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Insights & Perspectives

Deletions of DNA in cancer and their possible uses for therapy

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Abstract

Despite advances in treatments over the last decades, a uniformly reliable and free of side effects therapy of human cancers remains to be achieved. During chromosome replication, a premature halt of two converging DNA replication forks would cause incomplete replication and a cytotoxic chromosome nondisjunction during mitosis. In contrast to normal cells, most cancer cells bear numerous DNA deletions. A homozygous deletion permanently marks a cell and its descendants. Here, we propose an approach to cancer therapy in which a pair of sequence-specific roadblocks is placed solely at two cancer-confined deletion sites that are located ahead of two converging replication forks. We describe this method, termed "replication blocks specific for deletions" (RBSD), and another deletions-based approach as well. RBSD can be expanded by placing pairs of replication roadblocks on several different chromosomes. The resulting simultaneous nondisjunctions of these chromosomes in cancer cells would further increase the cancer-specific toxicity of RBSD.

KEYWORDS
cancer therapy, deletions in cancer, replication roadblocks

INTRODUCTION

Cancers harbor DNA alterations, including missense and other mutations of protein-coding regions in several (often many) genes, as well as mutations in regulatory or other genomic segments. Some of these mutations involve DNA amplification and translocation, as well as insertions or deletions of repetitive or nonrepetitive DNA sequences. Such alterations are relatively rare in normal cells but are frequent in most cancer cells, in part because some cancer-associated mutations decrease the fidelity of DNA repair and replication, and thereby facilitate alterations (e.g., mutations in tumor suppressor genes) that can lead to a symptomatic cancer.[1–8]

Therapies that aim to kill cancer cells selectively or at least preferentially face not only the complexity of cancer-relevant mutational changes but also the ongoing process of tumor progression, driven by genomic instability and genetic heterogeneity of most cancers. Tumors that are vulnerable to chemotherapy or radiation therapy can become resistant to an initially efficacious treatment, owing to mutations that increase resistance. A few cancers, such as testicular carcinoma, Wilm's kidney tumor, some leukemias, and some lymphomas, can often be cured through chemotherapy but require cytotoxic treatments of a kind that cause severe side effects and are themselves carcinogenic.[2] Recent advances, including the use of inhibitors of specific kinases and other enzymes, the use of retinoic acid/arsenoxide, the strategy of synthetic lethality, and new approaches to immunotherapy often decelerate the growth of tumors and can be, with some patients and some cancers, either completely or nearly curative.[9–20] Nevertheless, a majority of human cancers are still incurable once they have metastasized.

In 2007, one of us proposed an approach to cancer therapy that involves homozygous deletions of nonrepetitive DNA sequences.[21] Many studies have demonstrated that all or nearly all human cancers contain hundreds and often thousands of heterozygous and homozygous DNA deletions that are absent from normal cells.[22–41] Deletions in cancer cells occur particularly often at fragile (mutation-prone) sites in the genome and also in genes that encode tumor suppressors.[31,41] A majority of deletions in human cancers are heterozygous, but the number of cancer-specific homozygous deletions is also large.[31,33,40,41]

In contrast to other attributes of cancer cells, a DNA deletion cannot revert. Consequently, deletions that are confined to a specific cancer would be a particularly suitable target for therapy.[21] However, a deletion is an "absence" and therefore cannot be targeted using conventional molecular approaches. The deletion-specific strategy proposed in 2007 comprises a plasmid-based molecular circuit whose protein components "check" a cell for the presence of a DNA sequence that had been deleted in cancer cells.[21] If that DNA sequence is detected by the circuit, the latter "concludes" that it entered a normal cell and proceeds to self-destruct. A circuit that did not self-destruct would be classed as residing in a deletion-bearing cancer cell. After an elapsed period of time (sufficient for self-destruction of circuits that entered normal cells), a toxic protein, encoded by the same plasmid, is activated and selectively kills cancer cells.[21] This circuit is sound as a formal diagram,[21] but its elaborate design, stemming from having to ascertain whether a cell contains a specific deletion, made this approach unrealistic, thus far, for a clinically relevant therapy.

Despite this difficulty, the initial idea of using cancer-specific DNA deletions as immutable signposts for therapy[21] remains worthwhile. We now propose entirely different and more direct strategies for selective killing of cancer cells that are based on DNA deletions.

