DNA Damage Response Pathway and Replication Fork Stress During Oligonucleotide Directed Gene Editing

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Single-stranded DNA oligonucleotides (ODNs) can be used to direct the exchange of nucleotides in the genome of mammalian cells in a process known as gene editing. Once refined, gene editing should become a viable option for gene therapy and molecular medicine. Gene editing is regulated by a number of DNA recombination and repair pathways whose natural activities often lead to single- and double-stranded DNA breaks. It has been previously shown that introduction of a phosphorothioated ODN, designed to direct a gene-editing event, into cells results in the activation of γH2AX, a well-recognized protein biomarker for double-stranded DNA breakage. Using a single copy, integrated mutant enhanced green fluorescent protein (eGFP) gene as our target, we now demonstrate that several types of ODNs, capable of directing gene editing, also activate the DNA damage response and the post-translational modification of proliferating cell nuclear antigen (PCNA), a signature modification of our target, we now demonstrate that several types of ODNs, capable of directing gene editing, also activate the DNA damage response and the post-translational modification of proliferating cell nuclear antigen (PCNA), a signature modification of replication stress. We find that the gene editing reaction itself leads to transient DNA breakage, perhaps through replication fork collapse. Unmodified specific ODNs elicit a lesser degree of replication stress than their chemically modified counterparts, but are also less active in gene editing. Modified phosphorothioate oligonucleotides (PTOs) are detrimental irrespective of the DNA sequence. Such collateral damage may prove problematic for proliferation of human cells genetically modified by gene editing.

Introduction

Single-stranded DNA oligonucleotides (ODNs) can direct specific changes in the nucleotide sequence of DNA in a process known as gene editing. This technique has begun to emerge as a useful genetic tool for gene therapy. An increased understanding of the mechanism of action of gene editing has accelerated the progression toward application. Reaction parameters such as strand bias, suppression by certain mismatch repair proteins and the involvement of DNA replication in producing altered bases have been heavily investigated.1,2 While the focus of most workers remains, appropriately, on improving and stabilizing the frequency with which gene editing takes place (see ref. 3 for review), we have been examining DNA damage and its downstream consequences associated with the editing reaction.4,5 The development of double-stranded DNA damage appears tied to the slower progression of genetically altered cells through S phase.4–10 The slowing of DNA replication and the creation of strand breaks may be related, perhaps by the collapse of stalled replication forks during the gene editing process, a phenomenon known to occur under normal growth conditions when replication forks are impeded.16–19

Optimization of the ODN-induced gene editing reaction has led to the identification of factors that significantly enhance or impede the achievement of high levels of correction. Proteins involved in recombinational repair and regulation, which are elevated during S phase, are largely supportive of the gene editing reaction.4,20 Additionally, replication has been shown to positively influence the gene editing reaction. Slowing of replication, and the subsequent increase of the proportion of targeted cells in S phase, results in elevated correction levels; whereas prohibiting S-phase progression abolishes correction.3,10–13 Furthermore, the specific ODN has been shown to incorporate into the genome of corrected cells.14,15 The summation of current knowledge in the field thus far suggests a possible mechanism of ODN-integration in the context of replication. The associated DNA damage response seen in this context implicates replication stress as an outcome of gene editing and thus the incorporation of phosphorothioate ODNs may inhibit fork progression. In this article, we examine DNA damage and DNA breakage in greater detail with a particular emphasis on the specificity of the ODN and its chemical compositions as it relates to genotoxicity. To our surprise, we find that replication fork collapse21 can occur in the presence of electroporated ODNs even in the absence of gene editing activity. These results suggest that DNA damage induced by all forms of ODNs is a global effect of gene editing. Phosphorothioate linkages on the standard workhorse vector used to attain the highest frequencies of gene editing exacerbate these genotoxic effects.

