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Tools to live by: bacterial DNA structures illuminate cancer

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Abstract

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 DNA biology and cancer. HJ ChIP-seq showed 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: 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 HJ removers EME1 and GEN1, and human RecQ orthologs BLM and RECQL4—surprising potential pro-cancer roles for these known cancer-preventing proteins.

Keywords

BLM; cancer; *Escherichia coli*; Holliday junctions; RecQ; RECQL4

Engineered Proteins Trap HJs

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

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replication stalls, and the fork “reverses” (Box 1B and Figure 1, Key Figure, blue box). HJs are transient intermediates in reactions that underlie genome maintenance, and also 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] so that RDG “traps” HJs by binding them and preventing the action of other proteins on them (Figure 1, center). Biochemically, RDG binds four-way DNA junctions and inhibits EcoRI endonuclease and Flp high-affinity site-specific recombinase/**HJ resolvase** [3] activities at the junction [2]; i.e., RDG binds 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 with HJs from homology-directed DSB-repair (Box 1) in *E. coli* cells [2]. Repair of an *E. coli* chromosomal DSB made by **I-SceI site-specific endonuclease** (Figures 1 green box, 2 and 3A) increased the number of cells with RDG foci ~10-times 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 numbers of DSBs initiating repair with available repair templates. The DSB repair proteins **RecA** and **RecB**, which promote HJ formation [4, 5], are required for I-SceI-induced RDG focus formation (Figure 3A and Table 1) [2], supporting the interpretation that DSB-induced RDG foci indicate HJs from homology-directed DSB repair. Additionally, RDG foci were correlated with reparable 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 **ChIP-seq** in cells undertaking homology-directed repair of a DSB. Single site-specific DSBs were induced by I-SceI site-specific double-strand endonuclease at single engineered chromosomal sites [2] (Figure 1, orange circle). Proliferating cells have multiple chromosome copies and so can repair after DNA cleavage using an uncleaved sister chromosome as template [6, 7]. The map shows enrichment of RDG-bound DNA (HJs) near the induced DSB and downstream of it in the chromosome’s unidirectional replication path [2], and at the **replication terminus** [2] (Figure 1, orange circle, ter). These data suggest two modes of whole-genome control and co-directionality 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, Box 3). In it, HJs formed near the DSB during repair can sometimes be pulled behind the repair replication

fork, co-directionally 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 repairing two-ended DSBs [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**, proposed to keep repair replication going in the origin-to-terminus direction, 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, Figure 3B). The appearance of most spontaneous RDG foci requires strand-exchange protein RecA, and **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 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 supports focus occurrence at HJs. The origin of the 25% RecA-independent spontaneous RDG foci might be reversed replication forks (illustrated Figure 1, blue box, and Box 1B). Further evidence indicates that most 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], associated with single-stranded DNA gaps [15, 16], i.e., in single-strand-gap repair. A list of the requirements for HJ foci of various sorts is given in Table 1. The spontaneous DNA damage that necessitates homology-directed repair requires DNA replication, shown first by dependence of spontaneous RDG foci on replication-initiation protein DnaA, and second by their correlation with cell generations in microfluidic experiments [2]. Spontaneous HJs were 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 medium, respectively. A model for homology-directed repair of replication-generated single-stranded gaps is shown in Figure 3B (also see Box 1).

RecQ DNA Helicase Helps 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 accumulation of interchromosomal homology-directed repair intermediates held together by HJs in cells [21]. But 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 in *E. coli* [2]. Because RDG production did not affect RecQ/J-promoted accumulation RDG HJ foci, RecQ/J appear not to block HJ resolution, but rather to aid repair-HJ formation (Figure 1, purple box). RecQ also promoted 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 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 with its helicase activity, which is required for this role [22].

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

Human RAD51 is an ortholog of the *E. coli* RecA strand-exchange protein and is upregulated in most human cancers [23], which show elevated *RAD51* RNA. *RAD51* RNA is correlated 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 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 is correlated with decreased survival of lung-cancer patients [26]. Using human-cell lines with inducible RAD51, overproduction *decreased* repair of I-*SceI*-endonuclease induced DSBs, despite showing increased RAD51 foci [27]. The overproduced RAD51 also slowed replication-fork progression, visualized by DNA fiber assays [28], which label newly synthesized DNA [21]. These data indicate that increased RAD51 can inhibit homology-directed repair efficiency [27], slow replication, and cause genome instability—but how?

E. coli has a rich history of predicting important DNA biology of cancers [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 proliferate [2].

