

SURVEY AND SUMMARY

Initiation of homologous recombination at DNA nicks

Nancy Maizels^{1,2,*} and Luther Davis¹

¹Department of Immunology, University of Washington School of Medicine, Seattle, WA 98195, USA and

²Department of Biochemistry, University of Washington School of Medicine, Seattle, WA 98195, USA

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ABSTRACT

Discontinuities in only a single strand of the DNA duplex occur frequently, as a result of DNA damage or as intermediates in essential nuclear processes and DNA repair. Nicks are the simplest of these lesions: they carry clean ends bearing 3'-hydroxyl groups that can undergo ligation or prime new DNA synthesis. In contrast, single-strand breaks also interrupt only one DNA strand, but they carry damaged ends that require clean-up before subsequent steps in repair. Despite their apparent simplicity, nicks can have significant consequences for genome stability. The availability of enzymes that can introduce a nick almost anywhere in a large genome now makes it possible to systematically analyze repair of nicks. Recent experiments demonstrate that nicks can initiate recombination via pathways distinct from those active at double-strand breaks (DSBs). Recombination at targeted DNA nicks can be very efficient, and because nicks are intrinsically less mutagenic than DSBs, nick-initiated gene correction is useful for genome engineering and gene therapy. This review revisits some physiological examples of recombination at nicks, and outlines experiments that have demonstrated that nicks initiate homology-directed repair by distinctive pathways, emphasizing research that has contributed to our current mechanistic understanding of recombination at nicks in mammalian cells.

INTRODUCTION

Why study nicks?

Human cells experience tens of thousands of nicks each day, formed directly by DNA damage or generated as intermediates in essential nuclear processes and DNA repair pathways. Nicks interrupt a single strand of the DNA phospho-

diester backbone and must undergo repair to regenerate an intact DNA strand. Nicks carry clean 3'-hydroxyl ends that enable them to initiate repair synthesis or undergo ligation directly. In contrast, 'single-strand breaks' carry damaged ends that require specialized clean-up prior to ligation.

The first models of genetic recombination envisioned nicks as initiating events [reviewed in (1)]. However, the potential of nicks to initiate homology-directed repair (HDR) was largely ignored for several decades. Strathern *et al.* identified the two issues that have posed continuing challenges, in a paper that directly tested the ability of targeted nicks to initiate recombination in a eukaryotic cell (2):

'Nicks are more difficult than double strand breaks to test because nicks can be healed independent of recombination by simple ligation and because one method that nicks can be recombinogenic is by becoming double strand breaks.'

The emergence of enzymes that can introduce nicks at almost any DNA sequence in a large genome has recently made it possible to systematically compare repair of nicks and double-strand breaks (DSBs) at the same sites and in the same cell types. Those experiments have demonstrated that nicks can initiate recombination via pathways distinct from those that repair DSBs. Those experiments have also demonstrated that nicks can initiate gene correction accompanied by much less local mutagenesis than DSBs—making nicks invaluable for genome engineering and gene therapy.

This review outlines experiments that have formed our current understanding of HDR at nicks. Section 1 discusses nicks and how they differ from single-strand breaks (SSBs). Section 2 identifies physiological examples of nick-initiated HDR in both bacteria and eukaryotes. Section 3 presents early experiments designed to ask whether nicks can initiate HDR in eukaryotic cells. Section 4 focuses on the mechanism of nick-initiated HDR in mammalian cells, and discusses recent experiments that have used targeted nickases to demonstrate that HDR at nicks and DSBs occur by distinct mechanisms, determined in part by the structure of the donor for repair.

*To whom correspondence should be addressed. Tel: +1 206 221 6876; Fax: +1 206 221 6781; Email: maizels@u.washington.edu

DISCUSSION

Nicks are very simple DNA lesions

The simplest nick carries a 3' hydroxyl group adjacent to a 5' phosphate, ready for religation. Nicks with this structure initiate targeted HDR for some essential physiological processes and for genome engineering, via pathways in which the 3' end primes new DNA synthesis using a homologous donor as template. DNA ends bearing 3' hydroxyls also occur as intermediates in numerous pathways that maintain or modify genomic sequence and structure (3). They are generated in the course of repair of spontaneous deamination or depurination; during repair of R-loops and transcription bubbles; and in the base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) pathways; and they occur in DNA replication at the junctions between Okazaki fragments and adjacent replication forks, and at sites of replication fork collapse and arrest. In some cases, the 3' end is separated from the 5' end by a gap from a few to many nucleotides in length.

The term single-strand break (SSB) is typically applied to discontinuities produced by environmental lesions or aberrant physiological processes that damage the DNA 5'- and/or 3'-ends. SSBs are not immediately ligatable and are in this way distinct from nicks. Mechanisms of SSB repair are well characterized [reviewed in (4,5)]. Repair occurs upon a scaffold formed by XRCC1 and ligase 3, and other factors interact with this scaffold to regulate and carry out repair (6). Mutations in XRCC1 and other SSB repair factors are frequently associated with neurological disease (7). Debilitating modifications that affect DNA ends include 3'-phosphate or 3'-phosphoglycolate termini generated by oxidative damage; blocked 3' and 5' ends resulting from faulty release of factors, such as topoisomerases that form covalent bonds with DNA as obligatory transient intermediates; and 5'-AMP termini, formed in the course of abortive ligation. Subsequent processing is determined by the nature of the end modification. Polynucleotide kinase phosphorylase (PNKP) and AP endonuclease 1 (APE1) process SSBs with 3'-phosphate or 3'-phosphoglycolate termini to generate free 3'-hydroxyl ends that can prime repair synthesis. DNA polymerase β (Pol β) removes 5'-deoxyribose-phosphate (5'-dRP) moieties and then adds a single nucleotide to generate a substrate for ligation, in a reaction that is critical to base excision repair (BER). SSBs with 5'-AMP termini depend upon aprataxin (APTX) for clean-up (4). Two structurally distinct enzymes, tyrosyl DNA phosphodiesterase 1 (TDP1) and tyrosyl DNA phosphodiesterase 2 (TDP2) cleans up proteolytic fragments of topoisomerases trapped at 3' and 5' DNA ends, respectively (8).

The penultimate intermediate in SSB repair is a free 3'-hydroxyl end, which may undergo immediate religation or prime DNA synthesis using the undamaged strand as template, resulting in no change in genomic sequence. Alternatively, this free 3' end may participate in HDR using a homologous region of DNA as donor template, replacing the original sequence of the nicked DNA with that of the donor. Because SSBs produced by environmental lesions or physiological processes are so numerous, even infrequent repair by HDR could significantly contribute to genome instability.

Physiological examples of nick-initiated HDR

The section below provides examples of apparent nick-initiated HDR in six different physiological processes including viral replication, episome conjugal transfer, antigen variation in pathogenic microorganisms, gene conversion of fowl immunoglobulin (Ig) genes and retrotransposition of human LINE-1 elements. In some of these processes, the initiating nicks are targeted by sequence-specific nickases, while in others replication stalling or DNA repair appears to produce a free 3'-ends. In some cases, the possibility that HDR depends upon conversion of the nick to a replicative DSB has not been completely excluded: even when no DSB can be detected experimentally, the caveat remains that replicative DSBs are inherently transient and may elude detection.