Results

Gene editing in the HCT116-19 cell system

The model system employed herein is the human cell line HCT116 with a single copy of integrated mutant eGFP gene
(HCT116-19). Correction of the single-base nonsense mutation by specific ODNs results in the production of fluorescent eGFP protein, allowing phenotypic correction to be monitored via flow cytometry. As shown in Figure 1, the mutant eGFP gene contains a stop codon (TAG) in place of the wild-type tyrosine codon (TAC). The standard workhorse ODNs, 72NT, 72NT-U, 72NT-PM are 72-mers that are complementary to the nontranscribed (G>C) strand of the eGFP gene. The perfect-match ODN, 72NT-PM complements and binds to the nontranscribed strand, but does not create a mismatched base pair. The asterisks indicate three-phosphorothioate bonds on either end of the ODN. 72NT-U is the same length and sequence as 72NT, but does not have any phosphorothioate bonds (unmodified). The 72NS ODN has no known complementarity to the target region or the human genome as accessed via BLAST (NIH) and BLAT (UCSC) analyses. In contrast, 72NS does not support correction of the eGFP mutation as predicted and previously reported. These data validate the assay system used in this paper and display the specificity of the gene editing reaction.

Figure 2b illustrates one of the barriers to the implementation of gene editing as a therapeutic option; the progressive loss of percentage of corrected cells. This phenomenon has been widely reported (see refs. and references within) with reduced correction efficiencies easily observed at 96 hours. Here, we simply extend the time points at which the correction efficiency was determined as the percent of eGFP positive cells in the overall viable population. eGFP positive cells divided by the number of live cells in the population.

The experimental strategy of ODN-induced gene editing in this system has been previously reported and widely reviewed. Briefly, cells (2.5 × 10⁶) are electroporated with ODN after 24 hours of synchronization with aphidicolin, a drug that reversibly synchronizes cells in early S phase. Synchronization and release has been shown to enhance correction efficiency, most likely because it elevates the number of cells passing through in S phase as a population in the presence of the ODN. Extensive washing of the cells removes effective concentrations of aphidicolin, which as a reagent does not influence the gene editing reaction (see ref. 4). Cells are allowed to recover after electroporation in full growth media for the specified time frame before analysis. When 72NT is electroporated into the cells at various levels, a dose response of gene editing is observed (Figure 2a). In contrast, 72NS does not support correction of the eGFP mutation as predicted and previously reported. These data validate the assay system used in this paper and display the specificity of the gene editing reaction.

**Table 1.** Model system for gene editing in mammalian cells. The target enhanced green fluorescent protein (eGFP) sequence contains a TAG stop codon in place of the wild-type TAC tyrosine codon (bold and underlined). The mutant eGFP that is produced is truncated and nonfluorescent. Specific DNA oligonucleotides (ODNs) were designed that can hybridize to the nontranscribed (NT) strand (72NT, 72NT-PM) but not elicit a base-exchange event. The perfect-match ODN, 72NT-PM complements and binds to the nontranscribed strand, but does not create a mismatched base pair. The asterisks indicate three-phosphorothioate bonds on either end of the ODN. 72NT-U is the same length and sequence as 72NT, but does not have any phosphorothioate bonds (unmodified). The 72NS ODN has no known complementarity to the target region or the human genome as accessed via BLAST (NIH) and BLAT (UCSC) analyses.

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**Figure 2.** Gene editing in HCT116-19 cells. (a) HCT116-19 cells were synchronized with aphidicolin for 24 hours and then washed prior to the introduction of the indicated amount of DNA oligonucleotides (ODN); 72NT or 72NS. Gene editing activity was measured by the emergence of enhanced green fluorescent protein (eGFP) by flow cytometry analysis, 24 hours after the addition of the ODN. Correction efficiency (%) was determined as the percent of eGFP positive cells in the overall viable population. (b) HCT116-19 cells were synchronized for 24 hours and electroporated using standard conditions with 8 µmol/l 72NT. Time points were taken at 72, 96, 120, 144, and 168 hours respectively for analysis by FAC of eGFP (corrected cell) expression. Correction percentage was determined as the number of eGFP positive cells divided by the number of live cells in the population.
evaluation for correction efficiencies were undertaken. The results of this work herein may offer one solution to this problem.