REPLICATION BLOCKS SPECIFIC FOR DELETIONS (RBSD)

The method summarized below was termed *replication blocks specific for deletions* (RBSD).

1. By sequencing DNA of a specific cancer to be eliminated, one pinpoints and chooses two nonrepetitive DNA sequences of at least a few hundred bp that are present in normal cells of a patient but are deleted in cancer cells (Figure 1). Two DNA segments deleted in cancer cells can reside anywhere in the genome while satisfying the following constraints: (i) The two deleted sequences must be parts of two separate deletions, that is, they cannot be parts of a single deletion. (ii) DNA sites of these cancer-specific deletions can reside in two different but adjacent DNA replicons of a chromosome. They can also be a part of the same replicon, provided that the two deletions are not separated by an active origin of replication. Positions of cancer-specific DNA deletions vis-à-vis DNA replicons can be mapped using existing methods.[42–46] (iii) The pair of chosen DNA deletions must be present in all (or nearly all) copies of a relevant chromosome in cancer cells, which can be diploid or aneuploid (Figure 1).

Constraints 1i and 1ii ensure that sequence-specific roadblocks, once they are placed at the sites of two deletions, would delimit a long (as long as technically feasible) segment of unreplicated parental DNA between the roadblocks. A single roadblock would not yield a long segment of unreplicated parental DNA, since replication forks would converge on a roadblock from both sides. Hence two spaced roadblocks (Figure 1).

Constraint 1iii is about homozygosity of the chosen pair of cancer-specific deletions. Since the cancer-specific toxicity of RBSDS depends on deletions-specific roadblocks (Figure 1), it is important to make sure that the chosen pair of cancer-confined DNA deletions is present in all (or nearly all) copies of the relevant chromosome in a cancer cell population. This would preclude (or at least minimize) an accumulation of copies of this chromosome that lack one or both deletions. Since most cancers are genetically heterogeneous,[1,2] the requirement for homozygosity (or near-homozygosity) of two chosen deletions increases the probability that both deletions have been formed early in a cell lineage(s) that led to a symptomatic cancer.

Many, though not all, cancer-specific DNA deletions are neutral or nearly neutral vis-à-vis selection pressures that underlie the emergence of cancer from normal cell lineages.[22–41] Quasi-neutral homozygous deletions include those that occur at fragile (mutation-prone) DNA sites.[31] Other examples of cancer-specific homozygous deletions are DNA segments that encode tumor suppressor proteins. At least some cells bearing DNA deletions of this class are positively selected during tumor progression, as such deletions can facilitate an evolution of cell lineages that leads to a symptomatic cancer.[31,33,40,41] In sum, it would be important, for RBSD, to pinpoint and choose a pair of DNA deletions that are present in all or nearly all copies of a relevant chromosome in targeted cancer cells. Large numbers of DNA deletions, including homozygous ones, that are present in most cancers[22–41] should make it possible, in most cases, to identify a suitable pair of deletions despite the above constraints.

2. Using specific designs (see below), one places two sequence-specific, deletions-specific roadblocks in front of two converging DNA replication forks that move toward each other during S phase from two origins of replication in two adjacent replicons (Figure 1). The intent is to position two replication roadblocks as specifically as possible (minimizing off-target placements) at the two DNA sites of chosen cancer-specific deletions (Figures 1 and 2). (In vivo, some replication forks physically associate, forming DNA replication "factories".[44] Consequently, a description of forks moving on DNA is equivalent to descriptions of DNA segments that move relative to "immobile" forks.) As mentioned above (item 1ii), the sites of two cancer-specific deletions should reside in two different but adjacent DNA replicons of a chromosome. Two chosen deletions can also be a part of the same replicon, provided that the two deletions are not separated by an active origin of replication. Whether a candidate pair of cancer-specific DNA deletions satisfies these constraints can be ascertained by determining, using existing methods,[42–46] the positions of replication origins in cancer cells over a segment of chromosome that encompasses both deletions (Figure 1).
3. While a single-nucleotide mutation alters a DNA sequence, it does not change this sequence nearly as much as a significant (>>100 bp) deletion would. A deletion of nonrepetitive DNA joins two different and previously unlinked DNA sequences (Figure 2). A segment of the resulting junctional DNA that straddles the deletion's breakpoint can be used as a new (absent in normal cells) binding site for