These biological examples—especially Ig gene conversion in fowl—provided the impetus for recent mechanistic experiments that proved that nicks can initiate HDR (Section 4). Revisiting them from the perspective of current understanding shows that the physiological systems characterized thus far use pathways of HDR in which the 3' end of the nick primes DNA synthesis using a homologous donor as template. Aspects of these pathways may be similar to the pathway of HDR used by single-stranded donors complementary to the nicked strand (cN donors) (9,10), and to the synthesis-dependent strand annealing (SDSA) pathway of DSB repair (11).

Replication-associated HDR of the E. coli F factor. The *E. coli* F factor is a circular episomal DNA molecule which is transferred to a recipient cell by rolling circle replication. Replication is initiated by a nick targeted to the *oriT* element, which serves as the origin of transfer. Transfer depends upon both the *cis*-acting *oriT* element and on *trans*-acting factors encoded by the *tra* regulon, which include a 'relaxase' that nicks the DNA at a specific *nic* site in *oriT* to generate a free 3'-hydroxyl group and a 5' end covalently bound to the relaxase (12).

A mutant *lacZ* gene can recombine with the *lacZ* gene carried on the defective λ *plac5* phage to generate *lacZ*⁺ cells. Recombination was shown to be many-fold more efficient if the mutant *lacZ* gene resided on an episome rather than on the chromosome (13). Like transfer, enhanced recombination depends upon *oriT* acting *in cis*, and the transfer factors encoded by *traI* and *traY* (the relaxase that nicks *oriT* and a relaxase stimulatory factor), but not on transfer of episomal DNA to the recipient cell (14–16). Recombination with the F factor thus appears to take advantage of the nick generated by the relaxase to initiate rolling circle replication, as shown in Figure 1A. Sequence conversion may occur if replication switches to use of a homologous template provided by λ *plac5*, and HDR then transfers that sequence to the F42*lacZ* episome, correcting the mutation.

The evidence that the nick that initiates conjugal transfer can also stimulate HDR adds another layer to the genetic plasticity of conjugal transfer systems. These systems are already known to confer considerable genetic plasticity, as transfer can mobilize genes within or even between species. Genes that specify antibiotic resistance frequently reside on episomal elements and move between cells via

