sufficient to cause MMR defects and increase mutation rates at a CA17 microsatellite. MMR defects are likely due to decreases in MMR protein levels: these drop following activation of the DDR in DNMT1-depleted cell line, a normal diploid cell line immortalized by telomerase over-expression, in which we have carried out previous studies of MMR (38). This line has intact cell-cycle checkpoints and p53 responses and is non-tumorigenic (56–58). From this parental line, we generated novel isogenic derivatives with a range of DNMT1 expression levels. This was also important, since previous studies have indicated that the effects of DNMT1 loss on DDR and methylation are dependent on protein level (34,59).

RESULTS

Generation of isogenic normal fibroblast cell lines stably depleted in DNMT1

Human hTERT-1604 normal fibroblast cells (WT) were transfected with a pSilencer vector (Invitrogen) producing a short hairpin RNA (shRNA) known to target DNMT1 (60) and individual colonies containing the vector were isolated following growth in hygromycin. Quantitative PCR (qPCR) was carried out on total RNA isolated from individual cell lines to confirm depletion occurred at the RNA level. Clones varied in the extent of DNMT1 depletion, most likely due to insertion site effects, as previously seen (38). One clone with low (clone d1, 12.0% of WT), two with intermediate (d3, 31.4%; d15, 26.0%) and one with high (d10, 89.0%) levels of DNMT1 mRNA were isolated (Fig. 1A). A second, independent round of isolation and screening recovered a second clone (d24, 12.4%) with low levels. Due to the limited replicative potential of the most severely depleted lines (see in what follows), not all subsequent analyses were carried out on every cell line.

In order to determine if methylation levels in the cell lines showed matching changes, we carried out COBRA (combined bisulfite and restriction analysis) of the imprint control regions (ICR) for H19 and SNRPN. In normal somatic cells, these ICR show 50% methylation. Following sodium bisulfite treatment and PCR, products were digested with either HinfI (H19) or CfoI (SNRPN), whose recognition sites contain CpGs. The parental hTERT-1604 cells (WT) showed 50% methylation as expected: half the alleles had a methylated cytosine at the recognition site and could be digested into smaller fragments (Fig. 1B, m for methylated) while about half had an unmethylated cytosine which became a T following bisulfite treatment and PCR, leading to loss of the recognition site and failure to cleave (u for unmethylated). In the d1 cells, however, little or no cleavage was seen, indicating that both the H19 and SNRPN genes have lost most of their methylation (Fig. 1B). Methylation levels in the other lines examined also correlated well on the whole with DNMT1 mRNA levels. While COBRA avoids sampling and clonal bias, it can only assay a small number of CpGs, so bisulfite sequencing was also carried out on the same samples: this confirmed that levels of methylation across the ICR corresponded well with DNMT1 levels (Fig. 1C).

Cells with DNMT1 depletion show the hallmarks of MMR deficiency

Cells which are MMR deficient characteristically show resistance to 6TG. Figure 2A shows typical results for a clonogenic assay in the presence of the drug using HCT116 colon cancer cells, which are deficient in multiple MMR proteins. Parental hTERT-1604 (WT) which are MMR-proficient are sensitive to the drug, as expected. Cells with low levels of DNMT1 (d24) showed a clear survival advantage over WT and produced distinct colonies on the plates (Fig. 2A). The overall results of the clonogenic survival assays are summarized in Figure 2B: the DNMT1-depleted cells show almost the same number of colonies relative to their untreated controls as the MMR-deficient HCT116 cells, whereas the parental cells (WT) and cells containing a scrambled shRNA control (Scr) showed dose-dependent sensitivity.

To quantify mutation rates at microsatellites, we took advantage of a reporter which had been previously integrated into the parental hTERT-1604 (61), consisting of a neomycin-resistance cassette shifted out of frame by a CA17 microsatellite. The DNMT1-depleted cell lines typically showed greatly increased survival rates when grown in the presence of neomycin (Fig. 2C), indicating an increased slippage rate at the microsatellite. A Luria–Delbrück fluctuation assay was carried out as described (61) on the parental cell line and on the DNMT1-depleted derivatives and the results are summarized in Figure 2D. Mutation rates at the reporter were increased 3-fold in cells with intermediate levels of DNMT1 (d3, 31%) and 6- to 10-fold in lines with low levels (d1 and d24, 12%). The neo gene can be brought back in frame most parsimoniously by deletion of a single repeat (−2 bp) or insertion of two (+4 bp): while these were the most common mutations in the controls and DNMT1-depleted cells alike, the latter also showed an increase in other mutations, typically −8 bp deletions and +10 bp insertions (Fig. 2E, ‘other’).

Depletion of DNMT1 leads to decreases in MMR protein levels in normal and colon cancer cells

The combination of 6TG resistance and microsatellite mutation suggested MMR protein levels may be affected in these cells, which are deficient in multiple MMR proteins. Parental hTERT-1604 (WT) which are MMR-proficient are sensitive to the drug, as expected. Cells with low levels of DNMT1 (d24) showed a clear survival advantage over WT and produced distinct colonies on the plates (Fig. 2A). The overall results of the clonogenic survival assays are summarized in Figure 2B: the DNMT1-depleted cells show almost the same number of colonies relative to their untreated controls as the MMR-deficient HCT116 cells, whereas the parental cells (WT) and cells containing a scrambled shRNA control (Scr) showed dose-dependent sensitivity.

