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Review

Tools To Live By: Bacterial DNA Structures Illuminate Cancer

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Holliday junctions (HJs) are DNA intermediates in homology-directed DNA repair and replication stalling, but until recently were undetectable in living cells. We review how an engineered protein that traps and labels HJs in *Escherichia coli* illuminates the biology of DNA and cancer. HJ chromatin immunoprecipitation with deep sequencing (ChIP-seq) analysis showed the directionality of double-strand break (DSB) repair in the *E. coli* genome. Quantification of HJs as fluorescent foci in live cells revealed that the commonest spontaneous problem repaired via HJs is replication-dependent single-stranded DNA gaps, not DSBs. Focus quantification also indicates that RecQ DNA helicase plays dual roles in promoting repair HJs and preventing replication-stall HJs in an *E. coli* model of *RAD51*-overexpressing (most) cancers. Moreover, cancer transcriptomes imply that most cancers suffer frequent fork stalls that are reduced by the HJ removers EME1 and GEN1, as well as by the human RecQ orthologs BLM and RECQL4–surprising potential procancer roles for these known cancer-preventing proteins.

Highlights

HJs are DNA structures with four double-stranded arms that are formed as reaction intermediates during various biological processes, including meiosis, DNA repair, and DNA replication stalling.

HJs in cells could be a useful marker for genome instability that drives mutation and cancer, but their study has been hampered by the lack of sensitive methods to detect transient and rare HJs in living cells.

Newly developed engineered 'HJ-trap' proteins can trap, label, and allow mapping in genomes of HJs, and thus allow quantification and mapping of HJs in living cells. These engineered HJ-trap proteins have facilitated discovery of the main sources of DNA damage repaired by homologous recombination, and some surprising roles of DNA repair proteins in promoting cancer.

Engineered Proteins Trap Holliday Junctions

Four-way DNA junctions, or **Holliday junctions** (HJs, see Glossary) are central DNA intermediates in homology-directed DNA repair (Box 1A), and also form when DNA replication stalls, leading to **replication-fork reversal** (Box 1B and Figure 1, Key Figure, blue box). HJs are transient intermediates in reactions that underlie genome maintenance as well as genomic instability and evolution in all organisms [1]. Despite the centrality of HJs to universal biological processes, HJs have eluded direct observation in living cells, mostly because they are transient reaction intermediates.

An engineered synthetic protein, **RuvCDefGFP** (RDG), was created to detect HJs in living cells [2]. RDG is a catalytically defective, fluorescent-protein fusion of the four-way DNA-junction-specific **RuvC** HJ endonuclease of *E. coli* [2]. The bases that encode catalytic amino acids are substituted [2] such that RDG 'traps' HJs by binding to them and prevents other proteins from acting on them (Figure 1, center). Biochemically, RDG binds four-way DNA junctions and inhibits *Eco*RI endonuclease and Flp high-affinity site-specific recombinase/**HJ resolvase** [3] activities at the junction [2]; in other words RDG binds to and traps HJs. In living *E. coli*, production of RDG inhibits HJ processing by other HJ-interacting proteins, demonstrating HJ trapping in live cells (Box 2 for more on the bound HJ).

HJs Are Visible as Fluorescent Foci in Single Living Cells

RDG forms fluorescent foci (Figure 1, green box) that correspond to HJs from homology-directed double-strand break (DSB) repair (Box 1) in *E. coli* cells [2]. Repair of an *E. coli* chromosomal DSB produced by site-specific cleavage with **I-*Sce*I endonuclease**

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Box 1. Holliday Junctions Can Arise during Homology-Directed DNA Repair or Stalling of Replication Forks – Some of the Players

Homology-directed repair or homologous recombination is a conserved process [57] of repairing of broken DNA via the exchange of DNA strands between two DNA molecules (or regions) with stretches of identical DNA sequence. Strand exchange requires strand-exchange protein RecA in *E. coli* or its ortholog RAD51 in human. Strand exchange forms a region of **heteroduplex DNA** with strands from two different duplex DNA regions that basepair with each other (see Figure IA in Box 1, single-strand gap repair). Holliday junctions (HJs) can be an intermediate in homology-directed repair (see Figure IA in Box 1). The HJ is shown with crossed strands (left) and square planar (uncrossed) configuration (right), which illustrates the symmetry of the HJ. These repair HJs can be formed during repair of both single-strand DNA gaps (see Figure IA in Box 1) and DSBs (Figure 3A).

I-*Sce*I-induced repair HJs are formed during repair of DSBs (Figure 3A). This set of conserved molecular mechanisms requires RecA and its loader at DSBs, **RecBCD** in *E. coli*, and in human the RecA ortholog RAD51, which is loaded onto DNA by BRCA1/BRCA2 loaders at DSBs.

Most spontaneous repair HJs (those formed without exogenous or induced DNA damage) arise from repair of single-strand gaps (see Figure IA in Box 1) from DNA replication [2]. Figure 3B shows RecQ DNA helicase and RecJ single-strand-dependent DNA exonuclease promoting spontaneous repair HJs. RecQ and RecJ act before RecA strand-exchange protein, shown in living *E. coli* [2]. RecA and its loader at single-strand gaps, RecF, are required for the formation of most spontaneous repair HJs [2] (Table 1). RuvC, as well as RecG, a HJ branch-migrating DNA helicase, reduce steady-state levels of spontaneous HJs in cells (visualized as RDG fluorescent foci) by removing HJs [2].

HJs are also formed without homology-directed repair when replication forks stall and reverse. Fork reversal (B) occurs upon stalling because supercoils accumulate (from DNA unwinding) ahead of the fork and push back, reversing the fork (B).