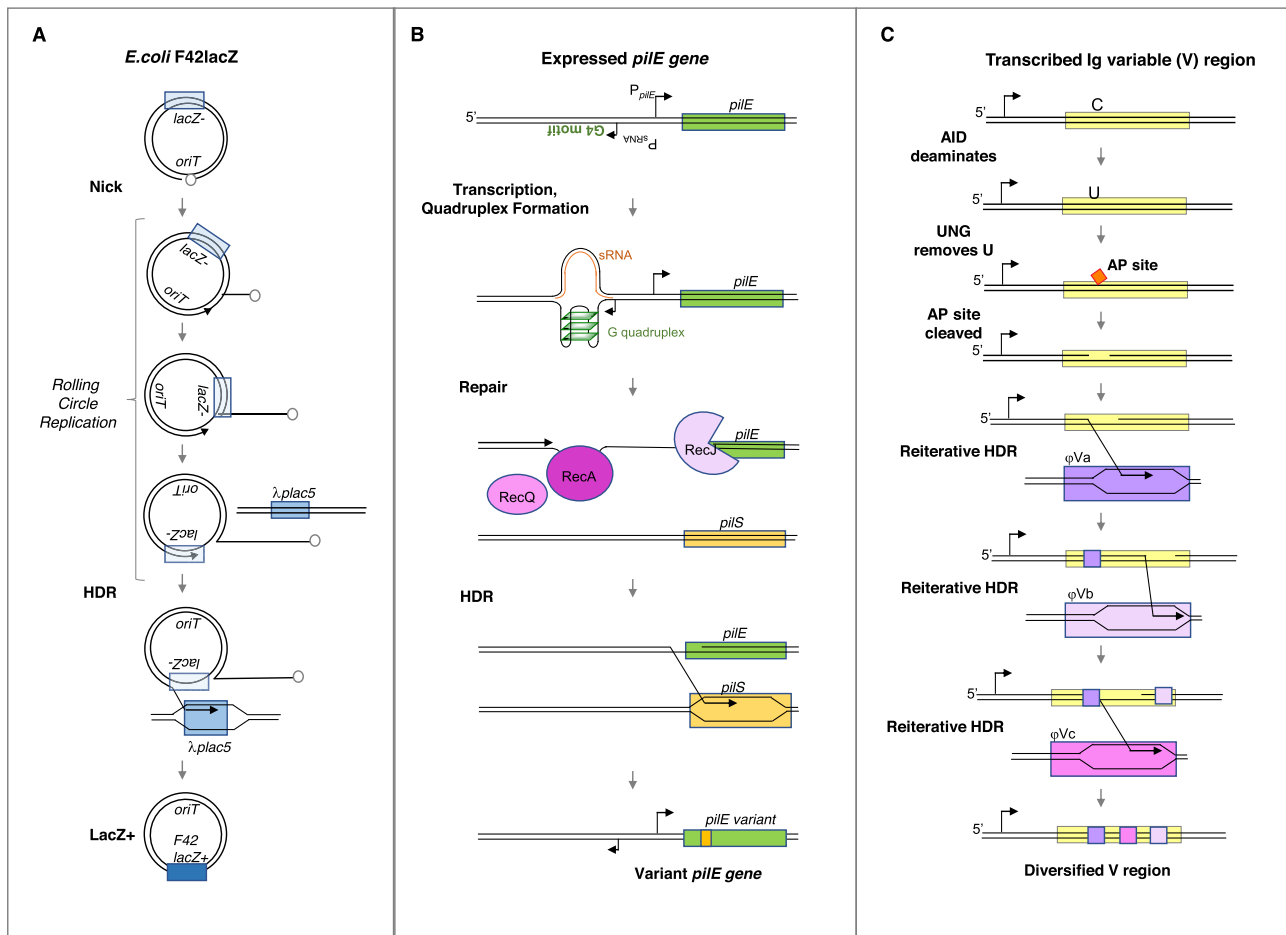


Figure 1. Nick-initiated HDR in *E. coli*, *N. gonorrhoeae* and chickens. (A) HDR repairs F42lacZ⁻ using λ plac5 as donor. Replication of the *E. coli* F factor initiates when *oriT* is nicked. The 5' end of the DNA remains bound to the enzyme that generated the nick during transfer, while the 3' end primes rolling circle replication. Recombination with a defective λ plac5 phage corrects the F factor lacZ mutation. Blue boxes, lacZ genes. (B) HDR enables immune evasion by the *N. gonorrhoeae* *pilE* surface antigen. Antigen variation requires that the *pilE* gene and an upstream G4 motif are transcribed from divergent promoters (P_{pilE} and P_{sRNA} , respectively), causing formation of a stable G-quadruplex that is bound by RecA. Repair of the structure at the quadruplex (shown) or replication arrest nicks the DNA at the quadruplex, RecQ unwinds it, and the 3' end of the replication fork traverses the region (arrowhead). HDR with a homologous *pilS* region transfers variant sequence to the expressed *pilE* gene. Indicated are *pilE* and *pilS* genes (boxes); proteins known to participate in HDR (circles); promoters (arrowheads). (C) AID initiates a nick that drives template HDR at immunoglobulin gene variable (V) regions. At the V regions (yellow) of transcribed chicken Ig genes (promoter, arrow), AID deaminates C to U, Uracil Nucleoside Glycosylase (UNG) removes U to create an abasic (AP) site (diamond), then the DNA is cleaved to generate a free 3' end that primes repair synthesis, using a pseudo-variable region donor (ϕ V, purple) as template. Repeated rounds of gene conversion using an archive of upstream ϕ V regions generate a diversified V region that is a patchwork of sequence.

conjugal transfer systems mechanistically related to the *E. coli* F factor transfer system; bacteria of the genus *Agrobacterium* use a conjugal transfer system to deliver DNA to the plant cells that serve as their eukaryotic hosts; and, while in some cases controversial, there is evidence for transfer by other bacteria to other eukaryotic hosts, including humans [reviewed in (17)].

G-quadruplex-regulated variation of the pilE surface antigen in Neisseria gonorrhoeae. The *pilE* gene of the pathogenic bacterium *Neisseria gonorrhoeae* encodes a cell surface antigen that is a target for the immune response. Antigen variation enables immune evasion, and this occurs by regulated HDR of *pilE* gene templated by homologous *pilS* donors. HDR depends upon a G-quadruplex (G4) structure specified by a G4 motif residing just upstream of the *pilE* gene,

which is essential for antigen variation, and mutations that impair formation of the quadruplex structure significantly reduce HDR (18,19). DNA nicks, and some DSBs, can be detected in the upstream region bearing the G4 motif, and mutations into the G4 motif that reduce HDR frequencies also reduced the density and distribution of the nicks. The source of the nicks may be arrest of replication at the quadruplex structure, which could generate a recombinogenic gap. Alternatively, the quadruplex could activate repair pathways that involve DNA nicking (19). The latter possibility is supported by evidence that the G4 motif is transcribed from a dedicated promoter divergent from the *pilE* promoter. Its transcription generates a small non-coding RNA (sRNA) complementary to the C-rich strand, and if the promoter for the sRNA is inactivated, or the direction of transcription is altered, antigenic variation is re-

duced (20). The quadruplex may promote persistence of a stable RNA/DNA hybrid (R-loop) between the sRNA and the transcribed C-rich strand. R-loops are resolved by two pathways in which nicks are intermediates, BER and MMR (21,22), and it is possible that HDR may leverage the free 3' ends borne by these intermediates to promote sequence variation at *pilE*.

Antigen variation depends on well-characterized repair factors: RecJ, a 5'-3' exonuclease; RecA, which promotes strand exchange; and RecF pathway proteins including RecQ, a G4 helicase (23,24). It does not depend on the RecBCD pathway that promotes HDR at DSBs (25). The model in Figure 1B shows RecJ resecting the nicked DNA, perhaps activated by the R-loop; RecA binding to the quadruplex, as *Neisseria* RecA has been shown to do at the *pilE* gene (26); and RecQ unwinding the quadruplex. Template switching, perhaps dependent on RecA, enables use of the homologous template provided by *pilS* to create a variant *pilE* gene.

What if no homologous donors were available to template repair? A possible insight is provided by analysis of nematodes, where a single quadruplex has been shown to persist through multiple generations, arresting replication to create a 3' end bordering a gap that is converted to a DSB during DNA replication (27).

Mating type switching in fission yeasts. Mating type switching in the yeast *Saccharomyces cerevisiae* uses a straightforward mechanism: the sequence-specific endonuclease encoded by the *HO* gene creates a DSB at the target *MAT* locus to initiate recombination with a homologous *mat* locus donor (28,29). This system provided the foundation for much of our understanding of DSB repair in yeast and other eukaryotes. However, endonuclease targeted DSBs may prove to be the exception rather than the rule.

Studies of two highly divergent fission yeasts, *Schizosaccharomyces (Sc) pombe* and *Sc japonica*, indicated that an epigenetic imprint at the *mat1* locus initiates mating type switching [reviewed by (30)]. While a DSB at *mat1* was reported in initial analyses, purification of intact chromosomes carrying the *mat1* locus indicated that the DSBs were artefacts generated during DNA preparation (31,32). Instead, more recent experiments have been interpreted to implicate either simple nicks (33) or strand specific ribonucleotide incorporation (34–36) as the epigenetic imprint at the *mat1* locus. Stalling of the fork progressing into this region leads to repair by the SDSA pathway (37) using sequence provided by a donor locus during recombination in G2/M phase.

DNA fragility-induced HDR promotes antigen switching in trypanosomes. The pathogenic microorganism, *Trypanosoma brucei*, is the agent of sleeping sickness. A variable surface glycoprotein (VSG) is a key target of host antibodies, and *T. brucei* evades the immune response by swapping new VSG cassettes from an archive of >1000 inactive copies into an actively transcribed subtelomeric expression site (38). DSBs were identified at the subtelomeric expression site and implicated in the mechanism of *T. brucei* antigen switching by experiments showing that switching was

stimulated upon targeted cleavage by I-SceI at a cut site introduced upstream of the transcribed VSG gene (39). However, several other models of initiation have found recent experimental support. Among these, it has been proposed that recombination reflects HDR at free 3' ends arising as a result of conflicts between the transcription and replication apparatus (40,41). This model is consistent with either a DSB or a nick as the actual initiating event.

Nicks induced by deamination initiate gene conversion at avian immunoglobulin loci. Immunoglobulin (Ig) gene diversification alters DNA sequence and structure to produce three distinctive mutagenic signatures: gene conversion (templated mutation), (somatic hypermutation (local mutagenesis), and class switch recombination (DNA deletion). Ig genes must diversify for an effective immune response, deficient diversification results in immunodeficiency.

Molecular understanding of the mechanisms of Ig gene diversification provided the first physiological example of HDR initiated by a DNA nick in vertebrates. Gene conversion is key to Ig gene diversification in fowl, which have a limited number of functional heavy and light chain V regions, and can thus produce a diverse pre-immune repertoire by combinatorial recombination using large archives of gene segments, as occurs in mice and humans (42). The donors for gene conversion are nonfunctional, 'pseudo-V' regions, located in arrays just upstream of the functional heavy and light chain V regions. Diversified V regions are patchworks of sequence from the germline V gene interspersed with tracts of sequence derived from the pseudo-V_λ donors.