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lines showed matching decreases in MLH1 protein levels (Fig. 3A), with MLH1 being low in d1, intermediate in d3 and d15 but high in d10. HCT116 colon cancer cells, which lack functional MLH1, are shown as a control. The mRNA levels for MLH1, PMS2 and a number of other repair proteins were not decreased in the stable lines (Supplementary Material, Fig. S1A–C), ruling out non-specific targeting: the scrambled control also showed no effects on MMR proteins.

In addition, analysis of the CpG island at the MLH1 promoter by pyrosequencing indicated that it remained unmethylated in the d1 cells, ruling out localized hypermethylation, which has been documented in colon cancer cells such as SW48, shown for comparison (Supplementary Material, Fig. S1D and E).

To determine if the opposite was true and targeting of MLH1 mRNA could lead to loss of DNMT1 protein as might be expected if they physically interacted, we took advantage of an hTERT-1604 cell line containing an MLH1 shRNA which we had previously generated (38). By western blotting, we could confirm that MLH1 protein levels were depleted by the MLH1 shRNA in M1 cells (Fig. 3B), but the decrease in MLH1 did not lead to a concomitant decrease in DNMT1 protein (Fig. 3B).
In order to confirm this effect in another cell line using another method, we expanded our study to colon cancer cells. To do this, we used a pool of four small interfering RNA (siRNA; Dharmacon ON-TARGETplus SMARTpool) to target DNMT1 in HT29 colon cancer cells, which are MMR-proficient and have normal levels of MLH1. Figure 3C shows a sharp drop in DNMT1 mRNA levels in cells transfected with the siRNA (+), while the mRNA for MLH1 is unaffected. qPCR indicated that DNMT1 mRNA levels as low as 9.6% of WT could be achieved (Fig. 3D). DNMT1 protein levels were decreased in line with mRNA levels (Fig. 3E). In this system too, knockdown of DNMT1 gave a concomitant decrease in MLH1 at the protein (Fig. 3E) but not the mRNA level (Fig. 3C, Supplementary Figure 2).
Material, Fig. S1F). We also found that levels of MSH6, another MMR protein, were markedly decreased in cells treated with DNMT1 siRNA (Fig. 3F).

**DNMT1 loss affects the steady-state levels of a number of DNA repair proteins**

To confirm that decreased levels of MLH1 in the DNMT1-targeted cells were affecting the MutLα repair complex, we examined the independently derived d24 hTERT-1604 line with stably integrated shRNA targeting DNMT1 and found by reprobing the same membrane that both MLH1 and PMS2 were decreased, compared with cells containing no shRNA (Fig. 4, compare MLH1 and PMS2 in lane 2 with WT in lane 1). This effect could be due to loss of MLH1 alone, since this is known to destabilize the complex, as we confirmed in the hTERT-1604 M1 cell line, which contains an shRNA targeting MLH1 (Fig. 4, compare levels of both components in lane 4 to lane 3). Targeting DNMT1 using transient siRNA transfection in HT29 gave similar results, i.e. a decrease in both MLH1 and PMS2 (Fig. 4, compare lane 7 with WT in lane 6). However, we had found DNMT1 depletion to affect MSH6 as well (Fig. 3F), whose stability is not dependent on MutLα components. We also found that MBD4 levels were affected in normal (Fig. 4, compare MBD4 in lane 2 with WT in 1) and cancer cells (compare lane 7 with WT in 6: note that the antibody used in lanes 3–9 was different from that in lane 2 with WT in lane 1).
shown at left. MLH1 and PMS2 bind to form the MutL complex as indicated: destabilizing one protein is known to cause a matching decrease in the other. The hTERT-1604 cell lines are as follows: lane 1, WT; lane 2, DNMT1 d24; lane 3, WT; lane 4, MLH1 M1; lane 5, MBD4 A6. The loading control was PCNA in lanes 1 and 2, GAPDH in lanes 3–9. The MBD4 antibody used in lanes 1 and 2 (Abcam) differed from that used in lanes 3–9 (Santa Cruz), the latter detecting both the target (arrowhead) and a non-specific band (asterisk). Each component blot was repeated at least once.

Figure 4. Comparison of the effects of targeting DNMT1, MLH1 and MBD4 in normal (hTERT-1604) and colon cancer (HT29) cells. Western blots of protein from hTERT-1604 lines (lanes 1–5) or HT29 cells (lanes 6–9) which were targeted using shRNA or pooled siRNA against the indicated genes (top). Matched control cells lacking siRNA and shRNA are also shown (–) beside the relevant sample. The same membranes were stripped and reprobed for each antibody shown at left. MLH1 and PMS2 bind to form the MutL complex as indicated: destabilizing one protein is known to cause a matching decrease in the other. The hTERT-1604 cell lines are as follows: lane 1, WT; lane 2, DNMT1 d24; lane 3, WT; lane 4, MLH1 M1; lane 5, MBD4 A6. The loading control was PCNA in lanes 1 and 2, GAPDH in lanes 3–9. The MBD4 antibody used in lanes 1 and 2 (Abcam) differed from that used in lanes 3–9 (Santa Cruz), the latter detecting both the target (arrowhead) and a non-specific band (asterisk). Each component blot was repeated at least once.

lanes 1–2 and only the bottom band is specific for MBD4). Since MBD4 interacts with MLH1 at the protein level, we also examined MLH1-depleted lines and could confirm that MBD4 stability is not dependent on levels of the MutL complex [Fig. 4, compare MBD4 levels (bottom band) in lanes 4 and 3].