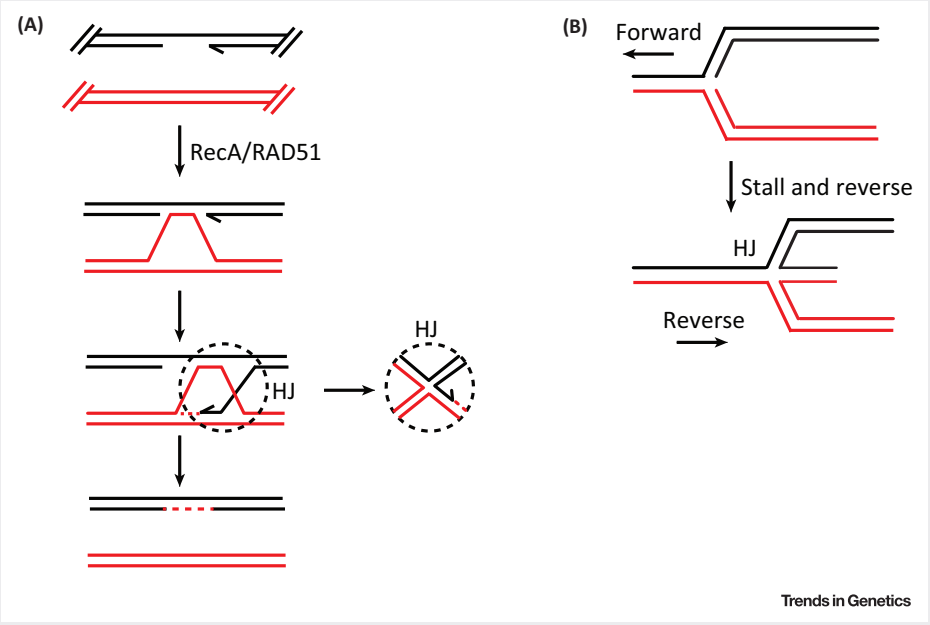

Figure I. Holliday Junctions from Single-Strand Gap Repair (A) and Replication Fork Reversal (B). (A) HJ generation by strand exchange. (B) HJ generation by replication fork reversal. Key: dashed lines, new DNA synthesis; half arrowheads, 3′ ends; lines, DNA strands.

(Figure 1, green box; Figures 2 and 3A) increased the number of cells with RDG foci ~10-fold above those of uncleaved control cells. Further, cleavage near the chromosomal replication origin, which has multiple DNA copies during exponential growth, caused more foci per cell than cleavage near the **replication terminus** (fewer DNA copies) [2]. These observations correlate RDG foci with the numbers of DSBs initiating repair with available

Glossary

Chi site: the DNA sequence 5′-GCTGGTGG-3′. Chi sites stop *E. coli* RecBCD exonucleolytic destruction of DSB ends and trigger RecBCD-mediated loading of RecA onto the resulting single-stranded DNA, which promotes strand exchange and DSB repair [4,5].
ChIP-seq: chromatin immunoprecipitation and deep sequencing. A technique to identify the DNA targets of DNA-binding proteins in cells.
Heteroduplex DNA: double-stranded DNA regions in which a DNA strand derived from one molecule or region is aligned and basepaired with its complement in a different DNA molecule or region. Heteroduplex DNA allows homology-directed repair to be 'homologous', holding together the crucial strand-exchange intermediate.
Holliday junction (HJ): a four-way DNA junction (illustrated in Figure 2C, D) in which four duplex DNA arms are joined at a central, symmetrical crossroad. Named for Robin Holliday, Holliday junctions were proposed as intermediates in homologous recombination via heteroduplex DNA [62].
Holliday junction resolvase: any of many proteins or protein complexes that remove four-way junctions from DNA, 'resolving' them to duplex DNA.
I-*Sce*I endonuclease: a site-specific double-strand DNA endonuclease that cleaves a specific 18 bp sequence [63] that is absent from most genomes.
PARP-inhibitor drugs: chemotherapeutic drugs that target poly(ADP-ribose) polymerase (PARP) and are used to kill cancer cells that lack homology-directed DSB repair. These include *BRCA*-deficient breast and ovarian cancers [64].
RecA: a bacterial strand-exchange protein required for homology-directed repair [4,5], orthologs of which function similarly in all species examined.
RAD51: a human RecA ortholog that performs strand exchange.
RecBCD: an *E. coli* DSB end-specific exonuclease (Exo V) that digests DNA using its helicase activity to unwind DNA from a DSB end and its two endonuclease

repair templates. The DSB repair proteins **RecA** and **RecB**, which promote HJ formation [4,5], are required for I-*Sce*I-induced RDG focus formation (Figure 3A and Table 1) [2], supporting the interpretation that DSB-induced RDG foci identify HJs generated by homology-directed DSB repair. In addition, RDG foci correlated with repairable DSBs produced by gamma rays, with an estimated 50% efficiency [2].

DSB Repair Captured by HJ-ChIP-Seq Is Directional in the Genome

The landscape of HJs in the *E. coli* genome was mapped by RDG chromatin immunoprecipitation with deep sequencing (**ChIP-seq)** in cells undertaking homology-directed repair of a DSB. Single site-specific DSBs were induced by I-*Sce*I site-specific double-strand endonuclease at single engineered chromosomal sites [2] (Figure 1, orange circle). Proliferating cells have multiple chromosome copies and therefore can repair after DNA cleavage using an uncleaved sister chromosome as the template [6,7]. The map shows enrichment of RDG-bound DNA (HJs) near the induced DSB and downstream of it in the unidirectional replication path of the chromosome [2], as well as at the replication terminus region [2] (Figure 1, orange circle, *Ter*). These data suggest two modes of whole-genome control and codirectionality of repair with replication in the chromosome (Figure 2). First, the occurrence of repair HJs at the replication-terminus region [2], megabases away from the DSB, supports a unidirectional replicative repair model [8–13] (Figure 2 and Box 3). In this model, HJs formed near the DSB during repair can sometimes be pulled behind the repair replication fork, codirectionally with normal replication, all the way to replication termination signals that are oriented to catch forks coming from the chromosome arm with the DSB (red brackets, Figure 2E). HJs pile up at those replication termination sites (Figure 2C–E). The pulse of RDG production induced might capture the HJs at their two most frequently occupied genomic spots during this process: at the site of DSB repair, and where the replisome ends its journey at the terminus. Alternatively, HJs in motion might be trapped poorly, making the beginning and end of the journey more detectable, or the terminal replication during repair might instigate DNA damage, the repair of which creates new HJs.

Second, HJs are distributed unevenly around two-ended DSBs under repair [2] (Figure 1 center, orange circle). There are more HJs downstream in the normal DNA replication path than upstream – on the replication-origin-proximal side in the chromosome [2] (Figure 1 center, orange circle). This skew supports a compelling but never-tested model [14] for control of DSB-repair replication directionality by recombination-promoting **Chi sites**, which are proposed to maintain repair replication in the origin-to-terminus direction, as described in Box 3 and Figure 2.