Recently, we observed double-strand break formation in response to the addition of ODNs designed to direct gene editing events. All three types of ODNs used in this study, 72NT, 72NS, and 72NT-PM, induced chromosomal damage albeit to various degrees. Double-stranded DNA breakage can result from stress on the replication fork caused by an impediment to S-phase progression. Ferrara and Kmiec showed that the introduction of ODNs into the cell in a gene editing reaction activates Chk1 and Chk2 and in turn, progression through S phase is slowed. A hallmark event that correlates with both DNA damage and the slowing of cells through S phase is the phosphorylation of H2AX. Ferrara and Kmiec showed that the introduction of ODNs into the cell in a gene editing reaction activates Chk1 and Chk2 and in turn, progression through S phase is slowed. A hallmark event that correlates with both DNA damage and the slowing of cells through S phase is the phosphorylation of H2AX. One might predict that H2AX activation will also be observed under our standard reaction conditions. Phosphorylation of H2AX at serine 139 by ATM is widely accepted as a cellular response to DNA damage. Thus, we electroporated 72NT, 72NT-PM, and 72NS into synchronized HCT116-19 cells and measured H2AX phosphorylation, 24 hours later. The results are displayed in Figure 3: all three ODNs induce phosphorylation of H2AX as judged by treatment with fluorescently conjugated antibodies directed against H2AX-r and visualized by fluorescence-activated cell sorting (FACS). As a positive control, hydroxyurea is used to induce this H2AX activation because it causes replication fork collapse. The three ODNs, independent of target specificity, induce a very consistent shift in the cellular status of H2AX with activation peaks partitioned to the right of the profile. These data align with our earlier hypothesis that the amount of ODNs required to direct gene editing is at a level that induces a significant DNA damage response in the cell.

If the targeting reaction is associated with a slowing of S-phase progression, and this slowing is attributable to the ODN causing DNA breakage, vis-à-vis replication fork stress, we would predict that a higher degree of DNA breakage would be seen during S phase. It has been previously demonstrated that the proliferating cell nuclear antigen (PCNA) changes cellular location throughout the cell cycle. During S phase, PCNA is distinctly nuclear, while during the rest of the cell cycle, PCNA appears more diffuse throughout the cell; this relocalization can be detected with immunofluorescence. It has been demonstrated that concurrent staining of γH2AX and punctate PCNA is indicative of DNA damage within cells positioned in S phase. In our experimental approach, HCT116-19 cells were synchronized in early S phase with aphidicolin, treated with ODN, and then allowed to recover for 20 hours before staining for PCNA (green) and γH2AX (red). This delay enables the dispersal of any lingering effects of the synchronization process. Hydroxyurea, known to cause replication fork collapse, and thereby double-strand break formation exclusively during S phase, served as a positive control for the punctate localization of nuclear PCNA and γH2AX. As can be seen in Figure 4, treatment of synchronized cells with an ODN (72NT) designed to modify the genomic sequence results in DNA damage during S phase; the appearance of distinct localization of punctate PCNA and γH2AX positive cells. Cells treated with a nonspecific ODN (72NS) however also show γH2AX positive cells at a level comparable to 72NT; the γH2AX positive cells also appear to have punctate, S phase-specific PCNA staining. In the absence of ODN, few cells exhibit H2AX activated staining. Thus, target specificity is not a differential factor in the DNA damage response during gene editing when cells are positioned in S phase.

**Figure 3** Fluorescence-activated cell sorting (FACS) analysis of the phosphorylation of H2AX. HCT116-19 cells synchronized with 2 µmol/l aphidicolin 24 hours before the addition of 2 mmol/l hydroxyurea (HU) or 4 µmol/l 72NT-PM, 72NT, or the 72NS DNA oligonucleotides (ODN). The reaction was allowed to proceed for 24 hours after which time the cells were processed for staining with antibodies directed against pH2AX. Analyses took place on a Guava EasyCyte 5HT Flow Cytometer (see Materials and Methods section) and phosphorylation is assessed by the degree of shift of the cell population to the right. The number in the upper right hand corner indicates the percentage of cells scoring positive for H2AX activation.