To determine if MBD4 might be a crucial intermediary linking the effects of DNMT1 depletion and loss of MLH1, we targeted the MBD4 mRNA itself in hTERT-1604 lines using shRNA in the same way as before and isolated a clone (A6) showing low levels (25.7%) of MBD4 (Supplementary Material, Fig. S2A and B). We also used transient transfection of siRNA pools (Dharmacon) to deplete MBD4 in the HT29 colon cancer cells to 14.0% of WT levels (Supplementary Material, Fig. S2C and D), as assessed by qPCR (data not shown). In both types of cells, only the MBD4 protein was affected, and no decreases in DNMT1, MLH1 or PMS2 were seen (Fig. 4, compare lane 5 with WT in lane 3, and lane 8 with WT in lane 9).

Loss of repair proteins follows activation of the DDR in DNMT1-depleted cells

As a number of repair proteins were affected in DNMT1-depleted cells and stabilization by direct protein–protein binding seemed therefore less likely, a view supported by the data from knockdown of individual components presented earlier, we surmised that loss of repair proteins in DNMT1 hypomorphs might instead reflect an indirect effect. Loss of DNMT1 is known to trigger the DDR and analysis of the cell-cycle profile in the various lines (Fig. 5A) indicated that as DNMT1 levels dropped, the cells spent an increasing amount of time in the G1 phase (d10 and d3), then in d1 cells with 12% of WT DNMT1 levels most cells appear to be in G2/M arrest. The G1 pause in d10 and d3 can be more clearly seen in the summary data presented in Figure 5B. If MMR proteins are predominantly expressed in S phase, the cell-cycle effects seen in the hypomorphs might lead to the apparent drop in MMR protein levels seen in those cells. To examine this, we synchronized WT cells in G1 using a double thymidine block and collected populations of cells at zero hours and at different times post-release. Untreated cells (u) show a normal cell cycle profile (Fig. 5C, top) with 62.9% of cells in G1, 16.8% in S and 20.3% in G2/M phases, respectively. Cells in S phase are most enriched at 4 h post-release (67.1%), those in G2/M at 8 h (79.7%) and cells in G1 at 12 h (81.6%). Western blotting of proteins from these timepoints confirmed that the MLH1 protein level appears highest at S phase and is lower in both G1 and in G2/M (Fig. 5C, bottom). As well as alterations in cell-cycle profile, the depleted clones also showed changes in cell morphology (Fig. 5D) and had higher levels of H2A.X phosphorylation, a signal for DNA damage (Fig. 5E). Although some evidence for changes in MLH1 subcellular location have also been reported following proteolytic cleavage (33), we could not detect any substantial changes in protein localization in the siRNA-treated cells (Supplementary Material, Fig. S1G).

Since low levels of DNMT1 interfere with cell-cycle progression and trigger damage signalling, we surmised that longer-term culture of the cells was leading to selection of those which have silenced or removed the shRNA, as we have seen previously (38). This indeed proved to be the case: analysis of cell-doubling times for the hTERT-1604 cell lines also indicated that the d1 and d3 cells grew significantly more slowly initially than the WT cells (Supplementary Material, Fig. S3A), though later appear to catch up. RT–PCR analysis of DNMT1 transcription showed recovery of mRNA levels in these surviving d1 and d3 cells by passage 7 (Supplementary Material, Fig. S3B) and the restoration of MLH1 protein levels (Supplementary Material, Fig. S3C). This indicates that there is strong selection against the expression of the shRNA. These cells also showed recovery of sensitivity to 6TG (data not shown), confirming that the primary cause of MMR defects was the lack of DNMT1 and that rare clones with mutations in MLH1 or other MMR components
had not been inadvertently isolated. Genomic PCR confirmed that the p7 cells still contained the integrated pSilencer vector (Supplementary Material, Fig. S3D), as expected from their continued growth in hygromycin. The cells which show recovery of DNMT1 levels fail to regain methylation at the paternal allele of the H19 imprint control region (Supplementary Figure 5.

Figure 5. Activation of the DNA damage response (DDR) in DNMT1-depleted cells. (A) Fluorescence-activated cell sorting (FACS) using propidium iodide staining of the cell lines indicated at left. The level of DNMT1 mRNA in each line from qPCR is indicated in parentheses, with WT set to 100%. G1, S and G2/M fractions are indicated: the experiment was carried out three times. (B) Graphical representation showing amount of time spent in each stage of the cell cycle (see key at right) as a percentage of the whole for the cell lines indicated. (C) FACS of WT cells which are either untreated (u) or following double thymidine block and release; hours after release are indicated above each profile (top). For untreated cells, 62.9% are in G1, 16.8% in S and 20.3% in G2/M phase. S-phase cells are most enriched at 4 h (67.1%), G2/M cells at 8 h (79.7%) and G1 at 12 h (81.6%). Western blotting for MLH1 (bottom) was carried out on proteins from the timepoints indicated: the same membrane was stripped and reprobed for GAPDH for loading. Synchronization was carried out once and western blotting three times. (D) Bright-field images of WT and d1 hTERT-1604 cell lines: magnification is ×100 and pictures are representative of results seen at different passage numbers. (E) Immunofluorescence staining for the cell lines in (C) using antibody against γH2A.X (green) and a DNA counterstain (TO-PRO 3-red). Representative images from duplicate experiments, taken with ×100 objective on a confocal microscope using identical settings, are shown. (F) Cell-growth kinetics and viability. HT29 cancer cells were transfected with a pool of 4 × siRNA against DNMT1 as before, or else mock-transfected. Cell numbers were assessed every 24 h (top), with error bars representing SD, not visible at all timepoints; *P < 0.015. Loss of viability was assessed by Trypan blue staining (bottom); cells which have taken up the dye are no longer viable. Error bars represent SD; *P < 0.03. The experiment was repeated three times. (G) HT29 cells were transfected with the siRNA against DNMT1 at 0 h, then harvested and mRNA extracted for RT–PCR at the times indicated. The same mRNA was used to assay each of the genes at right. The position of the 300 bp band on a 100 bp ladder is indicated for reference and the experiment was repeated at least four times. (H) Western blotting on protein samples at the indicated times after transfection with the siRNA. The same membrane was stripped and reprobed with each of the antibodies at right. PARP adds PAR groups to multiple nuclear targets, resulting in a ladder of bands as indicated. A product generated by proteolytic cleavage of PARP is shown by the arrowhead at right. Results are representative of at least three experiments.
Material, Fig. S3E), consistent with the need for germ-line passage of the allele to reset the imprint (62). In keeping with a loss of methylation at this ICR, the levels of IGF2 mRNA are reproducibly lower in d1 and d3 cells compared with WT (Supplementary Material, Fig. S3F).