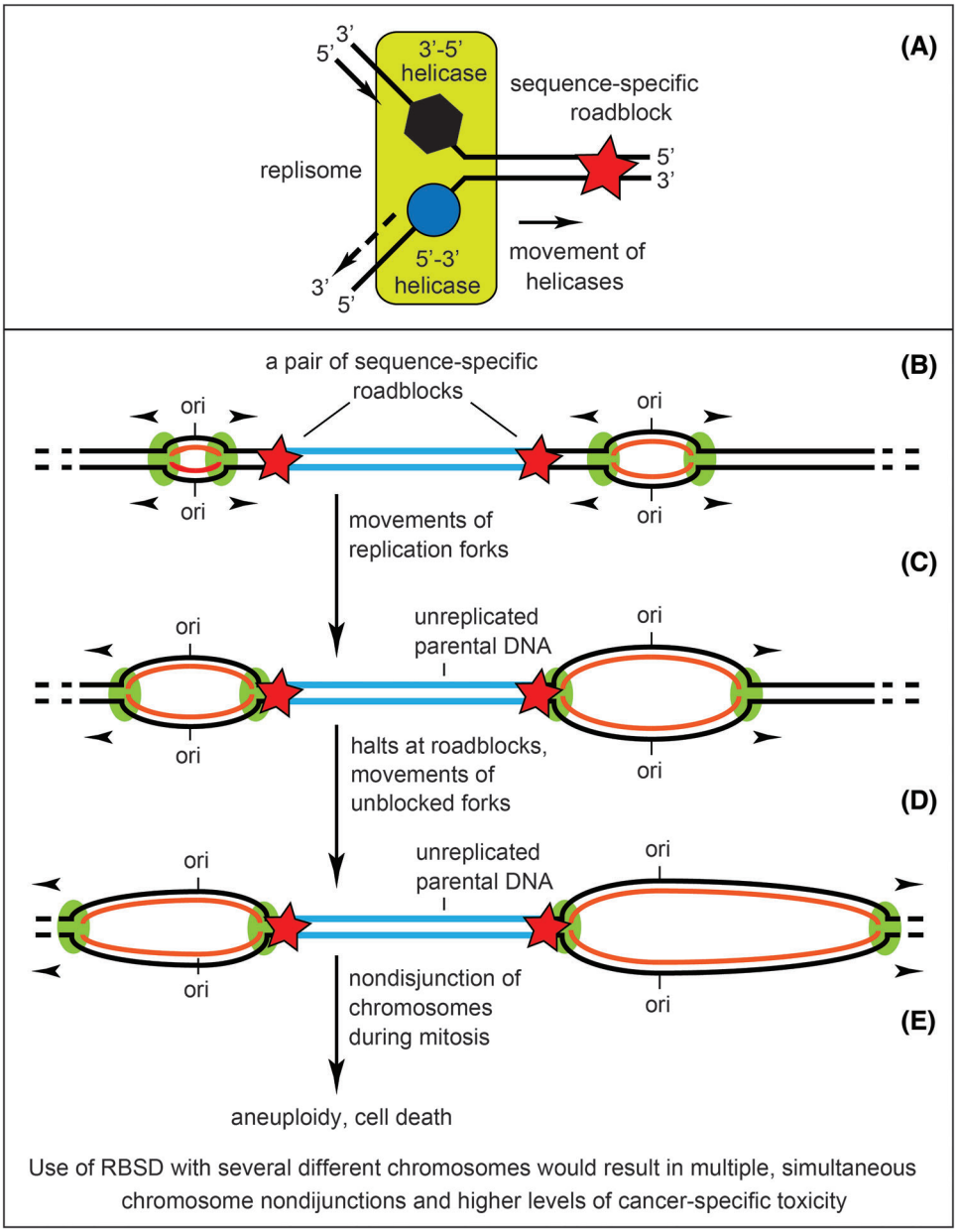

FIGURE 1 The concept of RBSD (replication blocks specific for deletions). (A) Replicative helicases, replication fork, and replisome, the latter a complex containing at least 50 dynamically associated proteins that mediate DNA replication and related processes. Only DNA helicases are shown. In eukaryotes, the main replicative helicase translocates from 3′ to 5′. The other replicase translocates from 5′ to 3′. Direction of synthesis of Okazaki DNA fragments is indicated by a dashed arrow. A sequence-specific replication roadblock (see the main text) is indicated by a red star. (B) In RBSD, two sequence-specific replication roadblocks are placed solely at two deletion sites that are specific to cancer cells and are located ahead of two converging replication forks. As described in Figure 2A and the main text, cancer-specific DNA sites for the binding of two roadblocks are present owing to two homozygous DNA deletions that are confined to cancer cells and were chosen for the placements of roadblocks. At an early stage, diagrammed in (B), two origins of replication, in two adjacent replicons, initiate the movement of four replication forks, two of which begin to approach two sequence-specific roadblocks. (C) A stage at which two converging replication forks (out of the four shown) collide with two roadblocks. (D) A stage at which two of the four replication forks continue to move, replicating DNA, whereas the other two forks are halted by two roadblocks, resulting in a segment of unreplicated parental DNA (in blue) between the roadblocks. (E) If a segment of unreplicated parental DNA persists until and during mitosis, it would lead to a nondisjunction of a chromosome. The latter would cause either aneuploidy or cell death, depending on cell types and other conditions. As described in the main text, RBSD can be expanded by placements of sequence-specific, cancer-confined pairs of replication roadblocks on several different chromosomes. The resulting simultaneous nondisjunctions of these chromosomes in cancer cells would further increase the cancer-specific toxicity of RBSD. Replisomes are denoted by green ovals. Pairs of arrowheads indicate directions of fork movements. Single strands of parental and daughter DNA are in black and orange, respectively. Unreplicated parental DNA is in blue.