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**Table:**

| Condition     | γH2AX Positive (%) |
|---------------|--------------------|
| No treatment  | 2.34               |
| Hydroxyurea   | 17.56              |
| 72NT          | 55.60              |
| 72NT-PM       | 53.48              |
| 72NS          | 51.62              |

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Termini modification of oligonucleotides can affect the degree of DNA breakage during gene editing

Historically, most ODNs used for gene editing have been designed with modifications that prevent nuclease degradation in the cell. These ODNs direct the highest levels of nucleotide exchange. As such, the ODNs used in the previous experiments contain three nonhydrolyzable phosphorothioate linkages on both termini. Recently, a number of independent labs demonstrated that unmodified ODNs support the gene editing reaction with a reduced level of cellular toxicity compared to ODNs containing the phosphorothioate linkages on both termini. Currently, most ODNs used for gene editing have been designed with modifications that prevent nuclease degradation. The use of unmodified ODNs has prompted us to examine the activity of the unmodified 72-mer (72NT-U) in our gene editing assays.

Synchronized HCT116-19 cells were treated with increasing amounts of 72NT or 72NT-U and we assessed the correction efficiencies of the two ODNs after 24 hours of recovery via flow cytometry. As seen in Figure 5a, the cells treated with unmodified ODN exhibit half as many correction events compared to ODNs containing the phosphorothioate modifications. Specifically, Aarts and te Riele showed that in delivery analyses of the unmodified ODN, low levels of DNA breakage are observed as judged by the neutral COMET assay. These observations, coupled with our own new findings surrounding the collateral damage of modified ODNs, prompted us to examine the activity of the unmodified 72-mer (72NT-U) in our gene editing assays.

Figure 4 Colocalization of PCNA and H2AX-r during gene editing. Confocal image of HCT116-19 cells synchronized with 2 µmol/l aphidicolin 24 hours before the addition of 2 mmol/l Hydroxyurea, 72NT or 72NS, all at 8 µmol/l final concentration. After recovery, cells were stained for γH2AX (red) and PCNA (green diffuse and punctate) to identify activated cells and viewed under confocal microscopy at 20× magnification.

Replication fork progression is inhibited significantly by the presence of 72NT in the cell, but less so by 72NT-U

Based on the data presented above, one would predict that an enhanced level of DNA damage response and replication stress induced by 72NT might account, in part, for the observed genotoxicity in targeted cells. This outcome might arise from the cessation of movement or stalling of replication forks as a function of the presence of 72NT. To measure the status of replication fork activity, we analyzed BrdU incorporation in treated cells by flow cytometry. Synchronized cells were released for 4 hours, at which time an ODN, 72NT, or 72NT-U, was delivered into the cells by electroporation; BrdU incorporation was then measured at 24, 48, and 72 hours, respectively. This assay provides a view of the number of actively replicating forks; it is not a measure of the number of cells in S phase per se. Here, we used a FlowCollect Biovariate Cell Cycle kit (Millipore, Temecula, CA) which identifies cells bearing actively replicating forks. The results are presented in Figure 6. In the untreated control panel, we observed a standard cell distribution with each phase of the cycle represented and S phase quantitated at ~12%. The addition of 72NT causes, over the time course of the experiment, a virtual loss of any cells bearing actively replicating forks.
Figure 5  Correction efficiency and DNA damage effects of the integrated enhanced green fluorescent protein (eGFP) gene as analyzed by flow cytometry and pulsed-field gel electrophoresis (PFGE). (a) HCT116-19 cells were synchronized with 2 µmol/l aphidicolin for 24 hours prior to electroporation with 2, 4, or 8 µmol/l of 72NT or 72NT-unm. After a 24-hour recovery period, cells were analyzed via flow cytometry analysis with propidium iodide being used to determine cell viability levels. Correction efficiency (CE) was determined as the percent of GFP positive cells out of the live cell population. *P < 0.05. (b) PFGE analysis of HCT116-19 cells treated with 2, 4, or 8 µmol/l 72NT or 72NT-unm for 24 hours. CPT (300 nmol/l for 24 hours) treatment is used as a positive control. The top and bottom of the gel are indicated as is the position of the loaded well. (c). Fluorescence-activated cell sorting (FACS) analyses of H2AX phosphorylation with samples of HCT116 cells treated with 30 nmol/l (CPT) or not treated (NT) or electroporated without DNA oligonucleotides (ODN). Electroporation only, also serves as a negative control. (D). All cell samples were treated with 8 µmol/l 72NT or 8 µmol/l 72NT-U, respectively. Cells were recovered for 16 hours or 24 hours in a 48-well plate. Cells were harvested by trypsinization, fixed, permeabilized, and stained with pH2AX, antibodies respectively and DNA damage analysis carried out by FACS. Phosphorylation was measured by the shift, to the right, in the histograms. The number in the upper right hand corner of each panel indicates the percentage of cells scoring positive for activation or staining with the H2AX-r antibody.
At 48 hours, the cells are piling up in G1 and by 72 hours, the total viable cell number has actually diminished dramatically; large percentages are nonviable at this time point. In contrast, cells treated with 72NT-U display a more modest reduction in the number of actively replicating DNA forks. There is clearly a detrimental effect with both ODNs, but cells treated with 72NT-U appear to induce a slower progression into G2/M but a significant number of cells remain active for replication. Thus, it appears that 72NT impacts active replication fork activity more dramatically than 72NT-U, perhaps by inducing replication fork collapse and ultimately double-stranded DNA breakage (see Figure 5b).