To examine the time-course of events following DNMT1 depletion, we looked at the response in HT29 cells following treatment with siRNA. In experiments where DNMT1 levels were decreased to below 15%, we saw marked inhibition of cell growth (Fig. 5F, top) due to increased cell death (Fig. 5F, bottom). To examine whether cell killing involved PARP or caspase-mediated mechanisms, we carried out western blots on extracts from the cells. Loss of the target mRNA at 24 h (Fig. 5G) triggers DNA damage signalling through the activation of PARP, visible as increasing levels of PARylation for a large number of target proteins, many of them present in chromatin (Fig. 5H). PARP activity peaked at 48 h, after which point it dropped sharply, a hallmark of substrate depletion in the cell (Fig. 5H). PARP itself is a target for caspases and while there was some evidence for caspase activation at 48 h, it is less marked, suggesting PARP activation might be the primary signalling event. MLH1 protein levels only started to drop appreciably at 72 h (Fig. 5H), suggesting it was consequent to PARP over-activation. We also examined methylation levels in these cells. Hypermethylation at the H19 ICR is seen in this cancer cell line like a number of other tumours (63,64), in contrast to the situation in the normal hTERT-1604 cells: nevertheless, a clear and progressive increase in the number of unmethylated alleles (u) is seen subsequent to siRNA treatment (Supplementary Material, Fig. S4A). Methylation at LINE1 interspersed retrotransposons, present at around $5 \times 10^5$ copies, also showed a decrease over time following exposure to DNMT1 siRNA (Supplementary Material, Fig. S4B), as measured by pyrosequencing. Notably, the decline in methylation levels out around 72 h, consistent with the triggering of cell death at this point in cells which are most affected.

**Inhibiting DNA damage signalling through PARP can relieve the effects of DNMT1 depletion**

To examine the effects of inhibiting the DDR, we used small molecule inhibitors to disrupt signalling (Fig. 6A). Normal

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**Figure 6.** Inhibition of DDR signalling through PARP relieves the effects of DNMT1 depletion. (A) Immunofluorescent staining was carried out for γH2A.X (green) on HT29 cells grown on coverslips and mock-transfected (mock) or transfected with siRNA targeting DNMT1 in the absence of any inhibitors (no inhib). The effects of DPQ (PARP inhibitor) or caffeine (ATM/ATR inhibitor) on siRNA-treated samples were also assessed as indicated. Samples were counterstained with Hoescht 33528 for DNA (blue). Scale bar indicates 50 μm, experiment carried out twice on multiple slides. (B) Effects of DNA damage response pathway inhibitors on cell survival. HT29 cells were allowed to recover for 24 h following transfection with DNMT1 siRNA before adding the inhibitors indicated. Pifithrin is an inhibitor of p53 and Boc-D-FMK is a broad-spectrum caspase inhibitor, DPQ and caffeine as earlier: siRNA-treated cells with no inhibitors (no inhib) are also shown. Mock-transfected cells were exposed to the same inhibitors. At 72 h, numbers of viable cells were assessed relative to the mock-treated control. Error bars represent SD. Statistically significant differences are indicated as follows: ***P < 0.001; **P < 0.01 and n.s., not significant. Experiment was carried out three times. (C) Western blot using protein samples from HT29 cells after transfection (siRNA) or mock-transfection (mock) as earlier. The same membrane was successively stripped and reprobed for the antibodies indicated at right. The locations of bands consistent with proteolytic cleavage of MLH1, detected on longer exposure, are indicated by arrowheads. The positions of bands on the size ladder are indicated at left in kiloDaltons (kDa). The intact MLH1 protein is 82 kDa, while its reported caspase cleavage products are around 45 kDa; the experiment was carried out at least twice.
HT29 cells which are mock-transfected (mock) show low levels of γH2A.X, but when transfected with DNMT1 siRNA in the absence of inhibitors (no inhibit) γH2A.X levels are greatly increased, as expected. This could be partially relieved by adding caffeine, an inhibitor of ATM and ATR (Fig. 6A) as previously reported. However, treating the siRNA-transfected cells with the PARP inhibitor DPQ also gave a clear reduction in H2A.X phosphorylation levels which appeared to exceed that seen with caffeine (Fig. 6A). In keeping with these observations, while treating DNMT1-depleted cells with an inhibitor of caspases (Boc-D-FMK) or p53 (Pifithrin) gave some recovery of cell viability (Fig. 6B), treatment with an inhibitor of PARP (DPQ) consistently showed a greater effect (Fig. 6B). Caffeine could also partially block cell death, in keeping with the findings stated earlier (Fig. 6B).