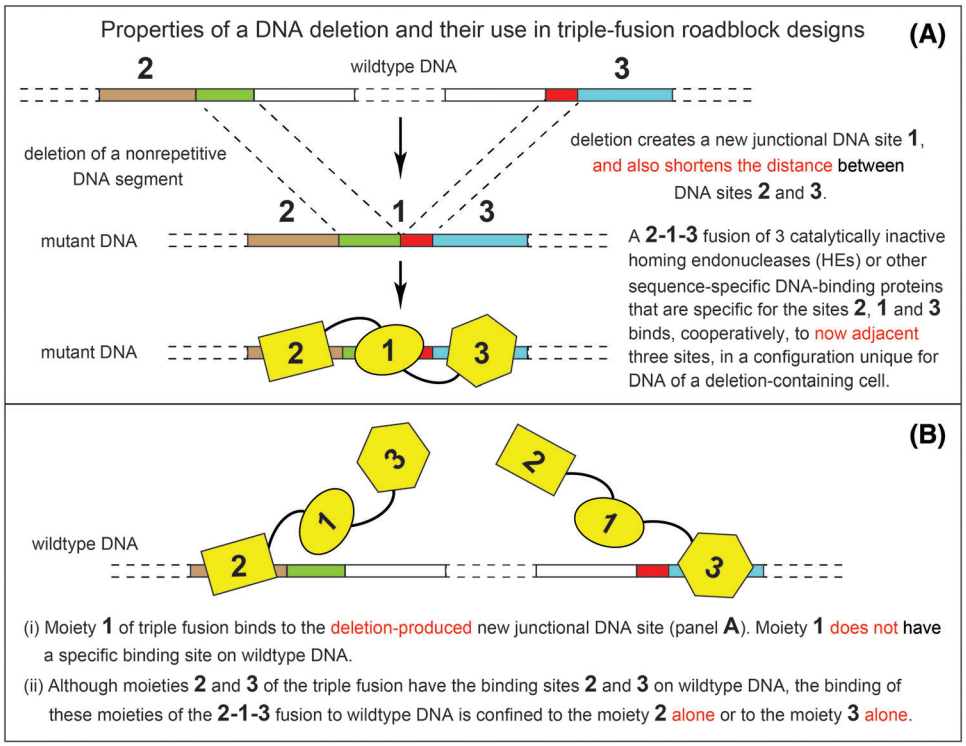

FIGURE 2 Properties of a DNA deletion and triple-fusion designs of replication roadblocks. As described in the main text, two short (~20 bp) DNA sequences that are located to the immediate left and immediate right of the ~20 bp deletion-generated junctional DNA segment become close to each other in DNA of deletion-bearing cancer cells, in contrast to a large distance between those sequences in DNA of deletion-lacking normal cells. The resulting configurational difference, characteristic of DNA deletions, makes possible triple-fusion protein designs that may result in higher-affinity (but still sequence-specific) replication roadblocks. (A) Top: DNA of deletion-lacking normal cells. The ~20 bp site-2 (brown) and the ~20 bp site-3 (blue) are far apart in wildtype DNA but become nearly adjacent upon a deletion of (nonrepetitive) DNA. The deletion also yields a new (junctional) ~20 bp DNA segment, denoted as site-1. It consists of two previously unlinked DNA segments (green and red, respectively). Segments of ~20 bp for protein-binding DNA segments were chosen because ~20 bp is a sufficient length, combinatorics-wise, for a DNA segment to contain a unique nucleotide sequence vis-à-vis the rest of human DNA (see the main text). In the mutant (deletion-derived) DNA, the sites 2, 1, and 3 (in that order, since the central junctional site is denoted as site-1) comprise a "composite" triple-segment DNA site that occurs only in cancer cells bearing this deletion. Consequently, a triple protein fusion, denoted by the linked yellow rectangle, oval and hexagon, can bind to this composite site through its three linked sequence-specific DNA-binding moieties (the main text, item 7i). The advantage of this design is a cooperativity-mediated increase of a sequence-specific DNA affinity, in comparison to otherwise identical but unlinked proteins. Possible protein components of triple fusions are mentioned in the main text (item 7i). (B) This diagram of deletion-lacking DNA in wildtype (normal) cells illustrates the fact that the above triple protein fusion, which recognizes three adjacent DNA sites in the deletion-derived mutant (cancer) DNA, would not be able to bind in the same mode to wildtype DNA, since the complete site-1 is absent there, while site-2 and site-3 are not adjacent in wildtype DNA, in contrast to mutant DNA.