**Discussion**

We and others have demonstrated that single- and double-stranded DNA breaks appear as a function of ODN-directed gene editing, raising concern about secondary effects and collateral damage in the genome.\(^5\,^6\) We demonstrate here that transient double-stranded DNA breaks appear during the gene editing reaction; breakage is seen to arise independent of the homology to the target site. These data suggest that, in general, ODNs activate the DNA damage response pathway and inhibit the progression of cells through S phase. Chromosomal breakage occurs on a global level, but the extent of cellular disruption appears to be related to chemical modifications.

Previous data suggest that certain proteins, important in the gene editing reaction, are activated during S phase.\(^6\,^8\,^10\) Correction efficiencies are higher in replicating cells than in cells not allowed to progress through S phase.\(^10\,\,13\,\,25\,\,34\) Proteins involved in homologous recombinational repair regulate the overall reaction with regard to the degree of correction.\(^5\,\,32\,\,37\) In contrast, proteins involved in nonhomologous end joining, appear to have little direct effect on the correction mechanism.\(^30\) These data, along with evidence of ODN integration into the genome,\(^14\,\,15\) suggest a mechanism wherein the ODN aligns in homologous register with its target sequence during replication. Once incorporated, the mismatch repair system addresses the mismatched base pair through recognition by
MSH2/MSH6/MLH1 etc. This complex signals the nuclease ExoI to remove a short stretch surrounding the mismatched base pair. A modified ODN would be resistant to removal by ExoI, thereby causing fork movement to be slowed or stopped. Under these circumstances, fork collapse can occur and a double-stranded DNA break can then ensue. Our previous data suggest that some of these breaks are repaired and the modified ODN incorporates into the double helix resulting in a new nucleotide in the genome. In the specific reaction, the degree of breakage seems to be related to the amount of 72NT that is designed to anneal to the target site. Dilution experiments show that ds breaks correlate to the level of specific ODN present in a gene editing reaction. This idea must be understood within the context of the HCT116 cell line used in this study. These cells lack some of the MMR proteins that form the complex initializing ExoI entry. Since PCNA and RFC are present, there may be enough degeneracy in the signals so that once the PTO-ODN aligns in homologous register, ExoI may be present to some degree to initialize nuclease activity. But again, HCT116 cells lack MLH1 and thus the attraction of ExoI to the target site could be problematic. Alternatively, other nuclease activities associated with polymerase editing functions could be involved directly with the PTO-ODN. Considering, however, that gene editing has been shown to be more active on the lagging strand of a replication fork, it is not unlikely that nuclease activity associated with the processing of Okazaki fragments could be involved in the reaction. Taken together, we now suggest that DNA damage and double-strand DNA breakage can develop as a function of the ODN interacting with and inhibiting the progression of the replication machinery.