MLH1 has previously been reported to be cleaved by caspase 3 into fragments of around 45 kDa by one group (33), though detection of the cleavage products appears to be difficult in some cell types (65). We examined protein extracts from HT29 cells transfected with DNMT1 siRNA in the presence or absence of the PARP inhibitor DPQ or the p53 inhibitor, neither of which inhibit caspase 3, and carried out western blots. Although no cleavage products were detected in the untreated cells or in the cells treated with siRNA alone, when cell death was delayed by the presence of the inhibitors, we could consistently detect the expected cleavage products for MLH1 on longer exposures (Fig. 6C, arrowheads). It is notable that while levels of full-length MLH1 (82 kDa) also appear higher in samples treated with inhibitors, consistent with inhibition of the DDR, levels of DNMT1 remain low here, suggesting that uncoupling of siRNA-mediated DNMT1 depletion and DDR-mediated MLH1 degradation may be occurring (Fig. 6C, compare lanes 4–5 with lanes 2–3).

**DISCUSSION**

In the work presented here, we set out specifically to address the question of whether loss of DNMT1 in differentiated normal human cells could lead to MMR deficiency and to look for possible mechanisms by which this could occur. To this end, we chose hTERT-immortalized normal human fibroblasts which have functional p53, intact cell-cycle checkpoints, maintenance of imprinting and a normal karyotype (56–58). Specifically targeting DNMT1 in these cells was sufficient to confer both resistance to 6TG and MSI at a CA17 reporter, two hallmarks of MMR deficiency (8). Mutation rates tracked with DNMT1 levels, going from 4.1-fold WT rates in cells with 31% of the normal protein level to ~10-fold WT rates in cells with 12% of WT DNMT1, the latter confirmed in two independently derived cell lines. The likely cause of the MMR defects in the DNMT1 hypomorphs is a decrease in the steady-state levels of MMR protein components, since MLH1, PMS2 and MSH6 were decreased in parallel with, but to a smaller extent than, DNMT1 levels. This is most likely due to the effects of activating the DDR. One effect of DDR activation is to slow the cell cycle to allow repair and we show that DNMT1 hypomorphic cell populations are deficient in cells in S phase due to delays in G1 or G2/M. Previous reports have indicated a small enrichment for MLH1 and PMS2 (66) as well as MSH6 (67) in S phase, which we confirmed for MLH1, and have found that MMR repair activity peaks in this cell-cycle phase (68). A second effect of DDR is the triggering of cell-death pathways and proteolytic cleavage of repair proteins, including MLH1 (33). It is thus likely that both pausing the cell cycle and triggering proteolytic cleavage contribute to the decreases in MMR activity and protein levels seen in DNMT1 hypomorphs. Effects at the protein level were unexpected, as DNMT1 normally acts to transcriptionally repress genes through methylation of their regulatory sequences (69). We therefore confirmed that this effect is not confined to one cell type or method of DNMT1 depletion, since the same effect on MMR proteins was obtained in colon cancer cells, where DNMT1 had been transiently targeted using pools of siRNA. Both the stable and transient knockdowns show WT mRNA levels for the repair proteins and we ruled out silencing of the MLH1 gene by gene-specific hypermethylation, seen in many colon cancers (70), since there was no increase in methylation at the MLH1 promoter.

Loss of multiple MMR proteins could potentially explain a number of MMR-related features of the DNMT1-deficient cell models generated here and by others. Several of the studies, like this one, found increased mutation rates at microsatellite repeats that do not contain any CpG sites either in the repeat itself (8,10,11,19) or nearby (9), indicating that changes in methylation could not be the primary cause of the instability. This was true of both integrated reporters (8,9,11) and endogenous repeats (10). The decreased levels of components of both the MutLα complex (i.e. MLH1 and PMS2) and the MutSα complex (i.e. MSH6) could potentially affect all microsatellite tracts, including trinucleotide repeats (11,19). We recently characterized MMR and DDR in a set of hTERT-1604 cell lines carrying stably expressed shRNA targeting MLH1 (38), including the M1 cell line used again here. No increase in microsatellite mutation rate was found in any of the cell lines, despite M1 cells having only 11.6% of WT MLH1 mRNA levels. This was in contrast to the DNMT1-deficient cells, where increased mutation rates at the reporter were seen even in d3 cells, which have only modest changes in MLH1 protein levels. When we compared these cell lines, it was clear that the loss of multiple repair components was only seen in the DNMT1 hypomorphs, offering an explanation for the greater rate of mutation here. The presence of a substantial number of larger indels at the microsatellite reporter also supports a model in which multiple repair components are affected. If correct, this hypothesis predicts that the effects of DNMT1 loss on MSI would be augmented in cells which already lack one repair component. It is interesting to note in this regard that in other DNMT1-deficient models where MMR has been assessed, an additive effect of the loss of DNMT1 and MLH1 on the trinucleotide class of microsatellite repeat has been reported (19), while the rate of microsatellite mutation in ES cells lacking both DNMT1 and BLM1 was greater than that in cells lacking DNMT1 alone (8).

While mouse ES cells can tolerate complete loss of DNMT1 (71), previous work in mouse fibroblasts and HCT116 colon
cancer cells showed that differentiated cells which lack the protein lose viability and enter G2/M arrest (34,36). It was suggested that a threshold effect may exist for DNMT1 levels in the latter: depletion to a certain level was consistent with viability, but cells would show loss of DNA methylation; below this threshold, cells lose viability too quickly to show extensive demethylation (34,59). Our results are consistent with these previous observations—decreases in DNA methylation and an increasing delay in G1 tracked with DNMT1 levels in our stable cell lines, with G2/M arrest being triggered when protein amounts were around 12% of WT DNMT1. It seems likely that the effects on viability in homozygous knockout mouse models preclude the observation of MMR defects. Resistance to 6TG and increased mutation rates were confined to a few passages of our stable knockdowns too, with longer-term culture selecting for cells which silence the shRNA epigenetically. This is almost certainly due to the negative effects of activation of the DDR in the knockdown cells.