Most Spontaneous Repair Handles Single-Strand Gaps from DNA Replication

In late exponential-/early stationary-phase *E. coli* cultures, most spontaneous RDG foci appear to result from homology-directed repair of spontaneous DNA damage, specifically single-stranded DNA gaps [2] (Figure 1, brown box, and Figure 3B). The appearance of most spontaneous RDG foci requires the strand-exchange protein RecA, and also **RecF** [2] – its loader for repair at single-strand gaps [15,16] – indicating that most spontaneous foci arise from homology-directed repair. Although purified RecA aids the formation of reversed forks biochemically [17], RecA is not required for formation of, or **RuvABC** action on, reversed forks in living *E. coli* [18] (Table 1). However, RecA is required for homology-directed repair [4,5] (Table 1). About 75% of spontaneous RDG foci required RecA, RecF, and RuvB, supporting their origin as spontaneous repair events in single-strand gap repair [2] (Table 1). The RuvB-dependence indicates that foci occur at HJs. The origin of the 25% of RecA-independent spontaneous RDG foci might be reversed replication forks (illustrated in Figure 1, blue box, and Box 1B). Further evidence indicates that most

activities to chop the unwound strands into oligonucleotides [4,5].
RecF: an *E. coli* RecA-loading protein at single-strand DNA gaps. RecF binds to the junction between single-stranded and duplex DNA at the gap (Figure 3Biii) [4,5].
RecQ DNA helicase: the *E. coli* founding member of the highly conserved RecQ family of DNA helicases, which are important in genome stability. They translocate on a DNA strand in the 3′ to 5′ direction, displacing the unbound 5′ end of the strand.
Replication-fork reversal: when a replication fork stalls, the two nascent strands can basepair with each other, and the two unwound template strands reanneal, forming a four-way DNA (Holliday) junction (Box 1B; Figure 3C,Diii).
Replication terminus: in *E. coli*, a replication terminus (*ter*) is a unidirectional sequence (red brackets in Figure 2E) that stops replication forks that cross the chromosome midpoint and proceed past it, into the other chromosome arm, towards the replication origin (*ori*).
RuvABC: an *E. coli* HJ-resolution complex that branch migrates (moves) HJs and resolves them by endonucleolytic cleavage.
RuvCDefGFP (RDG): an engineered protein that traps, maps, and labels HJs. RDG is a catalytically defective version of the *E. coli* RuvC HJ-specific endonuclease, fused to green fluorescent protein (GFP) [2]. RDG binds HJs specifically, and thus traps HJs, preventing the action of other proteins on the HJ DNA both biochemically and in live cells. RDG can be used to visualize HJs as fluorescent foci in living *E. coli* cells, and also to map HJs in genomes by ChIP-seq.

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Key Figure

DNA Biology from *Escherichia coli* to Cancer Revealed via Engineered 'Freeze-Frame' Proteins That Trap DNA Reaction Intermediates – A Holliday Junction (HJ) Trap Protein, RuvCDefGFP (RDG)