a sequence-specific DNA-binding protein or, alternatively, as a site for recognition of DNA by a guide RNA (gRNA) and its associated Cas9 protein (see below). An ~20 bp junctional DNA segment is long enough for its sequence to be, in general, unique in a random DNA sequence whose length is that of the human haploid genome ($\sim 3.2 \times 10^9$ bp). While two individual halves of a deletion-generated junctional DNA segment are also present in normal cells, the latter contain these halves as *two separate, unlinked* DNA segments, whereas the contiguous junctional DNA segment would be present solely in cancer cells (Figure 2).

4. Also and importantly, two short DNA sequences that are located to the immediate left and immediate right of the ~20 bp junctional (deletion-generated) DNA segment *become nearly adjacent* in DNA of deletion-bearing cancer cells, in contrast to a large distance between those two sequences in DNA of normal cells (Figure 2). DNA deletions, DNA insertions, and DNA translocations/fusions, which bring DNA sequences to the left and right of a breakpoint close together, exhibit this feature. Its presence makes possible a distinct class of roadblock designs (see below) that use, at a single deletion site, three different sequence-specific DNA-binding proteins that recognize three adjacent DNA segments in cancer cells, whereas no such DNA configuration is present in deletion-lacking normal cells (Figure 2).
5. A nondisjunction of a specific chromosome would cause aneuploidy and can be lethal, but not always so.[47,48] This would present a problem for a single-chromosome RBSD. However, RBSD can be expanded by placements of cancer-confined, deletions-specific pairs of replication roadblocks on different chromosomes,

as many of them as necessary and technically feasible. The resulting simultaneous nondisjunctions of several different chromosomes in cancer cells (but not in normal cells) would further increase the cancer-specific toxicity of RBSD. To our knowledge, there is no experimental evidence, at present, that directly compares cytotoxicity of a single-chromosome mitotic nondisjunction to cytotoxicity of simultaneous nondisjunctions of several different chromosomes. Given the current understanding of aneuploidy,[47,48] nondisjunctions of several chromosomes are expected to be much more toxic to a cell than a nondisjunction of a single chromosome.

6. The aim of RBSD is to preclude, through roadblocks at the sites of two cancer-specific deletions, the replication of a parental DNA segment between the roadblocks (Figure 1). The length of an unreplicated parental DNA segment, that is, the distance between two sequence-specific roadblocks, should be as large as technically feasible. As mentioned above (item 1i), the use of a single roadblock would not yield a long segment of unreplicated parental DNA, since replication forks would converge on a single roadblock from both sides.