Previous reports have shown that the loss of DNMT1 in differentiated cells triggers DDR signalling, with phosphorylation of H2A.X and p53, both downstream targets of the ATM/ATR kinases, and effects on the cell cycle (34,35). In DNMT1 null cells, programmed cell death is seen, which can be partly rescued by either blocking p53 or caspase activity (35–37). In addition to the cell-cycle effects, we also found increased levels of γH2A.X signalling in both stable and transient knockdown cells. Additionally, there were increased rates of cell death and we could confirm that inhibiting p53 ameliorated this effect (36). Our results are consistent with the reported roles for ATM/ATR and caspase-mediated cell death in mediating response to DNMT1 depletion (34,37), since caffeine could substantially reduce γH2A.X levels and could somewhat reduce cell death, as could caspase inhibition.

We also uncovered here a novel and substantial role for PARP-mediated cell death in the response to DNMT1 removal. PARP reacts to DNA damage by adding ADP-ribose polymers to many nuclear targets around the damage sites, including histones (40), utilizing nicotinamide adenine dinucleotide (NAD+) as a substrate. If damage levels are very high, this results in NAD+ depletion and mitochondrial membrane depolarization, leading to translocation of AIF to the nucleus (38,39). AIF causes chromatin condensation and DNA fragmentation and a late activation of caspases, leading to the death of the cell (39). DNA repair proteins are among the favoured targets for caspases, which cleave PARP itself (29), ATM (32), MLH1 (33) and BLM1 (28) among other repair components (30). Inhibition of DNMT1 led to steadily increasing levels of PARylation, followed by the sudden loss of PAR signal and the cleavage of PARP, suggesting the activation of cell death following substrate depletion (39,72). Consistent with a central role for PARP in responding to the loss of DNMT1, a PARP inhibitor could strongly decrease signalling through γH2A.X and markedly improve cell viability in the presence of the siRNA. It was also notable that loss of MLH1 was coincident with substrate depletion and PARP cleavage. We therefore considered the possibility that damage signalling through PARP played a substantial role in the loss of repair proteins, through release of AIF, activation of caspases or both. Supporting this hypothesis, we could detect proteolytic cleavage products for MLH1 in the knockdown cells, though these were only detectable when programmed cell death was slowed by the addition of PARP or p53 inhibitors. Due to the rapid elimination of DNMT1-deficient cells following PARP over-activation, more sophisticated cell models will be required to tease apart the sequence of events leading from loss of DNMT1 to cell death.

While it may be possible that DNMT1 itself plays a role in MMR either directly or through binding to another central regulator of repair, a number of other observations lead us to favour a model in which activation of the DDR and cell death pathways cause secondary and non-specific loss of MMR components. First, MMR protein levels do not decrease to the same extent as DNMT1 levels in the stable or transient knockdown cells (typically to an estimated 60% of WT in an efficient siRNA experiment), suggesting an indirect effect: compare this with targeted depletion of MLH1, which triggers an exactly matching loss in its binding partner PMS2 (38,73). Secondly, DNMT1 depletion causes loss of multiple repair protein components, including PARP and MBD4 which are part of the BER rather than MMR system. Thirdly, neither depletion of MLH1 or of MBD4 shows any effect on DNMT1 levels, which does not favour a mutual interaction. Lastly, MLH1- and DNMT1-depleted hTERT-1604 cells differ markedly in their damage responses: M1 cells (38) or other MLH1-deficient cells (74) show no cell-cycle defects and no DDR activation except when challenged by DNA-damaging drugs, whereas DNMT1-depleted hTERT-1604 cells (this study) or HCT116 cells (34,35) show clear cell-cycle defects and DDR signalling in the absence of extraneous treatment.

In conclusion, we have shown here that targeting DNMT1 in differentiated human cells reproducibly leads to secondary effects on MMR protein levels, drug resistance and MSI. This occurs through a mechanism involving activation of the DDR and cell-death pathways and we uncovered a novel and substantial role for the PARP enzyme in this process. Loss of MMR repair activity explains how depletion of DNMT1 can affect microsatellite sequences which do not contain CpG, the target of the methyltransferase enzyme. It has recently been shown that PARP activity may be normally required for programmed loss of DNA methylation in the early embryo (42). Demethylation triggered by the unscheduled loss of DNMT1 in our system instead appears to trigger overactivation of the enzyme and substrate depletion, leading to activation of cell-death pathways. The effects on members of both the BER and MMR family and our other findings here fit well with previous observations of a link between DNMT1 insufficiency and genomic instability and reinforce the idea that epigenetic and genetic stability are intrinsically linked.

MATERIALS AND METHODS

Cell culture and drug treatment

The colon cancer cell lines HCT116 (58) and HT29 were maintained in DMEM with 10% FBS and McCoy’s 5A
media supplemented with 10% FBS. All media supplies used were from Gibco/Life Technologies (Paisley, UK). The normal (WT) human fibroblast cell line hTERT-1604 (61) with a stably integrated (CA)17 microsatellite repeat (38) and their shRNA-expressing derivatives (d1, M1, etc.) were grown in high-glucose DMEM with 10% FBS and 2 x non-essential amino acids, with hygromycin (150 μg/μl) for the shRNA-containing cells. Selection was removed 24 h prior to any experimental analysis. For 6TG (Sigma, Poole, UK), medium was changed every 48 h with the drug present throughout. Other inhibitors were maintained in the media as follows: 3,4-di-hydro-5-[4-(1-piperindinyl)butoxy]-1(2H)-isoquinoline (DPO) at a final concentration of 30 nm; Boc-DFMK (Calbiochem, Nottingham, UK) at 10 mM; caffeine at 2 mM and pifithrin (Calbiochem) at 20 mM as previously described (38).