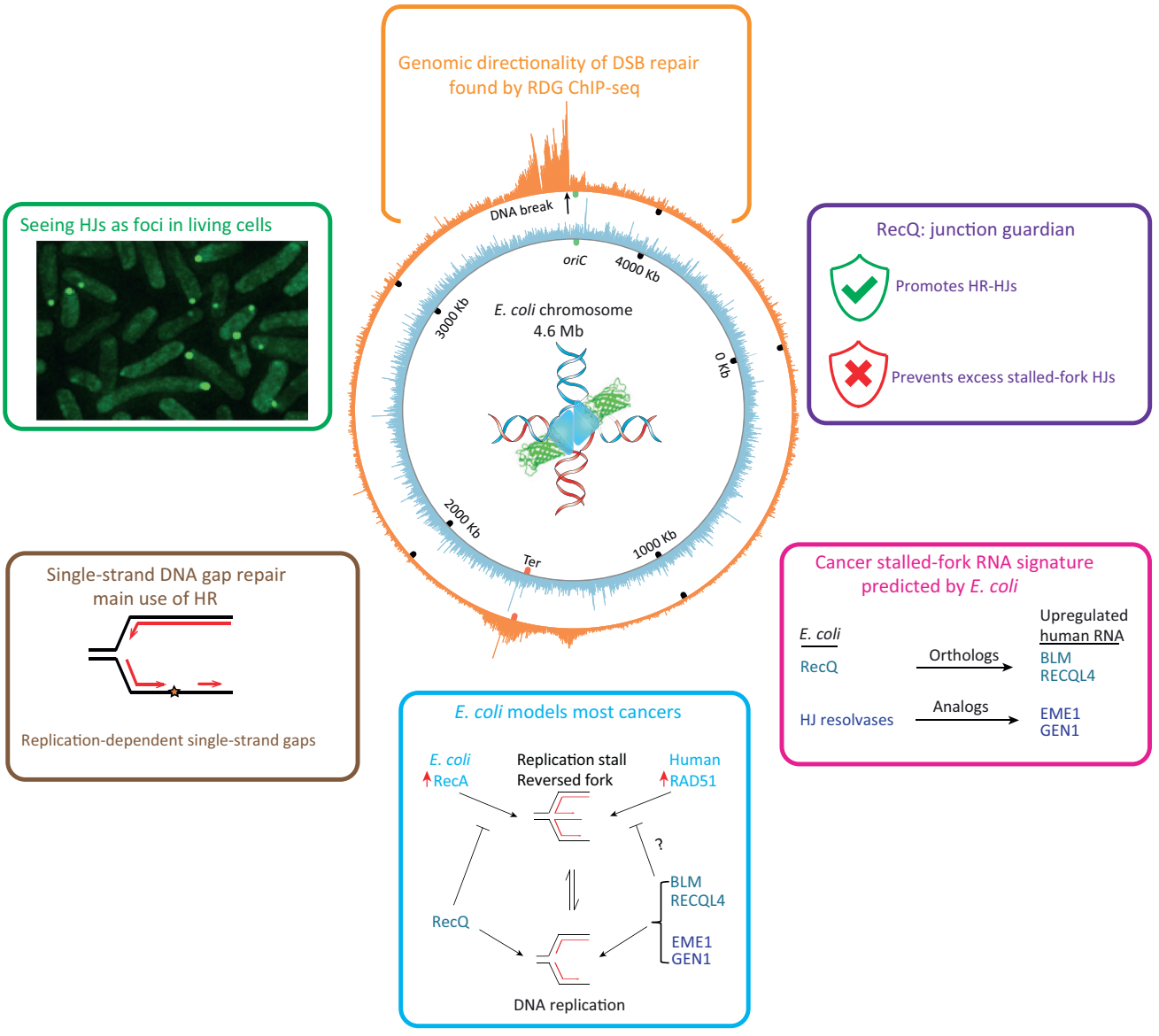


Figure 1. (Green box) RDG foci (green) in *E. coli* quantify HJs in single cells with repairing chromosomes after cleavage by I-*Sce*I endonuclease. (Orange) RDG chromatin immunoprecipitation with deep sequencing (ChIP-seq) maps reveal four-way DNA (Holliday) junctions in *E. coli* chromosomes cleaved by I-*Sce*I endonuclease, and show directionality of DNA-break repair. Two ChIP-seq maps of the 4.6 Mb *E. coli* chromosome generated using ChIP-seq analysis of the four-way DNA junction-specific RDG, which binds to and traps HJs, thus preventing their removal by other proteins. The orange wheel shows the RDG ChIP-seq map of chromosomes with a single chromosomal double-strand break (DSB, 'DNA break' in the diagram) induced by I-*Sce*I site-specific endonuclease in the living cells.

(Figure legend continued on the bottom of the next page.)

Box 2. Natural Holliday Junctions Are Mobile

Natural HJs are mobile, in that the particular bases paired at the junction can shift, which causes the junction itself to move along the DNA sequence (e.g., see Figure IB in Box 1). Whether RDG immobilizes the DNA at the junction itself during binding and protection from other proteins is not known, although RDG–DNA interaction, observed as fluorescent foci, is stable over 11 h in live cells tracked in microfluidic experiments [2].

repair RDG (HJ) foci result from single-strand-gap repair, not DSB repair (Box 1, Table 1). Whereas RDG foci induced by DSBs required RecB [2], part of the RecA loader complex at DSBs [19], and were blocked by the DSB-end trapping Gam protein of phage Mu [2], most spontaneous RDG foci were RecB-independent and unaffected by Gam [2] (Table 1). These data, and the requirement for RecF, demonstrate that most of these spontaneous repair-HJ foci formed independently of DSBs [2] and were associated with single-stranded DNA gaps [15,16], in other words in single-strand gap repair. A list of the requirements for HJ foci of various types is given in Table 1. The spontaneous DNA damage that necessitates homology-directed repair requires DNA replication, shown first by the dependence of spontaneous RDG foci on replication-initiation protein DnaA, and second by their correlation with cell generations in microfluidic experiments [2]. Spontaneous HJs correlated with replication forks, with a nearly constant spontaneous RDG-focus frequency per replication fork of 5.0×10^{-3} ($\pm 0.3 \times 10^{-3}$) and 4.2×10^{-3} ($\pm 0.6 \times 10^{-3}$) in rich and minimal media, respectively. A model for homology-directed repair of replication-generated single-stranded gaps is shown in Figure 3B (also Box 1).

RecQ DNA Helicase Helps to Generate Repair HJs in Living Cells

E. coli **RecQ DNA helicase** is the ortholog of five human cancer-preventing proteins that protect cells from genome instability [20]. Previously, *E. coli* RecQ was shown to promote the accumulation of interchromosomal homology-directed repair intermediates held together by HJs in cells [21]. However, whether RecQ promoted formation of HJs or deterred HJ resolution was unknown [21]. The RDG HJ trap blocks HJ-resolution routes, such that HJ formation alone affects HJ levels when RDG is produced. Timed-expression studies with production of either RDG first, then RecQ, or the converse, showed that both RecQ and its partner RecJ exonuclease promote the formation of spontaneous repair HJs from single-strand gap repair sites in *E. coli* [2]. Because RDG production did not affect RecQ/J-promoted accumulation of RDG HJ foci, RecQJ appear not to block HJ resolution, but instead aid repair-HJ formation (Figure 1, purple box). RecQ also promoted the accumulation of RecA–GFP foci in living cells, implying its action before RecA in homology-directed repair, as illustrated (Figure 3B), and RecQ was required for most spontaneous recombination events [2,21]. The data support a pre-RecA role of RecQ in promoting HJs intermediates in the repair of single-strand gaps caused by replication (model, Figure 3B). A role in the pre-RecA/**RAD51** stage of repair is also indicated for the human RecQ ortholog RECQL4 [22], which promotes 5′-end resection at DSBs via its helicase activity, which is required for this role [22].

RecQ Prevents Reversed Forks in an *E. coli* Cancer Model

Human RAD51, an ortholog of the *E. coli* RecA strand-exchange protein, is upregulated in most human cancers [23], which show elevated *RAD51* RNA. *RAD51* RNA correlates with RAD51 protein levels across many cancers (Spearman correlation $r = 0.53$, $P = 1.6 \times 10^{-59}$, data from depmap.org), implying that RAD51 protein is overproduced in most cancers. RAD51

The spikes indicate enrichment of DNA sequences bound by RDG. HJs accumulate near a repairable DSB (DNA break), and near the replication terminus. The blue wheel is the map of spontaneous HJs from RDG ChIP-seq in cells without an enzyme-induced DSB. Center, representation of RDG (blue and green) binding to a HJ. Data from [2]; illustration modified, with permission, from [65]. Other boxes summarize additional discoveries made using RDG. Abbreviation: HR, homologous recombination.