We have used modified ODNs (phosphorothioate) because they are resistant to nuclease activity and typically provide higher correction levels as compared to unmodified ODNs. Pulsed-field gel electrophoresis analyses herein revealed that double-strand break formation is present in cells treated with either modified or unmodified ODN, but the unmodified ODN induces less breakage. This difference in DNA breakage levels can be attributed to the degradation of the unmodified ODN. If we once again refer to the Aarts and te Riele model described above, one can imagine that an unmodified ODN incorporated near or at a replication fork would be, in most cases, efficiently removed by nuclease activity from some molecular process going on in the region. Thus, fewer forks stall and only a small amount of double-stranded DNA breakage would then be generated. This removal would also account for the lower number of successful gene editing events when unmodified ODNs are used. Modified ODNs may resist removal by long enough to direct the nucleotide exchange but can concurrently cause double-strand breakage by inducing fork collapse.

Our observations of gene editing-induced DNA damage lead us to believe that replication fork collapse is the most likely cause of DNA breakage, and perhaps the overall DNA damage response. By several benchmarks used in our system, DNA damage response and localization of PCNA occur at roughly the same level as seen when either 72NT or 72NT-PM is used as the targeting ODN. This is likely due to the overwhelming number of DNA free ends that enter the nucleus when the ODN is delivered by electroporation. Free ends ignite a DNA damage response which may, by itself, cause a stalling of replication forks and a wide-spread slowdown in S-phase progression. Fork collapse and double-strand breakage is likely under these circumstances even without gene editing activity because some forks simply don’t recover after the DNA damage response pathway is activated.

Thus, we believe that two distinct pathways account for DNA damage and double-strand breakage observed as a function of gene editing. As reported previously (and extended herein), specific or nonspecific ODN can induce a DNA damage response with the activation of H2AX, etc. But, the specific ODN (here 72NT) appears to induce DSBs at a level that is higher than its nonspecific counterpart. In this paper, we suggest two origins for the evolution of DSBs. The first is a relatively succinct and elegant fork collapse induced by the homologous alignment of the ODN to the target site. Inhibition of normal MMR activity by the modified ODN leads ultimately to the stalling of fork movement followed by development of double-strand breaks. This view is supported by a recent RNAi screen by Aarts and te Riele. Using an RNAi approach, they conducted a screening of proteins implicated in the ODN-induced gene editing reaction on a mouse embryonic stem cell system. Interestingly, knockdown of TLS proteins, involved in overcoming replication stress, resulted in a considerable depreciation of gene editing. The second pathway is more global in nature, based on a mass-action effect of large levels of ODNs, which overwhelms the cell. The presence of so many DNA free ends induces a protective response in a somewhat artificial way that can also lead indirectly to DSB. Hence, there may be a specific mechanism of double-strand breakage buried under a global nonspecific reaction, which by itself can lead to collateral damage.

Clearly, replication activity is least affected when unmodified ODNs are used for gene editing, although there is some negative impact. Induction of the DNA damage response pathway and double-strand breaks do occur but appear to be more moderate. Unmodified ODNs, however, direct <50% of the amount of gene editing seen with modified ODNs, again due in all likelihood to their efficient removal from the target site after alignment and incorporation. Thus, it may come down to a choice between efficiency of correction and the induction of collateral damage, a cost benefit analysis for consideration as gene editing moves closer to therapeutic application.

Materials and Methods

Cell line and culture conditions. HCT116 cells were acquired from American Type Cell Culture (Manassas, VA). The integrated HCT116 clone 19 (HCT116-19) was created by integrating a pEGFP-N3 vector (Clontech, Palo Alto, CA) containing a mutated enhanced green fluorescent protein (eGFP) gene, as described by Hu et al.