Stable and transient knockdowns

Overlapping primers incorporating an siRNA sequence previously reported to target DNMT1 (60) were made and ligated into pSilencer (Ambion, Huntingdon, UK) as per supplier’s recommendations. An siRNA oligonucleotide targeting MBDS4 was designed using an algorithm ([75] available at http://jura.wi.mit.edu/bioc/siRNAext/home/php, last accessed 31/5/2011), then overlapping primers Top 5′-GAT CCGCCTAGTGTGTGTGCTTTCCT CAAGAGAGAAA GC A CACACACTAGGCG3′ and Bottom 5′AGCTTTTCC AAAAAAGCCTAGTGTGTGTGCTTTCTC TCTTGAGGAAAGCACACACTAGGCG3′ made and ligated as for DNMT1. All constructs were verified by sequencing. The vector was linearized (Sall) and transfected either by electroporation with 1 μg of construct in PBS at 320 v and 500 μF into 1 x 10⁷ cells using the BioRad GenePulserII (d24, all MBDS4 clones) or by lipofection (d1–d15) with Lipofectamine 2000 following manufacturer’s recommendations (Invitrogen/Life Technologies). Medium containing serum was immediately added and the cells plated on 90 mm dishes at 5 x 10⁵ cells/dish before selection in hygromycin (150 μg/μl) for 10–14 days. Generation of the MLH1-depleted cell line has been previously described (38). DNMT1 and MBDS4 targeting in HT29 cells was done using pre-designed pools of four siRNA against each gene (ON-TARGETplus SMARTpool, Dharmacon/Thermo Scientific, Dublin, Ireland) as per manufacturer’s instructions. Briefly, 1 x 10⁵ HT29 cells were seeded in individual wells of a six-well plate and allowed to attach overnight, then treated the next day with 100 μl siRNA solution was replaced with nuclease free water.

Polymerase chain reaction

Total RNA was extracted using the RNeasy kit (Qiagen, Crawley, UK) using DNase treatment and cDNA made using oligo d(T)₁₅ and reverse transcriptase (Promega, Southampton, UK). qPCR was performed on a Roche LightCycler 2.0 using the relative standard curve method (76) with ACTB or HPRT as reference genes. Briefly, each 20 μl reaction contains 1 × QuantiTect SYBR Green PCR reagent (Qiagen), 0.5 μM of forward and reverse primers, 3 μl of cDNA and nuclease free H₂O. PCR reactions were started with an incubation at 95°C for 15 min to activate the Hot-Start Taq DNA Polymerase (Qiagen), followed by 50 cycles of 94°C, 15 s, annealing at 52°C, 20 s and 72°C, 15 s for elongation. Amplification specificity was confirmed by melting-curve analysis and gel visualization of final products. RT–PCR was carried out on cDNA using 1.25 U Taq, 1X buffer, 3.5 mM MgCl₂, 0.4 mM dNTP’s and 0.5 pmol primer (Invitrogen) at 94°C, 3 min, then 25 x 94°C, 1 min; 60°C, 1 min; 68°C 1 min and finally 72°C, 10 min. Primer sequences are available on request.

DNA methylation analysis

DNA was extracted from cells growing in the log phase essentially as previously described. Bisulfite modification and purification was carried out using the Epitect kit (Qiagen) according to the manufacturer’s instructions. For H19, a semi-nested PCR was carried out using primers designed by Kerjean et al. (77) under the following conditions: an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1 min with a final extension at 72°C for 7 min. A nested PCR was carried out for SNRPN using published primers (78) under the following conditions: an initial denaturation at 94°C for 1 min followed by 35 cycles (first round) or 25 cycles (second round) of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min. The second round was carried out under the same conditions but with only 25 cycles, using 5 μl of the first round PCR product and primers SNRPN F2 and R2. The PCR products were gel-extracted using a Wizard SV kit (Promega), then an equal amount, as judged by gel electrophoresis, was digested overnight at 37°C with the 10 U of the restriction enzyme Hinfl or CfoI (NEB) along with 1X buffer and 1X BSA in a total of 20 μl and run on a 3% agarose gel for visualization. There is one Hinfl site within the 227 bp H19 PCR product, resulting in two fragments of 148 and 79 bp while CfoI has three sites within the 384 bp SNRPN PCR product resulting in major bands of 248, 226, 114 and 92 bp, depending upon which sites have cut.

Primers for pyrosequencing of MLH1 and LINE1 were as supplied by Qiagen. Amplification was carried out on bisulfite-treated DNA in a PCR reaction containing 2.5 mM MgCl₂, 1 x Taq polymerase buffer, 0.4 mM dNTPs, 1 μM forward and reverse primers and 1 U Taq and the following cycle conditions: initial deannealing at 95°C, 15 min, followed by 50 cycles of 95°C, 20 s; 55°C, 20 s and 72°C, 20 s with a final 5 min elongation step. The products were run on 3% gels for verification. PCR products (20 μl) were bound to sepharose beads by mixing with 2 μl of beads and 40 μl of binding buffer (Qiagen) made up to a total volume of 80 μl with sterile water. Binding was facilitated by agitation at 1400 rpm for 5 min. Subsequently, the bead complexes were washed through a 70% ethanol solution, a denaturation
solution (Qiagen) and a washing buffer (Qiagen) in the Qiagen Pyramark Q24 workstation, as per manufacturer’s instructions. Bead complexes were transferred to a Pyramark Q24 plate containing 25 μl of 0.3 μm sequencing primer in each well. Samples were annealed to sequencing primer by heating to 80°C for 2 min, followed by cooling to RT. Annealed samples were analysed on the Pyramark Q24 Instrument.