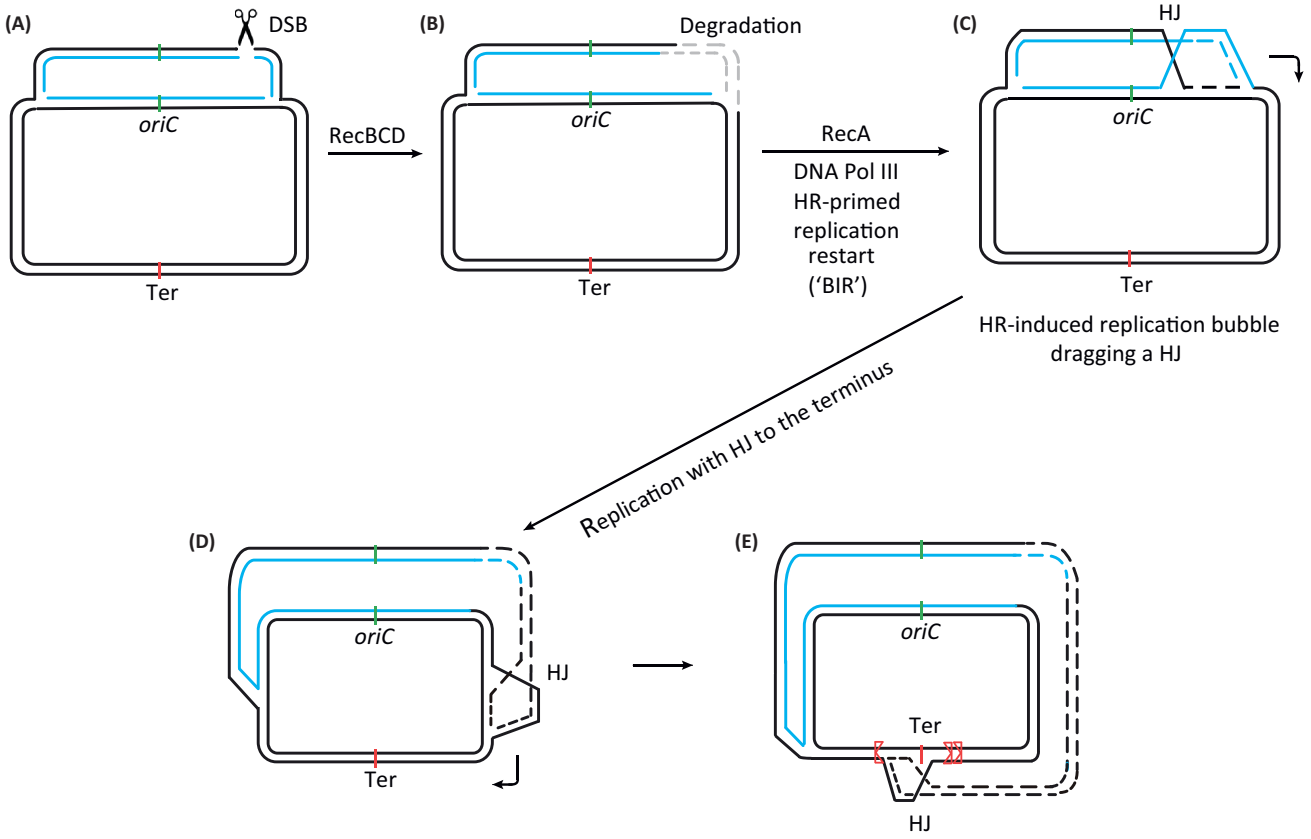
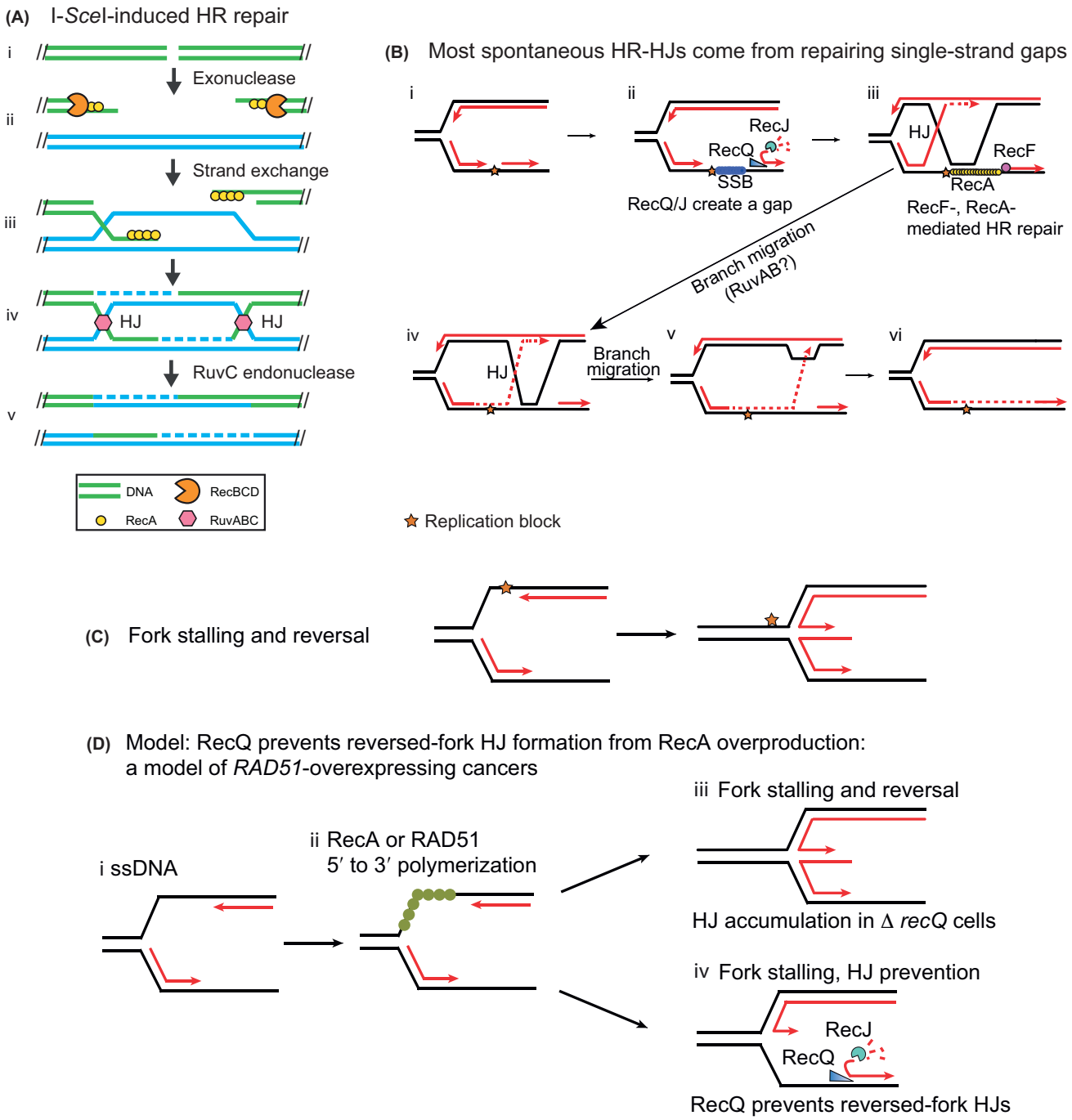