A mammalian replication fork, mediated by a replisome complex, is a transient and dynamic association of DNA and at least 50 different proteins, including DNA polymerases, primases, and helicases.[45,49–54] Many proteins of a replication fork deal not with DNA replication per se but with problems that a fork must solve in order to initiate and complete its movement from an origin to a terminus. In addition to sites of DNA damage (covalent modifications of DNA) that a moving fork must deal with, there are also encounters with noncovalently DNA-bound proteins and RNA. These include the histones of nucleosomes, DNA-associated transcriptional regulators, and transcribing RNA polymerases. Replication forks are known to bypass these and other naturally occurring roadblocks, usually with transient pauses in movement. Molecular mechanisms of bypasses are known only in part.[51,52,55–59]

The envisioned pair of cancer-confined, deletions-specific roadblocks would prevent the replication of a parental DNA segment between the roadblocks (Figure 1) and would also allow mitosis, thereby leading to a nondisjunction of either one chromosome or several of them, if several pairs of roadblocks are employed. Anticipated difficulties in achieving this aim include the requirement for a "sufficient" sequence specificity and robustness (including physical durability) of roadblocks. Other problems to solve in designing RBSD-suitable pairs of roadblocks (in addition to the delivery problem; see below) stem from a probable usefulness or, possibly, outright necessity of downregulating S-phase and mitotic checkpoints that deal with stresses caused by unreplicated DNA.[49–52,56–62]

A replisome of a replication fork contains, in particular, a 3′−5′ DNA helicase called CMG (Figure 1A). When a fork runs into a roadblock, the replisome can be stably stalled on DNA during the interphase, in that a halted fork is able to resume DNA synthesis once a roadblock had been removed or bypassed. However, if a roadblock persists, an eventual upregulation of a cyclin-dependent kinase that induces mitosis would also activate, directly or indirectly, the TRAIP E3 ubiquitin ligase. Activated TRAIP polyubiquitylates the fork-associated CMG helicase, causing its disassembly by the Cdc48/p97 ATPase.[49–52,56–62] These reactions cause a collapse of replication fork and other transitions that can include DNA rearrangements.[49,61,62] Given these and other properties of checkpoints, achieving the aims of RBSD (Figure 1) would require not only pairs of roadblocks specific for pairs of cancer-confined DNA deletions but also, likely (though not necessarily), a way to downregulate specific checkpoint reactions during mitosis and chromosome segregation. Interestingly, an "opposite" strategy can also be explored, the one that would upregulate the activity of a checkpoint that increases the probability of cell death upon a detection of unreplicated parental DNA.

7. Described below are outlines of RBSD designs. We do not consider here ways to downregulate (or upregulate) cellular checkpoints that are relevant to unreplicated parental DNA, not only because such measures might prove unnecessary but also because the construction and testing of deletions-specific roadblock pairs must be dealt with before anything else.

7i. *Pairs of RBSD roadblocks that use triple fusions of sequence-specific DNA-binding proteins.* A distinct feature of a DNA deletion is the presence of two previously unlinked DNA sites, to the immediate left and immediate right of an ~20 bp junctional DNA site, that become nearly adjacent upon a deletion (item 4 and Figure 2). In RBSD designs of this class, three sequence-specific DNA-binding proteins that recognize the three adjacent DNA segments can be joined through short linker peptides, yielding a triple fusion (Figure 2). An advantage of this design is a cooperativity-mediated increase of its sequence-specific DNA affinity, in comparison to unlinked protein moieties. The triple-site configuration of DNA sequences at a site of a cancer-confined deletion is absent in normal cells, which lack the deletion. Consequently, while a triple fusion-based replication roadblock would engage all three of its DNA-binding moieties at a single (deletion) site in cancer cells, only one such moiety at a time would be specifically bound to DNA in normal cells (Figure 2).

Possible components of envisioned triple-fusion roadblocks (Figure 2) include homing endonucleases (HEs), also called meganucleases.[63] Catalytically inactive (mutant) versions of these relatively small (<30 kDa) proteins can recognize ~20 bp long specific nonpalindromic DNA sequences through their binding largely to the minor DNA groove.[63] Previously developed methods have made it possible to produce HEs that recognize almost any predetermined ~20 bp DNA sequence,[64] a prerequisite for RBSD designs.