Microsatellite mutation rate analysis
The fluctuation analysis was performed as previously described (61), correcting for colony-forming efficiency. The Luria–Delbruck method of the mean was used to calculate mutation rate and statistical analysis was as described (61).

Clonogenic assays for drug resistance
Cells were seeded at 500 cells/90 mm dish and allowed to recover for 24 h before treating with 6TG as indicated above. After colonies became visible at ~14 days, plates were stained with crystal violet [0.25 (w/v) crystal violet in 50% methanol and 50% PBS] and colonies counted: numbers were expressed as a percentage of the colonies formed in the absence of the drug. The assay was carried out in triplicate and three independent experiments were completed.

Western blotting
Thirty micrograms of total protein from cells growing in log phase was resolved by SDS–PAGE, electroblotted onto nitrocellulose membranes and blocked for 2 h at room temperature (RT) in 5% non-fat dry milk. Membranes were incubated with anti-DNMT1 (Abcam, Cambridge, UK: ab54759), anti-β-actin (Abcam ab8226), anti-MBD4 [Santa Cruz, CA, USA: sc-10753 or Abcam (discontinued)], anti-MLH1 (BD Pharmingen, Oxford, UK: cat#51-1327GR), anti-GAPDH (Cell signalling Technologies, Herts, UK: cat#2118), anti-PMS2 (BD Pharmingen: cat#556415), anti-PARP (BD Pharmingen: 4C10-5), anti-PAR (BD Pharmingen: LP96-10) or anti-GAPDH (Cell Signaling Technologies: 14C10) overnight at 4°C, followed by HRP-conjugated secondary antibody for 2 h at RT before detection using ECL (Amersham, Amersham, UK).

Cell growth, viability and cell-cycle assays
Cells were seeded at a density of 1 × 10^4 cells/well of a six-well plate on day 0 and counted every 24 h for 4 days using a haemocytometer (Neubauer/Fisher, Loughborough, UK). To calculate the viability of cells, 50 μl trypan blue was added to 50 μl of the cell suspension. Numbers of viable and non-viable cells were calculated by counting the number of dead cells divided by the number of total cells to give the fraction of non-viable cells after allowing for the dilution factor. Cells in log phase (1 × 10^6) were harvested, resuspended in 2 ml ice-cold 70% ethanol and stored at −20°C for a minimum of 30 min before centrifuging at 300 g for 5 min, then resuspending in 400 μl PBS and passing through a 25 gauge needle. Fifty microlitres of 1 mg/ml RNase and 50 μl of 400 μg/ml propidium iodide were then added. Following incubation for 30 min in the dark at 37°C, cells were placed on ice before counting on a Becton Dickinson FACScalibur II (Oxford, UK).

Immunohistochemistry
Cells (0.5–1 × 10^5) were deposited on gelatin-coated, UV-sterilized superfrost slides (BDH) or coverslips (Menzel gläser/Fisher) and cultured in tissue culture dishes overnight to allow attachment. For HT29 cells, siRNA treatment was basically as described earlier. Slides/cover slips were washed for 3 × 5 min in PBS and fixed for 15 min in 4% ice cold paraformaldehyde, then washed 3 × 5 min in AB buffer (1% Triton X-100, 4% goat serum, 0.2% SDS in PBS). Blocking and permeabilization was in AB buffer for 3 h before incubation overnight with mouse anti-phospho H2AX antibody (Millipore, Consett, UK) at 1:300 dilution or anti-MLH1 at 1:500 dilution in AB buffer at 4°C. Slides/cover slips were then rinsed in AB buffer, 3 × 5 min. H2AX was visualized with either goat anti-mouse Alexa Fluor 488 (Molecular probes/Life Technologies) or donkey anti-mouse Alexa Fluor 546 (Molecular probes)at 1:400 for 1 h at RT, while MLH1 was detected with the former. Slides/cover slips were then rinsed as before with AB buffer. HT29 counterstaining was with Hoescht DNA stain (Sigma) at 1:200 in PBS for 15 min. hTERT 1604 counterstaining was for 15 min with TO-PRO 3 iodide 642/661 (Molecular probes)/Life Technologies) or donkey anti-mouse Alexa Fluor 546 (Molecular probes) at 1:400 for 1 h at RT, while MLH1 was detected with the former. Slides/cover slips were then rinsed as before with AB buffer. HT29 counterstaining was with Hoescht DNA stain (Sigma) at 1:200 in PBS for 15 min. hTERT 1604 counterstaining was for 15 min with TO-PRO 3 iodide 642/661 (Molecular probes)/Life Technologies) or donkey anti-mouse Alexa Fluor 546 (Molecular probes) at 1:400 for 1 h at RT, while MLH1 was detected with the former. Slides/cover slips were then rinsed as before with AB buffer. HT29 counterstaining was with Hoescht DNA stain (Sigma) at 1:200 in PBS for 15 min.

Statistical analysis
Results unless noted are presented as mean ± standard deviation for a given number of observations (n). Data from each set of observations were cross-compared using unpaired Student’s t-tests (Graphpad Prism, La Jolla, USA). Differences were considered significant if P < 0.05.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.
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