Diversification is initiated by targeted mutagenesis by the cytidine deaminase, Activation Induced Deaminase (AID), which converts C to U at transcribed Ig genes (43). Cytosine deamination occurs frequently in normal cells, and the resulting uracil in DNA is rapidly and faithfully repaired by enzymes in the BER or MMR pathways, which generate nicks as intermediates. In BER (Figure 1C), uracil nucleoside glycosylase (UNG) cleaves the glycosidic bond to generate an abasic (AP) site, which can be cleaved by APE1 or by DNA lyases and converted to a nick or gap with a free 3' end by subsequent processing. In MMR (not shown), MSH2/6 promotes endonucleolytic cleavage at sites flanking the U:G mismatch followed by helicase removal of the mutated region to expose a free 3' end adjacent to a short gap in the DNA duplex. Nicks generated by either pathway can then prime DNA synthesis templated by a pseudo-V donor (Figure 1C). Nicks have been identified at the diversifying Ig_λ locus of chicken B cells by a sensitive ligation-mediated PCR assay (44).

A nick initiates LINE-1 retrotransposition. Long Interspersed Element-1 (LINE-1) retrotransposons comprise nearly 20% of the human genome. While very few LINE-1s are actively mobile, mobility can result in human disease and is a significant source of genome variation (45). LINE-1 retrotransposition is initiated by a DNA nick targeted to a consensus 5'-TTTT/A-3' site, and the 3' end of the nick then anneals to the polyA at the 3' end of the LINE-1 mRNA which serves as a template for DNA synthesis

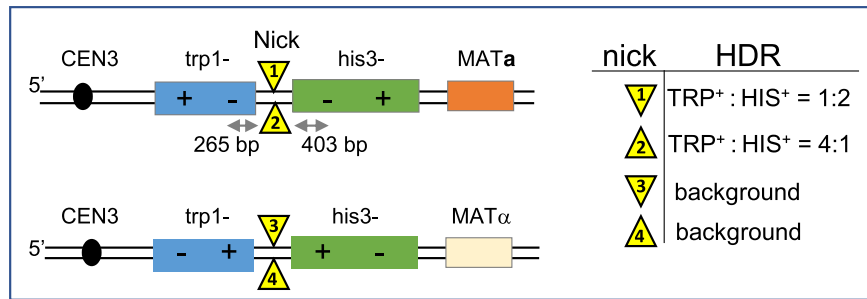


Figure 2. A targeted nick can initiate HDR in *S. cerevisiae*. Shown is a region of *S. cerevisiae* chromosome 3 engineered to carry mutant alleles of *trp1* (blue) and *his3* (green) on one chromosome and distinct mutant alleles of these genes on the homolog, with either *MATa* or *MATα* downstream [adapted from (2)]. Four strains were constructed, each containing the 32 bp recognition site for nicking by the *fl gene II* protein (yellow arrowhead) inserted into one of the strands on either homolog, and frequencies of HIS⁺ and TRP⁺ cells determined in cells expressing or not expressing phage *fl gene II* protein. The HIS⁺:TRP⁺ ratio is shown for configurations in which *fl gene II* protein expression increased recombination frequencies above background.

[reviewed in (46)]. Nicking, annealing and DNA synthesis (reverse transcription) are performed in a concerted manner by the LINE-1 encoded ORF2 protein. The resolution of LINE-1 retrotransposition, following second strand synthesis, is not well characterized but may require a second nick. Intriguingly, LINE-1 retrotransposition is inhibited by homologous recombination factors including RAD51 and BRCA2 (47). This could reflect stabilization of the nick in a fully annealed configuration by RAD51, as has been proposed for HDR at nicks supported by ssDNA donors [(10); and see below] and would inhibit mRNA annealing to the 3'-TTTT; or alternatively, RAD51 may promote competition for repair of the targeted nick by HDR with the sister chromatid or homologous chromosome.

Experimental tests of nick-initiated recombination

Prior to the emergence of enzymes that readily target cleavage to specific sites in large genomes, experiments using the phage *fl gene II* protein and nickase derivatives of the RAG proteins showed that nicks can initiate HDR, but did not address the question of whether HDR at nicks occurs by mechanisms distinct from HDR at DSBs.

Nicks initiate HDR in S. cerevisiae. Strathern and colleagues (2) provided the first direct experimental evidence that a nick can initiate HDR in eukaryotic cells, using *S. cerevisiae* strains engineered to carry a site for nicking by the coliphage *fl gene II* protein. Phage *fl* is a member of the F-specific filamentous (Ff) family of phages, which infect only male cells harboring an F factor, where they establish a chronic infection. Phage in this family, which also includes M13 and fd, have circular ssDNA genomes which replicate via rolling circle intermediates, initiated by sequence-specific nicking of a circular duplex replicative form (48). (Note the similarities with *E. coli* F factor replication, Figure 1A) The multifunctional protein encoded by phage *fl gene II* nicks the plus strand of the dsDNA replicative form, displaces the 5' end of that strand to enable the 3'-end of the nick to prime new DNA synthesis, and then cleaves and religates the displaced plus strand, circularizing the unit length DNAs for packaging and extrusion from the infected cell.

To determine whether nicks can initiate HDR, *S. cerevisiae* was engineered to carry the 32 bp recognition site for

nicking by the *fl gene II* protein on one copy of chromosome 3 between mutant alleles of *trp1* and *his3*. The homologous chromosome bore distinct *trp1* and *his3* mutations (Figure 2). Expression of the *gene II* protein stimulated HDR, increasing the frequencies of TRP1⁺ or HIS3⁺ cells from 8- to 20-fold relative to spontaneous events. Conversion occurred in both directions, but 2- to 4-fold more efficiently if the 3' end of the nicked strand pointed toward the marker. The bidirectional nature of some of these events suggests that either significant 3'-5' processing of the nick occurred prior to invasion of the homologous duplex, or that a heteroduplex formed by branch migration of Holliday junctions is resolved to generate recombinants.

The *gene II* site increased the frequencies of TRP1⁺ or HIS3⁺ cells if inserted on the chromosome carrying *MATa* but not on the corresponding site on the chromosome carrying *MATα* (see Figure 2). This reflects the positions of the mutant sequences (-) and the sequences necessary for correction (+) relative to the nick site. On the chromosome carrying *MATα*, continuous transfer of sequence from the nick will include mutant (-) rather than wild type (+) regions of the *MATa* chromosome, preventing HDR that corrects the mutations to generate wild-type alleles.

The results of this analysis showed clearly that the un-nicked chromosome served as donor and the nicked chromosome as the recipient, and that sequence at the site of the nick was not retained. This contrasts with an early model for how nicks might initiate recombination via Holliday junction-like intermediates (49). Strathern and colleagues propose a model in which the strand bearing the 3' end of the nick is transferred to a homologous region of the donor chromosome to prime synthesis templated by the donor for recombinational repair (2). Aspects of this model are similar to both the SDSA pathway of DSB repair (11) and the annealing-dependent strand synthesis pathway that incorporates sequences from single-stranded donors complementary to the nicked strand at targeted nicks in human cells (9,10), discussed below.