Other choices of DNA-recognizing protein moieties in triple-fusion designs (Figure 2) include zinc-finger proteins, which can be made to order for recognizing a predetermined ~20 bp sequence,[65] and transcription-activator-like effectors (TALEs), which can also be constructed to recognize a predetermined ~20 bp sequence.[66] In contrast to HEs,[63] zinc-finger proteins and TALEs recognize DNA sequences largely through the major DNA groove. Any one of these proteins (catalytically inactive HEs, zing-finger proteins, TALEs) can be chosen for testing as moieties of triple-fusion roadblocks (Figure 2).

The aim, in all triple-fusion designs, is to maximize the efficacy of a sequence-specific roadblock through DNA affinity enhancements that are afforded by features of DNA deletions mentioned above (Figure 2). It should be mentioned that designing and testing triple fusions of these type as replication roadblocks would be worthwhile not only because of RBSD (Figure 1) but also because an efficacious sequence-specific roadblock would be of value as a tool for studies of DNA replication, chromosome segregation, and associated checkpoint pathways.

7ii. *Pairs of RBSD roadblocks that use CRISPR-Cas*. This technology is based on the ability of a conditional endonuclease Cas9 (or other Cas proteins) to be "guided" to a predetermined site on DNA by a Cas9-associated gRNA, whose nucleotide sequence defines the sequence specificity of a "programmable" interaction between Cas9 and DNA.[67–69] Useful derivatives of Cas9 include a Cas9 nickase, which produces a nick rather than a double-strand DNA break (DSB), and a catalytically inactive ("deactivated") Cas9, termed dCas9, in conjunction with Cas9-associated gRNAs.[67,69,70]

In 2019, Whinn et al.[71] have shown that dCas9-gRNA can be used as a sequence-specific replication roadblock in the settings of in vitro DNA replication systems that were assembled using, in particular, purified yeast (*Saccharomyces cerevisiae*) proteins. Another work, in 2021, has shown that dCas9-gRNA can act, in *S. cerevisiae*, as a transient in vivo replication roadblock; its efficacy, including its residence time on DNA, remains to be examined.[72] Both studies[71,72] used dCas9-gRNA as a tool for analyzing DNA replication or recombination, that is, these investigations viewed roadblocks in contexts unrelated to either DNA deletions or cancer therapy.

Pairs of triple-fusion DNA-binding proteins (item 7i) as well as pairs of dCas9-sgRNA roadblocks can be initially explored using *S. cerevisiae*. In both cases, a pair of conditional roadblocks (expressed from inducible transcriptional promoters) could be placed, sequence-specifically and at a variable distance between the two roadblocks, on a circular low-copy (*CEN*-based) yeast plasmid containing a single bidirectional origin of replication, followed by measurements of the fidelity of plasmid's retention during cell divisions in the presence or absence of roadblocks. Readouts would include the in vivo levels of plasmid DNA and also the levels of a short-lived fluorescent EGFP protein that can be constitutively expressed from the plasmid. A negative control is an otherwise identical plasmid (present in the same cells) that bears mutations in roadblock-targeted DNA sites and expresses a different (RFP) short-lived fluorescent protein. Since a low copy yeast plasmid contains a centromere and is cosegregated with chromosomes during mitoses, such assays can be an initial testing ground for RBSD that are free, at that stage, of delivery problems.

8. Inasmuch as pairs of sequence-specific replication roadblocks would be expected to assemble largely or solely in cells that bear pairs of chosen cancer-specific deletions, normal cells would not be expected to be harmed by entry of RBSDS components (Figures 1 and 2). Consequently, RBSD can be delivered to both cancer and normal cells. These expectations would become verifiable upon development of efficacious and sequence-specific roadblock pairs. Since RBSD involves protein-sized effectors (Figures 1 and 2), their delivery into cells (as proteins or mRNAs, or via plasmids/viruses) would have to be addressed and optimized later, if and when RBSD is shown to work in model settings that lack delivery problems.