Figure 2. Model of One-Ended Double-Strand Break (DSB)-Induced Repair Replication Forks Dragging Holliday Junctions (HJs) to the *Escherichia coli* Replication Terminus. Panels (A–C) follow the model of Kuzminov [14]; panels (C–E) are adapted, with permission, from [10]. (A) Double-strand cleavage by I-*Sce*I endonuclease makes a two-ended DSB. (B) Chi sites, which attenuate RecBCD double-strand exonuclease activity [4,5], and promote recombination thereby, occur asymmetrically in the genome [14]. A two-ended DSB is likely to be degraded extensively towards the terminus preserving the *ori*-proximal chromosome arm, which has many active Chi sites. (C,D) The nondegraded origin-proximal DSB end could initiate DSB repair by strand exchange, and prime a replication fork that would run in the natural *ori*-to-*ter* direction of the chromosome (dashed lines indicate newly synthesized DNA). (D,E) Parts C–E of the model were offered previously [10] in support of observations that most homology-directed DSB repair requires the major replicative DNA polymerase (Pol III) and that the new strands are segregated conservatively [10], as observed subsequently in yeast break-induced replication [66]. We suggested that the replication bubble proceeds towards the terminus, dragging an unresolved HJ behind it in a displacement- or D-loop, which forces the new DNA strands (dashed lines) out of the bubble, causing the observed conservative segregation of new strands [10]. (E) Forks, trailing HJs behind them, that were initiated on one side of the terminus will pause and accumulate at the terminus. Some that overshoot the midway point will be stopped at the unidirectional *ter* sites(s) (red, angled sides stop oncoming forks) on the opposite side of the chromosome from which the bubble began. This pattern of HJ accumulation is seen in the chromatin immunoprecipitation and deep sequencing (ChIP-seq) data (Figure 1, orange circle) [2]. Figure reproduced, with permission, from [2], modified from [10]. Abbreviations: BIR, break-induced replication; HR, homologous recombination; *oriC*, origin of replication; Ter, termination region.

upregulation destabilizes genomes by unclear mechanism(s) [24], and promotes tumor resistance to chemotherapeutic **PARP-inhibitor drugs** by enhancing RAD51-dependent homology-directed DSB repair [23]. Increased RAD51 supports breast cancer metastases [25], and correlates with decreased survival of lung cancer patients [26]. Using human cell lines with inducible RAD51, overproduction decreased the repair of I-*Sce*I endonuclease-induced DSBs, despite showing increased RAD51 foci [27]. The overproduced RAD51 also slowed replication fork progression, as visualized by DNA fiber assays [28] which label newly synthesized DNA [27]. These data indicate that increased RAD51 can inhibit homology-directed repair efficiency [27], slow replication, and cause genome instability – but how?



Trends in Genetics

Figure 3. Common Sources of Holliday Junctions. (A) Model of I-*Sce*I induced-DSB repair by homologous recombination (HR) (Box 3). Pink hexagons, RuvC on HJs; dashed lines, new DNA synthesis. Figure modified, with permission, from [2]. (B) Model: most spontaneous HJs arise from repair of single-strand gaps. Modified, with permission, from [2], original ideas from Rupp and Howard-Flanders [67]. Lines, DNA strands; red lines, new strands; black lines, old strands; dashed red line, new DNA synthesis. (i) When a lagging strand 3′ end (half arrowhead) encounters a lesion (star) that blocks DNA replication, (ii) the adjacent Okazaki fragment can be unwound by RecQ DNA helicase, which exposes a 5′ single-stranded (ss) DNA end [33], the substrate of RecJ 5′ single-strand-dependent exonuclease. The resulting single-strand gap is coated by single-strand-binding protein SSB. (iii) At the 5′ end of a single-strand/duplex DNA junction [16], RecF displaces SSB and loads RecA

(Figure legend continued on the bottom of the next page.)

Table 1. Similarities and Differences between Sources of, and Phenotypes of Cells with, HJs Detected by RDG[a]

RDG foci	HR or non-HR[b]	Replication-dependence	Instigating damage	Proteins that promote	Proteins that reduce or prevent	Not required
I-*Sce*I-induced HJs	HR	Partly yes[c]	DSBs	RecA, RecB, RuvB		RecF, RuvA
Most spontaneous HJs	HR	Yes	Single-strand gaps	RecA, RuvB, RecF, RecQ, RecJ, DnaA	RuvC, RecG	RecB, RuvA, SOS
RecA overproduction-induced HJs	Non-HR	Not known	Reversed forks	RecA	RecQ, RecJ	RecB, RecF, SOS response

[a]Summary of data from [2].
[b]HR denotes HJs formed as intermediates in homology-directed repair, and requiring RecA strand-exchange protein and its loaders, either RecBCD (at DSBs) or RecF (at single-strand gaps).
[c]These foci are more abundant when DNA is cleaved at an I-*Sce*I cutsite near the replication origin, where there are multiple DNA copies, than when cleaved at an I-*Sce*I cutsite near the replication terminus, where there are fewer chromosome copies in proliferating *E. coli* cells.

Box 3. Two Modes of Control and Directionality of DSB Repair

Break-induced replication (BIR) is double-strand DNA replication initiated by a strand-exchange intermediate in homology-directed DSB repair (Figure 2). BIR initiates a single unidirectional replication fork from the DSB end, copying the homologous molecule (Figure 2C–E). BIR produces conservative segregation of the new DNA strands (Figure 2C–E, dashed lines), and is found in *E. coli* [10] and yeast [58]. In cancer cell lines [59] and mice [60], BIR is also implicated in repairing replication-fork collapse and breakage caused by overproduction of the cyclin E oncoprotein, which drives cancers when overproduced [61]. Some component of this 'replication stress' observed could potentially relate to HJ pile-up events such as those seen in *E. coli*.

Replication initiated by homology-directed DSB repair in *E. coli* appears, at least sometimes, to continue from the DSB site to the replication terminus [10], dragging its associated HJ with it (Figure 2) [2] (as predicted [10]). The picture suggests HJ pile-up at replication termination signals that are oriented to catch forks coming from the chromosome arm containing the DSB, supporting this model (Figure 2C–E). In addition, the distributions of HJs are skewed around two-ended DSBs under repair (Figure 1, central orange circle) [2]. There is more HJ signal downstream of the DSB site than upstream, nearer the replication origin, in the chromosome replication path (Figure 1, central orange circle) [2]. This skew supports the Kuzminov model [14] for Chi-site control of DSB-repair replication. Chi sites are the stop signs for the RecBCD exonuclease. Chi is an asymmetrical sequence that is oriented asymmetrically in the chromosome. Considering a two-ended DSB (e.g., Figure 2A), there are more Chi sites that stop degradation upstream in the replication path than downstream (Figure 2B), which results in preservation of the upstream origin proximal chromosome arm, while RecBCD degrades the downstream terminus proximal DNA duplex (Figure 2B). The origin-proximal DSB end exchanges strands with the sister chromosome template causing a HJ (Figure 2C) which results in repair replication being primed preferentially in the usual *ori*-to-terminus direction [14] (dashed lines indicate newly synthesized DNA, Figure 2C–E). This model predicts the observed accumulation of HJs at the replication-stopping signals (Figure 2E, red brackets) that are oriented to 'catch' forks coming from the chromosome arm with the DSB (Figure 2C,D) [4,5].