Initiation of recombination by RAG1/RAG2 mutant enzymes/targets. During B and T cell development, the RAG1/RAG2 complex orchestrates regulated recombination of V, (D) and J segments necessary to construct the regions that encode antigen receptor heavy and light chains

(50). RAG1/RAG2 first nick at recombination signal sequences (RSSs), then form a stable post-cleavage synaptic complex in which hairpins protect the ends of DNA segments that will be joined to generate the functional gene, and adjacent DSBs at the ends of the signal sequences promote their circularization. Mutational analysis identified a variant RSS at which the initiating nick was not efficiently converted to a DSB; and conversely, also identified RAG protein derivatives that form an unstable post-cleavage complex generating nicks but not DSBs (51,52). The RAG nickases and the nick-only RSS were shown to support recombination of a fluorescent reporter integrated in the chromosome of Chinese hamster ovary (CHO) cells (52).

Pathways of recombination at targeted nicks

Nickases make it possible to study the mechanism of recombination at nicks. Mechanistic studies of HDR at nicks have depended upon robust ‘nickases’ (Figure 3). The first of these was derived from the *S. cerevisiae* meganuclease, I-SceI, which had powered studies of the mechanism of DSB repair in *S. cerevisiae* itself and in mammals [reviewed by (53)]. Meganucleases, which are found in eukaryotes, archaea and bacteria, create DSBs at target sites 12–40 bp in length to produce recombinogenic ends bearing short 3′ overhangs that enable duplication of a mobile element by gene conversion (54). I-SceI proved very adaptable to other organisms, and DSBs targeted by I-SceI were shown to initiate HDR in tobacco cell protoplasts (55), monkey cells (56) and murine cells (57). Like many meganucleases, I-SceI is a monomer with two active sites, each of which cleaves a different DNA strand at a site within the 18 bp target (Figure 3). An I-SceI ‘nickase’ was generated by mutation of one of the two active sites, and shown to exhibit unaltered sequence-specificity (58). This provided a paradigm for generating nickases from other meganucleases, such as I-AniI (59).

Meganucleases drew some interest as possible tools for genome engineering despite the fact that the few hundred characterized meganucleases fell considerably short of the millions necessary to provide useful coverage to the size of the human genome. Some efforts focused on computational redesign that would enable cleavage of sites near high-profile disease genes, but this proved very challenging. Attention quickly shifted to other classes of enzymes that can target specific sequences in the human genome with great specificity.

Zinc finger (ZF) proteins and transcription activator-like effector proteins (TALEs) are transcriptional regulators that bind to specific sites in promoters, using modular domains to recognize 3 nt or 1 nt of DNA sequence, respectively (Figure 3). ZFs and TALEs can be readily engineered to generate sequence-specific nucleases (ZFNs and TALENs) by fusing the correct series of recognition modules to the cleavage domain of FokI, a type IIS bacterial restriction enzyme which generates DSBs with 4-nt 5′ overhangs (60–64). The resulting dimeric enzymes can be converted to nickases by mutation of the active site in one of the two FokI domains. ZFNs and TALENs were both briefly embraced by genome engineers, then largely abandoned in favor of RNA-guided endonucleases, like CRISPR/Cas, in which

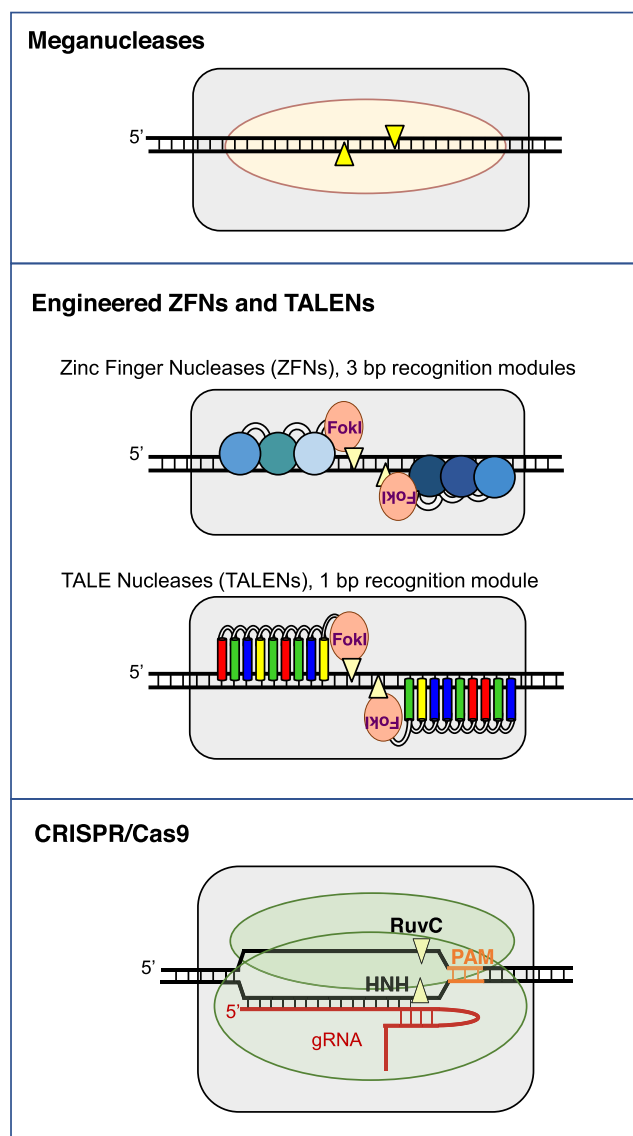


Figure 3. Engineered nickases. Three classes of enzymes have been converted to nickases by inactivation of one active site. Meganucleases like I-SceI naturally generate DSBs at target sites 12–40 bp in length. Modular enzymes, like zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs), are engineered by fusing a series of domains that specifically recognize 3 bp (ZFNs, blue balls) or 1 bp (TALENs, colored cylinders) of DNA sequence to the cleavage domain of FokI restriction enzyme. RNA-guided endonucleases, like CRISPR/Cas9, are ribonucleoproteins composed of a guide RNA (gRNA) that hybridizes to a 20–22 nt complementary target sequence bearing a 5′ protospacer adjacent motif (PAM), and a polypeptide that bears structurally distinct RuvC and HNH cleavage domains (green ovals), each of which nicks one strand of the target. Coordinated cleavage produces a DSB with blunt ends. Yellow triangles, cleavage sites.

a short CRISPR guide RNA (gRNA) directs the Cas9 polypeptide to cleave a complementary target sequence.

CRISPR/Cas systems are key components of the adaptive immune systems of bacteria and archaea. The host maintains memory of prior infections by bacteriophage and mobile genetic elements by storing sequence segments in its own genome, then transcribes these segments formatted as

CRISPR guide RNAs which hybridize to their complementary targets upon subsequent infection and cause them to be cleaved by Cas polypeptides (65).