The validity of RBSD (Figures 1 and 2) rests on the assumption, remaining to be verified, that the ability of a DNA replication fork to bypass a roadblock is far from unlimited, that is, that a pair of cancer-confined, deletions-specific roadblocks can be designed that would robustly block a pair of converging replication forks and yield a long segment of unreplicated parental DNA. A subsequent expansion of RBSD to cancer-confined pairs of roadblocks on several different chromosomes would result in simultaneous nondisjunctions of these chromosomes, thereby further increasing the cancer-specific toxicity of RBSD.

In 2017, Chen et al.[19] used a version of CRISP-Cas9 to insert a DNA cassette that expressed a conditionally toxic HSV1-tk enzyme into a breakpoint DNA sequence that resulted from a large deletion and yielded an oncogenic fusion protein. Such an insert made possible a selective destruction of HSV1-tk-expressing malignant cells.[19] We cite this advance to propose that an insertion-based strategy does not have to be confined to oncogenic fusion genes. As discussed previously,[21] deletions as targets can encompass a multitude of DNA breakpoint junctions that are specific to cancer cells. Most of these deletions are functionally neutral.

AN APPROACH SPECIFIC FOR DNA DELETIONS AND DISTINCT FROM ROADBLOCKS

Described below is yet another way to use deletions as targets. DSBs can occur during transcription, replication, or recombination, and can also be caused by stresses such as, for example, ionizing radiation.[73,74] DSBs are cytotoxic and can be lethal if not repaired. Mechanistically distinct and functionally overlapping systems that mediate DSB repair include the homologous recombination (HR) pathway and the nonhomologous end joining (NHEJ) pathway.[73,74] The usually efficacious repair of even multiple DSBs in a mammalian cell can preclude or at least minimize lethal effects of unrepaired DSBs. CRISPR-based methods for multiplex genome engineering use Cas9 and arrays of several coexpressed gRNAs to produce multiple sequence-specific DSBs in a mammalian cell.[75,76] If cell's DSB repair pathways are intact, CRISPR-mediated DSBs can cause mutations but no significant cytotoxicity. However, the disposition can change if such DSBs would be produced in a cell with partially inhibited systems of DSB repair.

The approach below, termed "*unrepaired breaks at deletions*" (UBD), would selectively target cancer cells by combining a partial inhibition of DSB repair (through the use of previously characterized small-compound DSB repair inhibitors[77,78]) and a CRISPR-mediated multiplex array of, for example, 20 different coexpressed gRNAs. (This number of simultaneously targeted genomic sites is within the range of current methods.[75,76]) Each of these Cas9-associated gRNAs would be designed to specifically hybridize, in vivo, with distinct junctional (breakpoint's) DNA sequences (Figure 2) that resulted

from 20 (pinpointed and chosen) heterozygous or homozygous deletions of nonrepetitive DNA in cancer cells of a patient that are absent in patient's normal cells. (As described above, most cancers contain hundreds and often thousands of cancer-confined DNA deletions.[22–41])

In this approach, targeted cancer cells would encounter, over a short (few hours) time interval, the stress of ~20 CRISPR-mediated additional DSBs (in addition to much fewer (<<10) of DSBs that would naturally form in such cells at the same time[73,74]), in contrast to the absence of *additional* DSBs in patient's normal cells. As described above, the CRISPR-mediated generation of (e.g.) ~20 additional DSBs would be accompanied by the presence of small-compound inhibitors of DSB repair. The levels of added inhibitors would be adjusted, in preliminary experiments, to downregulate repair of DSBs while remaining below inhibitors' cytotoxic levels vis-à-vis normal cells. (The latter would lack additional ~20 DSBs.) In this design, the additional DSBs may prove to be selectively lethal to cancer cells, given the presence of *both* additional DSBs and DSB repair inhibitors in these cells, in contrast to inhibitors only in normal cells.

While distinct mechanistically, both the RBSD (Figures 1 and 2) and UBD strategies aim at achieving selectivity for cancer cells by relying on cancer-confined DNA deletions. Experimental verification of these approaches is feasible and is beginning.

AUTHOR CONTRIBUTIONS

Alexander Varshavsky conceived RBSD. Alexander Varshavsky and Kim Lewis conceived UBD. Alexander Varshavsky, Kim Lewis, and Shun-Jia Chen discussed these strategies, with Shun-Jia Chen contributing to designs of replication roadblocks. Alexander Varshavsky, Kim Lewis, and Shun-Jia Chen wrote the paper.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data derived from public domain resources.

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