E. coli has a rich history of predicting important DNA biology in cancer [29–31]. Data from *E. coli* predict that the problem with *RAD51*-overexpressing cancers is replication fork stalling and reversal, and imply that human RecQ orthologs help those cancers to proliferate [2].

The majority of common cancers, including most TP53^{-} cancers, have increased *RAD51* RNA levels [23]. These were modeled in *E. coli* by overproducing the RAD51 ortholog, RecA. The

onto the single-stranded DNA [15] forming a RecA–DNA nucleoprotein complex [4,5]. The RecA–DNA complex promotes strand exchange and HJ formation. (iv) HJ branch migrating to the right, passing the lesion. (v,vi) Further branch migration returns the 3′ end to the original duplex and removes the D loop and HJ. (C) Diagram of spontaneous reversed-fork HJ formation. When forks stall, the accumulated negatively supercoiled DNA ahead of the fork can spontaneously push it backwards [68], independently of RecA [18,68] (Table 1). (D) Model of overproduced RecA-promoted fork reversal, and reversed-fork prevention by RecQ/RecJ. With RecA overproduction, forks might reverse spuriously because of RecA polymerization on single-stranded DNA at the fork (i,ii) instigating strand exchange with the nascent sister duplex. (ii) RecA loaded onto single-stranded DNA at a fork is extended 3′ from the fork junction to the single-stranded/duplex DNA junction at the 3′-leading-strand end, independently of RecA loader proteins [18,69]. The RecA–DNA complex could promote (iii) fork reversal (Table 1). (iii) Without RecQ or RecJ, reversed-fork HJs will accumulate. (iv) RecQ and RecJ could prevent reversed-fork HJs. Figure reproduced, with permission, from [2].

overproduced RecA caused a significant twofold increase in four-way junction foci [2], that were shown not to arise by repair but instead by replication fork stalling and reversal (Figure 1, blue box, and Box 1). The extra four-way junction foci form independently of the RecA-loading proteins used in homology-directed repair [2] (Table 1). Because the spontaneous repair-RDG foci (which remain present when RecA is overproduced) still required the loader [2], the overproduced RecA has not merely made repair independent of the loader proteins. Instead, the additional foci produced with extra RecA appear to be reversed replication forks, indicating replication stalling.

Although the formation of repair-HJ foci requires RecQ and RecJ (per Figure 3B), RecQ and RecJ prevent the accumulation of the HJ foci caused by overproduced RecA (Figure 1, purple box, blue box) [2]. This indicates a role for RecQ in preventing reversed forks and promoting replication after a stall. Figure 3C,D shows a model for how increased RecA may promote stalled, reversed replication forks, and how RecQ and RecJ can prevent their accumulation.

Courcelle *et al*. suggested a post-HJ role of RecQ and RecJ in removing reversed replication forks caused by UV-induced accumulation of plasmid-based double-Y junctions inferred to be HJs [32]. Data that quantify RDG-labeled HJs suggest instead (or additionally) that RecQ and RecJ prevent the formation of (RecF-independent) reversed-fork foci induced by RecA overproduction. In this work, removal of RecQ and RecJ increased reversed-fork HJ foci even after RDG was produced [2]. Because HJ removal is blocked by RDG, this implies a HJ-preventing rather than a HJ-removal role for RecQ/J [2]. *E. coli* RecQ and RecJ could prevent the HJ stage of fork reversal by unwinding and digesting the lagging strand, as shown in Figure 3Div. Lagging-strand removal/digestion is supported by RecQJ biochemistry [33] as well as by DNA degradation in RecQ/J-proficient cells under replication arrest [34–36]. Moreover, the role of *E. coli* RecQ in preventing reversed-fork HJs may be shared by the budding yeast RecQ homolog Sgs1. 2D gel electrophoresis of DNA from *sgs1*-deficient cells showed an increase in X-DNA structures near an engineered replication-fork barrier, supporting a role for Sgs1 in reducing reversed forks [37].

E. coli Predicts Cancer-Promoting Roles of BLM, RECQL4, and HJ Resolvases

The *E. coli* data suggest that cancers that overproduce RAD51 might also face replication stalling and fork reversal (Figure 1, blue box). This is paradoxical because fork reversal stops DNA replication, and cancers are, by definition, champions at replicating DNA. Most cancers upregulate RAD51 [23]. If RAD51-high cancers contend with reversed forks, they might co-upregulate HJ-reducing proteins with RAD51, allowing their replication. This hypothesis is supported by transcriptome data from human cancers [2], and also by the correspondence of *RAD51* transcripts with RAD51 protein across many cancers (reviewed above).

Similarly to *E. coli* that overproduces RecA, RAD51-high cancer cells could experience replication-fork stalling and reversal, as suggested by the following evidence [2]. If RAD51-overproducing cancers had excessive replication stalling and fork reversal, they might upregulate proteins that reduce the HJs that result, which would allow replication to resume. A search of cancer RNA data in The Cancer Genome Atlas (TCGA [38]) revealed strong correlations between upregulated *RAD51* RNA and upregulated RNAs that encode two known HJ-resolution proteins: EME1 and GEN1 [2]. With the caveat that RNA and not protein levels are available for EME1 and GEN1, these data support the hypothesis that increased RAD51 in cancers promotes HJs. Moreover, the data imply that increased EME1 and GEN1 might actually promote DNA replication in cancer cells by clearing increased reversed-fork HJs – an

unexpected and potential cancer-promoting role. However, at this point, with very limited proteomic data, we can only assume that these transcripts are translated into active proteins. Unlike RAD51, these DNA nucleases and helicases are low-abundance proteins, below the detection limit of, for example, global proteomics applied to cancer research in recent years, and are also not among the limited number of proteins (150–400) covered in the current reverse-phase protein array (RPPA) data in TCGA.