There is considerable diversity among CRISPR/Cas systems [reviewed in (66)], but the widely used *Streptococcus pyogenes* (*Spy*) Type II CRISPR/Cas9 highlights key features of mechanism (67,68). CRISPR/Cas9 is an RNP, composed of RNA and polypeptide components. The *Spy* Cas9/gRNA RNP complex targets cleavage by hybridizing to a complementary genomic site 20 nt in length bearing a 5' protospacer adjacent motif (PAM) with the sequence NGG (Figure 3). The Cas9 polypeptide employs two structurally distinct domains to generate a DSB. The C-terminal HNH domain binds to the strand complementary to the guide RNA, causing a conformational change that facilitates binding of the N-terminal RuvC domain to the other strand (69,70). (Taking the gRNA's perspective, the two strands of the DNA duplex are sometimes referred as the target and non-target strand, respectively.) Coordinated cleavage by these two domains produces a DSB with blunt ends.

Cas9 nickase derivatives that support HDR in mammalian cells have been generated by mutation of each of the two active sites of Cas9 (71,72). The Cas9D10A nickase, with an inactive N-terminal RuvC domain, uses the HNH domain to nick the DNA strand of the RNA/DNA hybrid (the target strand). The Cas9H840A or Cas9N863A derivatives, with inactive HNH domains, use the RuvC domain to nick the non-target DNA strand (Figure 3).

Targeted nicks initiate HDR with low levels of local mutagenesis. Comparisons of mechanisms of repair at nicks and DSBs has shown that nicks can employ distinctive pathways of repair, determined by the structure of the repair donor and — in the case of single-stranded donors — whether the donor is complementary to the intact or nicked strand of the nicked DNA (Figure 4). The first direct comparison of

frequencies of HDR at nicks and DSBs targeted to the same site used derivatives of the monomeric meganuclease I-AniI (from *Aspergillus nidulans*), which recognizes a 20 bp sequence in DNA and cleaves to produce 3' overhangs. The natural cutting site of I-AniI does not appear in the human genome, so experiments with I-AniI have relied on reporters bearing an embedded I-AniI site. Some initial analyses of the outcomes of I-AniI cleavage used the DR-GFP reporter, devised decades ago (73) and still widely used to study pathways of HDR at both nicks and DSBs (74). In this reporter, homologous segments of a defective GFP gene flank the cleavage site, and nuclease expression initiates HDR by SDSA. Presence of target and donor sequences in a single construct is experimentally convenient, and their proximity may enhance HDR frequencies. More complex reporters came into use in response to growing awareness that mutations — especially insertions/deletions (indels) — could accompany targeted cleavage. The Traffic Light reporter (75), for example, carries a defective GFP gene bearing a 38 bp insert which is corrected by replacement with 17 bp of sequence from an exogenous homologous donor; and an out-of-frame mCherry gene which moves to the correct reading frame as a result of +2 indels. This enables HDR and mutagenic end-joining (mutEJ) to be scored within a single cell population by two-color flow cytometry that quantifies GFP+ and mCherry+ cells, respectively.

Repair outcomes can also be assayed by PCR or deep sequencing of the targeted regions. These approaches are advantageous if the goal is to engineer specific genes or promoters, or to optimize outcomes of gene targeting in a specific cell type. However, reporters may be preferable for studies of mechanism, as they provide a defined context in which to study contributions of a variety of factors or conditions to a repair pathway, and the high sensitivity of flow cytometry allows reproducible assays of events that may only

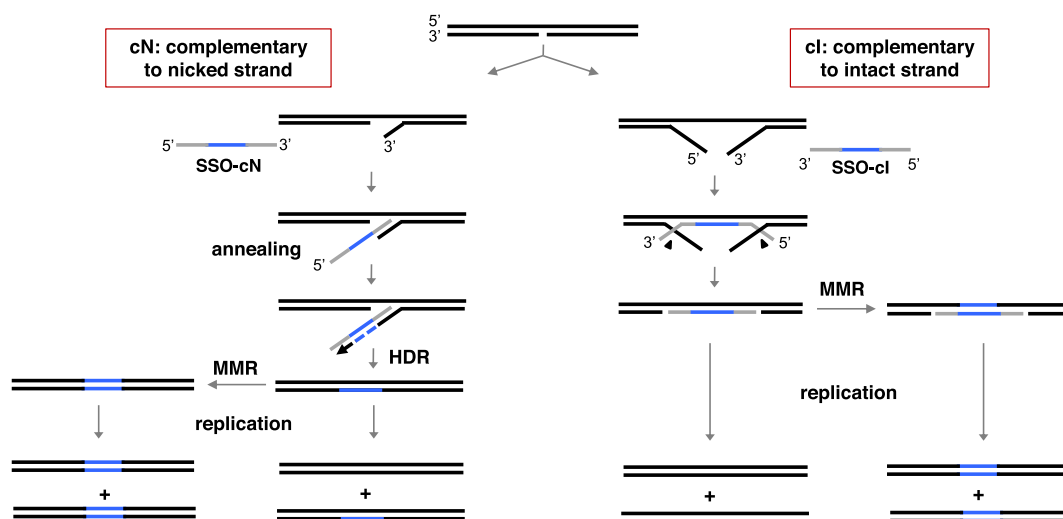


Figure 4. Strand asymmetries in nick repair. A nick can be repaired by homologous SSOs complementary to either the nicked (cN) or intact (cI) strand. In repair via the cN pathway, the 3' end of the nicked target primes new DNA synthesis using the annealed donor as template, transferring sequence exclusively in one direction. In contrast, in repair via the cI pathway, sequences on both sides of the nick may undergo conversion (9,10). Note that heteroduplex DNA is formed in both pathways. The heteroduplex can be corrected by mismatch repair (MMR) or persist and be replicated and then segregated at the next cell division.