The mutated eGFP gene has a nonsense mutation at position +67 resulting in a nonfunctional eGFP protein. For these experiments, HCT116-19 cells were cultured in McCoy’s 5A Modified medium (Sigma–Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum, 2 mmol/l l-glutamine, and 1% penicillin/streptomycin. Cells were maintained at 37 °C and 5% CO2.
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Oligonucleotide designs and eGFP targeting. ODNs were synthesized by Integrated DNA Technologies (Coralville, IA). The correcting oligonucleotide is a 72-mer that complements the nontranscribed strand of the target mutant eGFP gene. It has a centrally positioned mismatch that directs conversion of the mutant stop codon to the wild-type eGFP tyrosine, thereby allowing expression of functional eGFP. A perfect-match ODN was also designed as a 72-mer to target the nontranscribed strand, but would not induce a correction event. A nonspecific ODN bears a DNA sequence that has no homology to the target gene. Each ODN, unless otherwise noted, has three-phosphorothioate linkages on either end to help prevent nuclease degradation: 72NT-U does not have any phosphorothioate linkages.

Before eGFP targeting, cells were treated with aphidicolin for 24 hours in complete growth medium (unless otherwise noted). Cells were then trypsinized and harvested by centrifugation. Cells were resuspended to a concentration of 2.5 × 10^6 cells/ml in serum-free medium and 100 µl transferred to a 4-mm gap cuvette (BioExpress, Kaysville, UT). The respective ODN was added to a final concentration of 8 µmol/l (unless otherwise noted) and the cells were electroporated (250 V, 13 ms, 2 pulses, 1-second interval) using a BTX Electro Square Porator ECM 830 (BTX Instrument Division, Holliston, MA). The electroporated cells were then transferred to a 100-mm dish and allowed to recover in complete growth medium for 24 hours (unless otherwise noted) at 37 °C.

Flow cytometry. EGFP fluorescence was measured by a BD FACSARia II flow cytometer with FACSDiva (BD Biosciences, San Jose, CA) 24 hours after electroporation with ODN. Cells were harvested by trypsinization, washed once with phosphate-buffered saline (PBS), and resuspended in buffer (0.5% bovine serum albumin, 2 mmol/l EDTA, 2 µg/ml propidium iodide in PBS−/−). Correction efficiency was then calculated as the percentage of eGFP positive cells out of the live cell population.

Fluorescence of eGFP was also measured by FACS analysis using a Guava EasyCye SHT Flow Cytometer (Millipore). Cells were harvested by trypsinization, washed once with 1× PBS−/− and resuspended in buffer (0.5% bovine serum albumin, 2 mmol/l EDTA, 2 µg/ml propidium iodide in PBS−/−). eGFP fluorescence was calculated two ways: the percentage of the total live eGFP positive population over the total live population and the percentage of the total eGFP positive population (live + dead) over the total cell population. Error bars are produced from three sets of data points generated over three separate experiments.

Analysis of DNA damage activity. HCT116-19 cells were synchronized with 6 µmol/l aphidicolin for 24 hours, released for 4 hours and targeted with ODN (with electroporation) allowed to recover in a 48-well plate in complete growth media for specific amount of time. Cells were then treated with the FlowCollect DNA Damage Kit (Millipore) specific for phosphorylated Histone H2AX. It uses a fluorescently labeled antibody optimized for analysis using flow cytometry. Briefly, 1 × 10^6 cells were harvested by trypsinization, washed with 1× wash buffer, fixed for 20 minutes on ice, washed again with wash buffer, and permeabilized for 20 minutes on ice. After permeabilization, 200,000 cells were transferred to a V-bottom 96-well plate, washed with assay buffer and then resuspended in 85 µl of assay buffer. The antibody (γH2AX) was added (5 µl) to the cells and allowed to incubate for one hour at room temperature. After incubation, the cells were washed with assay buffer and then resuspended in 200 µl of assay buffer and analyzed on a Guava EasyCye SHT Flow Cytometer (Millipore). The position along the x-axis correlates to the amount of phosphorylated H2AX, with a shift further to the right signifying a positive signal—phosphorylated H2AX. The percentage (out of total population) of phosphorylated H2AX is determined by the gating imposed by the positive and negative controls (nontranscribed and CPT, respectively). Anything under the right gate (R5) is considered to be positive and everything to the left is considered to be negative, according to the manufacturer.