The majority of common cancers, including most P53⁻ cancers, have increased RAD51 RNA levels [23]. These were modeled in *E. coli* by overproducing the RAD51 ortholog, RecA. The overproduced RecA caused a significant two-fold increase in 4-way junction foci [2], shown *not* to arise by repair, but rather by replication-fork stalling and reversal (Figure 1, blue box; Box 1). The extra 4-way junction foci form independently of RecA-loading proteins used in homology-directed repair [2] (Table 1). Because the spontaneous repair-RDG foci, which remain present with RecA overproduced, still required the loader [2], the overproduced RecA has not merely made repair independent of the loader proteins. Rather, the extra foci with extra RecA appear to be reversed replication forks, indicating replication stalling.

Although formation of repair-HJ foci requires RecQ and RecJ (per Figure 3B), RecQ and RecJ *prevent* accumulation of the HJ foci caused by overproduced RecA (Figure 1, purple box, blue box) [2]. This indicates a role for RecQ in prevention of reversed forks and promotion of 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 that 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 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 (Figure 3D iv). Lagging strand removal/digestion is supported by RecQJ biochemistry [33], and by DNA degradation in RecQ/J-proficient cells under replication arrest [34–36]. Moreover, the role of *E. coli* RecQ in prevention of 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 with overproduced 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 the correspondence of *RAD51* transcripts with RAD51 protein across many cancers (reviewed above).

Like *E. coli* that overproduce RecA, RAD51-high cancer cells could experience replication-fork stalling and reversal, 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 RNA data from cancers in The Cancer Genome Atlas (TCGA [38]) revealed strong correlations of upregulated *RAD51* RNA with 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, 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 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), both orthologs with very robust significance [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 of these data is that, currently, mRNA 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, while EME1 and GEN1 cleave the reversed forks creating DSB ends that cycle through cleavage, repair and replication. The *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 so 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 with too much potentially driving DNA replication in cancer cells, and too little causing cancer via genome instability. The upregulation of *EME1* with *RAD51* in breast, lung, AML, kidney, thyroid, and prostate cancer patients, and *GEN1* with *RAD51* in breast and lung cancers suggest 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 with RAD51 upregulated—most 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 co-localize with and prevent repair HJs [44, 45], and was suggested to disrupt stalled replication forks [46]. RECQL4 binds HJs with high affinity and specificity suggesting a HJ processing role [47]. As demonstrated for *E. coli* RecQ, which promotes 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 so is expected to promote repair-HJ formation. Both could additionally prevent reversed forks, playing dual roles, like *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 learn 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 of reducing their numbers—a necessity for maintaining DNA replication. Increased RNAs of BLM, RECQL4, EME1 and GEN1 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 co-delivered with BLM inhibitor to target *RAD51*-overexpressing cancers.

The use of engineered “freeze-frame” protein traps of DNA reaction intermediates facilitates studies of DNA biology greatly (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, and 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, which facilitates dissection of the stages at which repair proteins act. Although 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 of direct study in living cells. Following them with quantification, mapping, and trapping are powerful approaches.

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Glossary

Chi sites:

the DNA sequence 5'GCTGGTGG. 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 sequencing. A technique to identify 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 critical strand-exchange intermediate

Holliday junctions:

four-way DNA junctions, illustrated Figures 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-SceI-double-strand endonuclease:

a site-specific double-strand DNA endonuclease that cleaves a specific 18-basepair sequence [63] that is absent from most genomes

PARP-inhibitor drugs:

cancer chemotherapeutic drugs that target Poly-ADP-Ribose Polymerase (PARP) are used to kill cancer cells that lack homology-directed DSB repair. These include *BRCA*-deficient breast and ovarian cancers [64]

RecA:

Bacterial strand-exchange protein required for homology-directed repair [4, 5] orthologs of which function similarly in all species examined

RAD51:

human **RecA** ortholog that performs strand exchange

RecBCD:

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 endonucleases to chop the unwound strands into oligonucleotides [4, 5]

RecF:

E. coli **RecA**-loading protein at single-strand DNA gaps. RecF binds the junction of single-stranded with duplex DNA at the gap (Figure 3C iii) [4, 5]

RecQ DNA helicase:

E. coli founding member of the highly conserved RecQ family of DNA helicases, important in genome stability. They translocate on a DNA strand in the 3' to 5' direction displacing the 5' end of the strand not bound

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 1, B; Figure 3C,D iii)

Replication terminus (of *E. coli*):

unidirectional sequences (red brackets Figure 2E) that stop replication forks that cross the chromosome midpoint and proceed past it, into the other chromosome arm, towards *ori*

RuvABC:

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 so traps HJs, preventing the action of other proteins on the HJ DNA biochemically and in live cells. RDG can be used to visualize HJs as fluorescent foci in living *E. coli* cells, and to map HJs in genomes by ChIP-seq

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Outstanding Questions

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

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 cancers with the RNA signature of reversed-fork reduction, the majority of cancers, which overexpress RAD51, be treated with drugs that inhibit reversed-fork reducing proteins as cancer-specific or biased DNA-replication inhibitors?