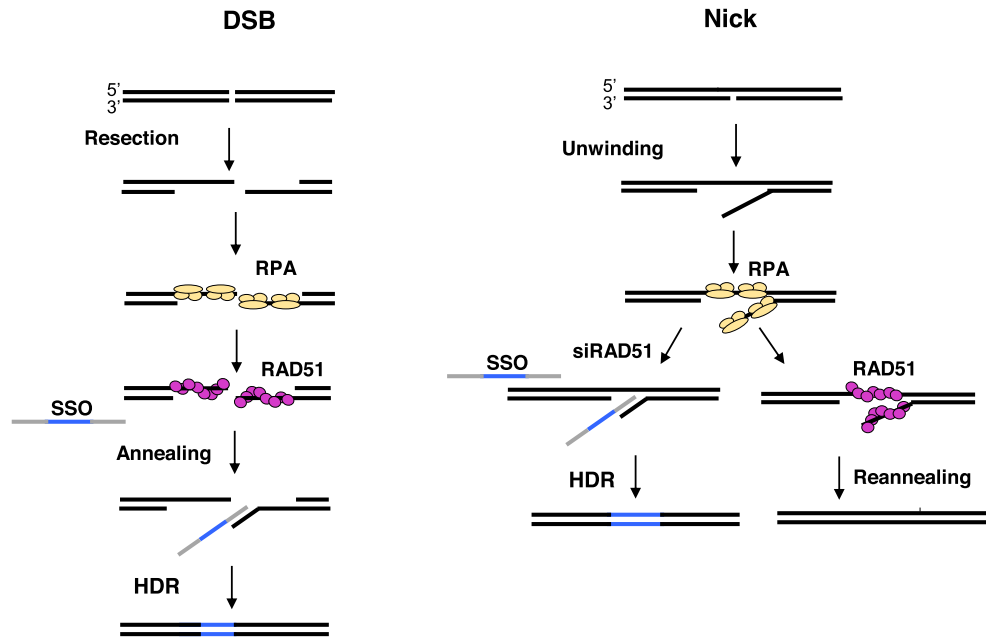


Figure 5. SSO donors use alternative HDR pathways. Left, SSO donors support HDR at DSBs independent of RAD51. While RAD51 may be present on the resected ends at a DSB it cannot promote reannealing of the (non-complementary) ends which remain available to anneal to a donor SSO. Right, in contrast, HDR at nicks is suppressed by RAD51, which may promote reannealing of complementary DNA ends and thus prevent hybridization of the SSO donors. (Shown for cN pathway only.)

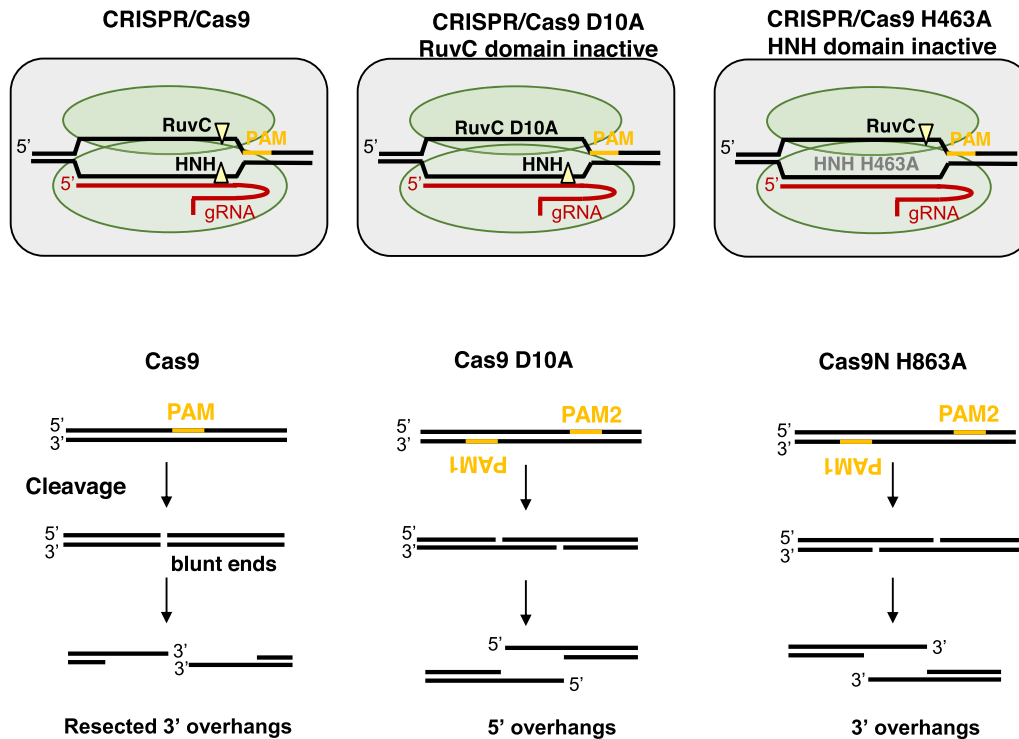


Figure 6. Paired nicks. Above, cleavage by wild type CRISPR/Cas9 generates a blunt end, while cleavage by CRISPR/Cas9 D10A or CRISPR/Cas9 H463A generates nicks on the strand annealed to the gRNA or the free strand, respectively. RuvC and HNH domains are shown; guide RNA (gRNA) red; PAM gold; cleavage sites, arrowheads. Below, paired nicks produce 5' overhangs (Cas9D10A) or 3' overhangs (Cas9H463A).

occur at low frequency to preserve the dynamic range of an assay (76).

Analysis of HDR frequencies using an episomal DR-GFP reporter in human HEK293T cells showed that nicks stimulated HDR to a level only 3-fold lower than DSBs targeted to the same site (59). Similar experiments using a chromosomally integrated reporter and an exogenous donor gave similar results (59). This established that targeted nicks can initiate HDR in human cells. The Traffic Light reporter was subsequently used to show that HDR initiated by the I-Anil nickase is accompanied by about 50-fold less mutEJ than HDR initiated by DSBs (77). Low levels of mutEJ have also been documented at nicks produced by zinc finger nucleases (ZFNs) (78,79) and CRISPR/Cas9 (71,72,80,81). The evidence that nicks are intrinsically less mutagenic than DSBs has led to considerable interest in use of nicks for genome engineering and gene therapy.

Strand asymmetries characterize pathways of nick repair. The transient nature of DNA nicks and DSBs means it is essentially impossible to prove directly that HDR at nicks does, or does not, depend on a DSB as an obligatory intermediate. Proof that nicks initiate pathways of HDR distinct from those initiated by DSBs required a different strategy.

Nicks are inherently asymmetric, as they occur on only one strand of the DNA duplex. The nicked and intact strands would be expected to interact differently with donor DNA molecules. Nicks might therefore be expected to display asymmetries in repair outcomes that are distinct from DSB repair. Strand asymmetries of nick-initiated HDR became evident when single-stranded oligonucleotide (SSO; also called ssODN) donors were used to support HDR (9,10). Very efficient HDR can be achieved using SSO donors from 70–200 nt in length, which are cheap to synthesize and easy to use. SSO donors are especially suited for genome engineering because they are short-lived and do not readily integrate into the genome.

Comparison of HDR by SSO donors complementary to the nicked (cN) or intact (cI) strand showed clear evidence of strand asymmetry. A cN donor supports HDR via a pathway in which the 3' end of the nicked target forms a hybrid with sequences at the 5' end of the donor, then primes new DNA synthesis using the annealed donor as template (Figure 4). Sequence is transferred preferentially in one direction, bounded on one side by the 3' end of the target and limited on the other by the 5' end of the donor.

The cN pathway for nick repair takes advantage of the ability of the 3' end of a nick to prime new DNA synthesis – just as in a PCR reaction. This pathway is analogous to physiological nick repair illustrated in Figure 1. A pathway analogous to the cN pathway at nicks also supports HDR by SSO donors at DSBs (10): sequence is transferred preferentially in one direction, bounded by the 3' end of the target and templated by the 5' region of the donor that remains single-stranded after formation of the duplex between the SSO and the target strand. The cN pathway shares some features with the 'template intermediate model' for repair of DSBs by SSOs in yeast (82). Genome engineering efforts that employ the cN pathway must take this directionality of transfer into account when designing SSOs to support repair.