The cancer RNA data also imply that the increased HJs in *RAD51*-overexpressing cancers are probably reversed forks, not repair intermediates, because the human RecQ orthologs *BLM* and *RECQL4* are co-upregulated with *RAD51* in those cancers [2] (Figure 1, blue box). *BLM* RNA was co-overexpressed with *RAD51* RNA in eight of the eight most common cancers: breast, lung, acute myeloid leukemia (AML), colon, kidney, thyroid, bladder, and prostate. *RECQL4* RNA was co-overexpressed with *RAD51* in four of the eight most common cancers (lung, breast, AML, and prostate), and both the *BLM* and *RECQL4* correlations with *RAD51* were of robust significance in cancer [2]. RecQ in *E. coli* prevents reversed-fork HJs and not repair HJs [2], implying that the extra HJs in these cancers may reflect reversed forks, and that BLM and RECQL4 might also combat or prevent reversed forks in cancer cells (Figure 1, blue box). Again, a caveat to these data is that, currently, only mRNA- and not protein-level data are available for *BLM* and *RECQL4* in these cancers.

Reversed forks are genome-destabilizing and could drive the cancer state [39]. BLM and RECQL4 may prevent reversed-fork HJs, whereas EME1 and GEN1 cleave the reversed forks, creating DSB ends that cycle through cleavage, repair, and replication. *RAD51*-overexpressing cancer cells require rapid DNA replication, but *RAD51* overexpression could cause reversed forks, which block replication. The data suggest that, like RecQ, both BLM and RECQL4 may prevent reversed forks in *RAD51*-overexpressing cancers, and thus promote cancer (Figure 1, blue box). These data are surprising because loss of function of any of the four HJ reducing protein-coding genes causes genome instability and cancer via the apparent loss of DNA repair. Loss-of-function mutations are associated with xeroderma pigmentosum (*EME1* or *GEN1*) [40], Bloom (*BLM*), and Rothmund–Thompson (*RECQL4*) cancer-predisposition syndromes. Apparently, the levels of these proteins must be tightly balanced, because an excess potentially drives DNA replication in cancer cells, whereas too little causes cancer via genome instability. The upregulation of *EME1* with *RAD51* in breast, lung, AML, kidney, thyroid, and prostate cancers, and of *GEN1* with *RAD51* in breast and lung cancers, suggests heavy loads of HJs in these cancers. These four proteins act in separate pathways [41,42], suggesting that there are at least four redundant means of reducing reversed forks in cancers in which RAD51 is upregulated – namely in the majority of cancers [23].

In support of the hypothesis that RAD51 increases reversed forks which are reduced, separately, by BLM, RECQL4, EME1, and GEN1, BLM unwinds HJs efficiently [43], can colocalize with and prevent repair HJs [44,45], and was suggested to disrupt stalled replication forks [46]. RECQL4 binds to HJs with high affinity and specificity, suggesting a HJ processing role [47]. As demonstrated for *E. coli* RecQ, which promotes the formation of repair HJs and prevents reversed-fork HJs [2], the human RecQ orthologs might act similarly and play different roles in different cellular environments or on different substrates. Biochemically, purified BLM can promote fork reversal [48]. RECQL4 promotes the earliest pre-strand-exchange stage of homology-directed repair in living cells – DNA-end resection [22] – and therefore is expected to promote repair-HJ formation. Both could additionally prevent reversed forks by playing dual roles, similarly to *E. coli* RecQ [2].

Concluding Remarks

The conservation of basic biology of DNA across the tree of life allowed us to experiment in *E. coli* bacteria and explore the general biology of DNA replication-fork reversal and homology-directed repair that appears to apply to human cancer [2]. The results provide surprising insight into unexpected potential tumor-promoting proteins. The data suggest that reversed forks are a vulnerability of most human cancers, which overexpress *RAD51*, and that cancer cells employ multiple redundant ways to reduce their numbers – a necessity for maintaining DNA replication. Increased *BLM*, *RECQL4*, *EME1*, and *GEN1* mRNA levels may be a potential reversed-fork (reduction) signature of human cancers (Figure 1, pink box) – a hypothesis that awaits testing – and a surprise given the known tumor-preventing roles of properly expressed BLM and RECQL4 [49].

The data suggest that human BLM, RECQL4, EME1, and GEN1 might be useful potential drug targets to inhibit tumor growth in *RAD51*-overexpressing cancers. A small-molecule inhibitor that targets BLM DNA-binding activity was identified from a chemical library screen and, in agreement with our prediction, cells targeted by the BLM inhibitor exhibit reduced proliferation [50]. Although there is currently no inhibitor of RECQL4, future RECQL4-targeting drugs might be codelivered with a BLM inhibitor to target *RAD51*-overexpressing cancers.

The use of engineered 'freeze-frame' protein traps of DNA reaction intermediates greatly facilitates studies of DNA biology (Table 1), although many problems remain unsolved (see Outstanding Questions). The GamGFP trap for DSB ends works in *E. coli* and human cells [51–53] because DNA structures are universal. The RDG HJ trap enables real-time monitoring and quantification of HJs as foci in *E. coli* single cells, as well as mapping of their genomic locations in cell populations by ChIP-seq. RDG, or engineered proteins like it, are expected to work also in other organisms including human cells or mice, and efforts to optimize or adapt RDG for use in human cells are needed. RDG is far more sensitive than previous methods for observing HJs including 2D gel electrophoresis [54] and electron microscopy [55,56]. The freeze-frame protein traps also block downstream reactions of their substrates through multiple pathways, and this facilitates dissection of the stages at which repair proteins act. Although studies on the molecular mechanisms of DNA biology have focused on the enzymes that catalyze the reactions, the DNA reaction intermediates themselves define the molecular mechanisms, and are compelling subjects for direct study in living cells. Following them by quantification, mapping, and trapping is a powerful approach.

Outstanding Questions

Can the numbers of HJ foci in homology-directed repair-deficient *E. coli* cells be used as a marker for replication stalling?

Can the genomic locations of HJs be tracked through time during repair or repair-induced DNA replication?

Can RDG or other HJ-trap proteins be applied to other organisms, including human and other eukaryotic cells?

How do RuvC-derived HJ traps compare with traps engineered from other HJ-binding proteins?

Can drugs that inhibit reversed-fork-reducing proteins be used as cancer-specific or biased DNA-replication inhibitors to treat cancers with the RNA signature of reversed-fork reduction (the majority of cancers overexpress *RAD51*)?

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