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 based paired with each other (A, single-strand gap repair). Holliday junctions (HJs) can be an intermediate in homology-directed repair (A). The HJ (A) 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 (A) or DNA double-strand-breaks (DSBs, Figure 3A). Dashed lines, new DNA synthesis; half arrowhead, 3' end; lines, DNA strands.

I-SceI-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 uses 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 (A) 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 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 accumulated ahead of the fork, from DNA unwinding, push back, reversing the fork (B).

Box Fig 1.

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., Box 1B). 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 hours in live cells tracked in microfluidic experiments [2].

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 (see Figure 2). BIR initiates a single unidirectional replication fork from the DSB end copying the homologous molecule, shown Figure 2C–E. BIR produces conservative segregation of the new DNA strands (Figure 2C–E, dashed lines), 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 like those seen in *E. coli*.

The 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], (predicted [10]). The picture suggests a HJ pile-up at **replication termination signals** that are oriented to catch forks coming from the chromosome arm with the DSB, supporting this model (Figure 2C–E). Additionally, the distributions of HJs are skewed around repairing two-ended DSBs (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 of the **RecBCD**-exonuclease. They are oriented in the chromosome asymmetrically, with more that can preserves origin-proximal DSB ends, while RecBCD erodes terminus-proximal DSB ends (Figure 2B, “degradation”). 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 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].

Highlights

- Holliday junctions (HJs) are DNA structures with four double-stranded arms, 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 formation and cancer, yet 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 so 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 surprising roles of DNA-repair proteins in promoting cancer.

Figure 1. Key Figure:

DNA biology from *E. coli* to cancer revealed via engineered “freeze-frame” proteins that trap DNA reaction intermediates—a Holliday-junction trap protein, RuvCDefGFP. Summary. Green box: RuvCDefGFP (RDG) foci (green) in *E. coli* quantify HJs in single cells with repairing chromosomes after cleavage by I-SceI endonuclease. Orange: RDG ChIP-seq maps reveal 4-way DNA (Holliday) junctions in *E. coli* chromosomes cleaved by I-SceI endonuclease, and show directionality of DNA-break repair. Two ChIP-seq maps of the 4.6Mb *E. coli* chromosome made using chromatin-immunoprecipitation and sequencing (ChIP-seq) of the 4-way-DNA-junction-specific RDG, which binds and traps HJs, 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 diagram) induced by I-SceI site-specific endonuclease in the living cells. The spikes indicate

enrichment of DNA sequences bound by RDG. HJs accumulate near a reparable 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 a HJ. Data from [2], illustration modified from [65]. Other boxes summarize additional discoveries made using RDG.

Figure 2.

Model of one-ended DSB-induced repair replication forks dragging HJs to the *E. coli* replication terminus. Parts A-C follow the model of Kuzminov [14], parts C-E are from [10], (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 non-degraded origin-proximal DSB end could initiate DSB repair by strand exchange, and prime a replication fork that would run in the chromosome's natural *ori*-to-*ter* direction (dashed lines, newly synthesized DNA). (D-E) The model parts C-E 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 ChIP-seq data (Figure 1, orange circle) [2]. Figure from [2], modified from [10].

Figure 3.

Comparison of different sources of HJ formation and resolution, and their key players, modified from [2].

(A) Diagram of I-SceI induced-DSB repair by homologous recombination (see Box 3). Pink hexagons, RuvC on HJs; dashed lines, new DNA synthesis.

(B) Model: most spontaneous HJs arise from repair of single-strand gaps. Modified 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 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 onto the single-stranded DNA [15] forming a RecA-DNA nucleoprotein complex [4, 5]. The RecA-DNA complex promotes strand exchange and Holliday junctions (HJs). (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 negative 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 from [2].

Table 1.

Similarities and differences between sources of 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-SceI-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

^aSummary of data from [2].

^bHR 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) (Table 1).

^cThese foci are more abundant when DNA is cleaved at an I-SceI cutsite near the replication origin, where there are multiple DNA copies, than when cleaved at an I-SceI cutsite near the replication terminus, where there are fewer chromosome copies in proliferating *E. coli* cells.