In contrast, donors complementary to the intact strand (cI donors) can convert sequence on both sides of the nick (9,10). The mechanism of sequence conversion by the cI pathway is not yet understood. In principle, a cI donor that has hybridized to the intact strand may be incorporated into daughter molecules if unhybridized flaps are cleaved and ligation joins it to adjacent chromosomal DNA (Figure 4). If flaps formed during HDR are indeed cleaved, this suggests that repair of some SSB with blocked or damaged ends may occur by flap cleavage that would remove the damaged 5' or 3' end, permitting HDR without canonical SSB repair. It has been reported that donor SSOs used by the cI pathway can become physically incorporated into the chromosome (83), but it has not been established whether this accounts for a significant fraction of recombinants.

The HDR pathways that use cN and cI donors both involve formation of a heteroduplex intermediate. In the cN pathway, a heteroduplex will form when the newly synthesized strand releases the donor template and anneals to the complementary strand of the chromosome. In the cI pathway, a heteroduplex will form upon annealing of the donor to the target. In either case, the heteroduplex can be corrected by MMR; and if not corrected it may persist and undergo replication, allowing segregation of the new sequence upon cell division.

MMR is not required for HDR at nicks, which is active in MMR-deficient cell lines, including the embryonic kidney derived cell line HEK293T (9,10) and the HCT116 colon cancer line (83). The absence of Ku70 does not affect the frequency of HDR at nicks, suggesting that there is no obligatory DSB intermediate (84), presuming that Ku loads on replicative DSBs.

It is possible that the cN and cI pathways differ in their dependence upon helicases that unwind the nick and endonucleases that cleave the regions of the nick exposed by unwinding or donor hybridization. However, no differences of this sort have as yet been reported. It may be that these activities are redundant and thus undetected by standard screening approaches.

'Alternative' HDR dictated by donor structure. In canonical HDR, such as HDR at a DSB by a dsDNA donor, a newly resected DNA 3' end is bound by RPA, then replaced by RAD51 to enable homology search and invasion of the dsDNA donor (85). A number of 'alternative' HDR pathways do not involve RAD51-mediated strand invasion [reviewed by (86)], among them HDR supported by SSO donors. At nicks, HDR by SSO donors requires RPA, but is stimulated dramatically (10-fold or more) upon depletion of RAD51 itself or factors that promote loading of RAD51 on DNA, including BRCA2 and its binding partners PALB2 and SHFM1; or by expression of the dominant negative RAD51K133R mutant or the inhibitory BRC3 peptide (9,10,87).

An insight into the mechanism of inhibition of HDR by RAD51 was provided by the finding that at DSBs, HDR by SSO donors is not inhibited (or facilitated) by RAD51 (10). DSBs are resected immediately after cleavage, while nicks appear to undergo unwinding rather than resection. Resection at DSBs eliminates the complementary strand and leaves an exposed 3' tail available to hybridize with

an SSO donor or invade a duplex donor, but without the possibility of re-annealing in cis. In contrast, unwinding at a nick produces a gap at which binding by RAD51 may promote re-annealing to the complementary intact DNA strand. RAD51 may thereby prevent annealing of the target to an SSO donor, thus inhibiting HDR by SSO donors at nicks (Figure 5).

Duplex circular plasmid DNA molecules can serve as donors for HDR at both nicks and DSBs. If these donor molecules are intact, HDR occurs by the canonical RAD51-dependent pathway, presumably because RAD51 is required to mediate strand invasion. HDR is stimulated if donor plasmids are nicked on either strand upon nuclear entry; and further stimulated upon inhibition of RAD51 activity or RAD51 depletion (9,88).

Thus, alternative pathways support HDR at nicks by both SSO and nicked circular donors. Alternative HDR has also been shown to occur in physiological nick repair in LINE-1 transposition, as discussed above.

Paired nicks are repaired like DSBs. The strategy of cleaving DNA by targeting nicks to closely spaced sites ('paired nicks') was based on the notion that requiring cooperative cleavage at two sites would increase specificity and diminish off-target cleavage by CRISPR/Cas9, a concern for all genome engineering and especially for gene therapy in human cells (80,89–93). This approach complemented efforts to improve cleavage specificity by engineering Cas9 based on reaction parameters or structural analysis (94–97). Paired nicks proved to diminish off-target cleavage, and to undergo repair by pathways previously described for DSB repair.

The globin locus is of considerable interest for gene therapy, as mutations in β -globin are responsible for sickle cell disease and a number of severe thalassemias, currently treated by transplant (98). The human hemoglobin beta (HBB) and delta (HBD) genes are homologous direct repeats of nearly identical sequence spaced 6 kb apart, reminiscent of the organization of target and donor sequences in the DR-GFP reporter. HDR at paired nicks targeted to the HBB gene was analyzed in human U2OS osteosarcoma cells (87) which provide a convenient model for studying targeted HDR although they do not express globin genes. Paired Cas9D10A or Cas9N863A nickases were targeted to sites spaced to generate ends 47 nt in length and bearing 5' or 3' overhangs respectively (Figure 6). If an exogenous SSO donor was provided, HDR frequencies were very similar at 5' and 3' overhangs, and at blunt ends; and HDR was independent of RAD51/BRCA2, as documented for HDR at other DSBs by SSO donors (10). If no exogenous donor was provided, there were some indels at the target site, and also clear evidence of gene conversion that used the homologous delta globin (HBD) gene located 6 kb downstream as donor. At 5' overhangs, about one-third of events involved gene conversion; while at 3' overhangs, there was essentially no gene conversion. Gene conversion depended upon RAD51 and BRCA2, as does HDR at DSBs in the DR-GFP reporter (84). Thus the structure of the donor determines whether HDR proceeds via the canonical or alternative pathways at diverged genomic loci as well as at reporters.

CONCLUSION

The ability of nicks to initiate recombination has become widely accepted. We now look forward to a growing roster of newly appreciated physiological examples of recombination initiated by nicks, and to scarless gene editing enabled by the application of nick-initiated recombination to genome engineering and gene therapy.

Future questions

The ability of nicks to initiate HDR in physiological and experimental contexts raises a number of questions to be answered in the future.

- Does recombination at nicks pose a threat to genomic stability? Are nicks that arise during normal DNA transactions a potential source of deletion, insertion or loss-of-heterozygosity?
- Do nicks induce DNA damage signaling? If so, what factors are involved? Does this occur via pathways shared with DSBs or via novel pathways yet to be characterized?
- Do frequencies of HDR and mutEJ at nicks vary through the cell cycle?
- What helicases, endonucleases and polymerases support HDR at nicks?
- What factors promote mutEJ at nicks?
- Can the frequency of mutEJ at nicks, which is normally low (1–5% the frequency of mutEJ at DSBs), be further reduced to enhance the utility of nicks for genome engineering?

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