Immunofluorescence. Electroporated cells were immediately plated in 8-well chambers (100,000 cells/well) and allowed to recover for 20 hours. Control cells were treated with 2 mmol/l hydroxyurea, which is known to cause replicated fork collapse and double-strand breaks exclusively in S phase for 20 hours. γH2AX and PCNA: Cells were then washed for 5 minutes at 37 °C in PBS. Cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature. After washing with PBS, cells were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. Cells were incubated in 1% normal goat serum for 10 minutes and then for 30 minutes in a 10% milk solution. γH2AX and PCNA antibodies were added at a dilution of 1:100 in 10% milk and kept at 4 °C overnight. γH2AX: Cell Signaling, Boston, MA; PCNA: Santa Cruz Biotechnology, Santa Cruz, CA). The next day, cells were washed four times for 10 minutes each in 0.1% Triton X-100. The secondary antibodies were then added for 1 hour at room temperature (γH2AX: α-rabbit, goat-Cy3; PCNA: α-mouse, goat-Alexa Fluor 488). Cells were again washed four times for 10 minutes each in 0.1% Triton X-100. Cells were visualized with a BioRad/Zeiss MRC 1,024 on an inverted Nikon Diaphot 300 (Nikon, Tokyo, Japan).

Pulsed-field gel electrophoresis. Targeted cells were allowed to recover for specified times in 60 mm dishes. Cells were harvested by trypsinization and 1 × 10^6 cells were isolated by centrifugation at 1,500 r.p.m. for 5 minutes. Cells were washed once in PBS and resuspended in 50 mmol/l EDTA. Cells were combined in a 1:1 ratio with 1% low melt agarose ( Gibco; Invitrogen, Carlsbad, CA) in 50 mmol/l EDTA and transferred to plug molds. Plugs were allowed to cool at 4 °C for ~30 minutes before being transferred to lysis solution (50 mmol/l EDTA, 1% N-lauroylsarcosine, 1 mg/ml proteinase K). Cells were kept in lysis solution at 50 °C for 24 hours while shaking. Plugs were then washed four times in 1× TE buffer before being inserted into a 1% pulsed field certified agarose gel (Bio-Rad, Hercules, CA). The gel was run for 24 hours using a 120° field angle, 60–240 s switch time, 4 V/cm at 14 °C. The next day the gel was stained for 1 hour in ethidium bromide prior to imaging on an Alpha Innotech Fluorchem Q (Cell Biosciences, Santa Clara, CA).

Measurement of BrdU incorporation by flow cytometry. HCT116 cells were synchronized with 6 µmol/l aphidicolin

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for 24 hours, released for 4 hours and targeted with 6 µmol/l 72NT, 72NT-U by electroporation and allowed to recover in a 6-well plate in complete growth media for specific amount of time. Cells were then treated with the FlowCeltect Bivariate Cell Cycle Kit for DNA Replication Analysis Kit (Millipore). This kit identifies cells undergoing replication in S phase of the cell cycle by employing a directly conjugated Anti-BrdU Alexa Fluor 488 antibody plus propidium iodide, a DNA-binding dye. This combination allows for the bivariate detection in two dimensions without the need for software modules and, therefore, can follow labeled cells through the cell cycle. Briefly, after recovery, cells were labeled with 1× BrdU for 60 minutes and then harvested by trypsinization followed by centrifugation, washed with 1× wash buffer, and fixed for 20 minutes on ice. After fixation, 250,000 cells were washed again with wash buffer, transferred to a V-bottom 96-well plate, and permeabilized for 20 minutes on ice. The cells were washed with assay buffer and then DNase I was added at a concentration of 300 µg/ml and incubated for one hour at 37 °C. The DNA denaturation reagent was removed by centrifugation and then washed with assay buffer. The cells are then resuspended in 95 µl assay buffer and 5 µl of the anti-BrdU Alexa Fluor 488 antibody was added and incubated on ice for 1 hour. After the incubation, the cells were washed with assay buffer and the DNA was stained with a freshly prepared solution of propidium iodide/ RNase and allowed to incubate for 30 minutes at room temperature. The cells were then analyzed on a Guava EasyCyte 5HT Flow Cytometer (Millipore).

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