Holliday junction trap shows how cells use recombination and a junction-guardian role of RecQ helicase

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DNA repair by homologous recombination (HR) underpins cell survival and fuels genome instability, cancer, and evolution. However, the main kinds and sources of DNA damage repaired by HR in somatic cells and the roles of important HR proteins remain elusive. We present engineered proteins that trap, map, and quantify Holliday junctions (HJs), a central DNA intermediate in HR, based on catalytically deficient mutant RuvC protein of *Escherichia coli*. We use RuvC-DefGFP (RDG) to map genomic footprints of HR at defined DNA breaks in *E. coli* and demonstrate genome-scale directionality of double-strand break (DSB) repair along the chromosome. Unexpectedly, most spontaneous HR-HJ foci are instigated, not by DSBs, but rather by single-stranded DNA damage generated by replication. We show that RecQ, the *E. coli* ortholog of five human cancer proteins, nonredundantly promotes HR-HJ formation in single cells and, in a novel junction-guardian role, also prevents apparent non-HR–HJs promoted by RecA overproduction. We propose that one or more human RecQ orthologs may act similarly in human cancers overexpressing the RecA ortholog RAD51 and find that cancer genome expression data implicate the orthologs BLM and RECL4 in conjunction with EME1 and GEN1 as probable HJ reducers in such cancers. Our results support RecA-overproducing *E. coli* as a model of the many human tumors with up-regulated RAD51 and provide the first glimpses of important, previously elusive reaction intermediates in DNA replication and repair in single living cells.

**INTRODUCTION**

Elucidating the enzymatic mechanisms of biological processes by in-cell genetic and in vitro biochemical analyses can be daunting because of the transient and elusive nature of reaction intermediates, which define the reaction mechanisms. Although genomics reveals the stable end products of reactions underlying genome instability and evolution—the new DNA sequences created—the intermediates themselves are seldom studied directly, especially in living cells. Here, we examine reactions of DNA repair by homologous recombination (HR), which can provoke genome instability/evolution. We report the engineering and use of synthetic proteins to detect, quantify, trap, and map a central DNA intermediate in HR, the Holliday junction (HJ): a four-way DNA junction [Fig. 1, A (iv) and B]. We use these engineered proteins to address fundamental questions in genome stability and replication in single living somatic cells: What is the primary use of/need for HR and how does a model RecQ family protein promote genome stability?

HR mends damaged DNA (text S1 and Fig. 1A) and in doing so also promotes genome instability that drives cancer (1) and evolution (2), adaptation of pathogens to our immune systems (3), and immune defenses (4). Most of the cancer-driving roles of HR occur in somatic cells repairing (mostly) endogenous DNA damage (5). Although HR commonly occurs between identical sister chromosomes in somatic cells (6), nonidentical homologous chromosomes sometimes recombine, which can cause “loss of heterozygosity” of alleles that promote cancer when mutant (7). Also in somatic cells, HR between repeated sequences promotes some cancer-driving chromosomal rearrangements, including large and small deletions and duplications (copy number alterations) and translocations (5, 8). In addition, human RAD51, the ortholog of *Escherichia coli* RecA—the conserved, ubiquitous, central HR catalyst—is overexpressed in diverse human tumors and associated with poor prognosis (9, 10), implicating HR in the maintenance or progression of cancer (11).

Here, we discover the primary uses of HR in single living somatic or vegetative (nonsexual) cells: the main DNA damage types that necessitate HR and the main cellular processes that cause them. We assess how frequent HR is in somatic cells. The importance of HR would be different whether used once per genome replication [estimated for *E. coli* and similarly per base pair for human DNA (12–14)] rather than once per hundred replications. We also demonstrate a genome-scale control and directionality of double-strand break (DSB) repair in the *E. coli* chromosome and address the functions of a model RecQ family protein in single living cells.

Five human orthologs of *E. coli* RecQ DNA helicase are genomestabilizing cancer prevention proteins (15) important to human health, but their precise functions have been elusive in living cells.
Fig. 1. RDG traps HJs, inhibiting recombination, repair, and protein action on HJs biochemically and in living cells. (A) Example of an HR model that includes HJs (with pink hexagons, RuvC, on them; see text S1 for HR and this model). Paired parallel lines, base-paired DNA strands; dashed lines, new DNA synthesis. (B) Illustration of RuvC (blue triangles) binding to HJ, adapted with permission from West (32). (C) Design of chromosomal regulatable ruvCDegfp gene (see also fig. S1). P\textsubscript{N25tetO}, doxycycline-inducible promoter (39; arrows, directions of transcription). (D) RDG protein is induced with doxycycline (doxy; Western blot). (E) RDG protects HJ DNA (not linear duplex DNA; fig. S2D) from Eco RI cleavage in solution. Left: Eco RI site in synthetic immobile HJc DNA, 3 bp from the HJ center. Middle: Representative digestion of HJc by Eco RI inhibited by prebinding (fig. S2B) of RuvC\textsubscript{GFP} or RDG. Right: DNA band intensities normalized to that of HJc at time 0 of Eco RI treatment (means ± SEM, three experiments). (F) RDG production causes dominant-negative UV light sensitivity, implying failed DNA repair. The data imply that RuvC\textsuperscript{+} protein cannot act on HJs trapped by RDG. Native ruvC locus, either ruvC\textsuperscript{+} or deleted (ΔruvC\textsuperscript{−}L), and the protein produced from the chromosomal transgene (right): P\textsubscript{N25tetO}-RDG, P\textsubscript{N25tetO}-RuvC\textsubscript{GFP}, or P\textsubscript{N25tetO} P\textsubscript{ΔruvCDE+} promoter only. (G) In-cell titration of RDG/RuvC\textsuperscript{+} ratios shows that RDG remains dominant-negative, implying HJ trapping, when RDG levels are reduced to allow many RuvC\textsuperscript{+} homodimers. RuvC homodimers/RDG homodimers at 2.3 (black line) determined by Western blots. Right: percentages of RuvC homodimers and RuvC/RDG heterodimers expected at this ratio. RuvC and RDG levels controlled by IPTG-inducible P\textsubscript{ tac} and doxycycline-inducible PN25\textsubscript{tetO}, respectively. MW, molecular weight. (H) RDG inhibits HR in a phage P1 transduction assay in the presence (RDG) or absence of native RuvC or RecG HJ resolution proteins and in the absence of both (RDG, RecG, and RuvC, respectively). RDG transcription induced (green) or repressed (gray). \textsuperscript{†} or \textsuperscript{‡}, frequency \textless 1 \times 10\textsuperscript{−7} colony-forming units (cfu) per particle; \textsuperscript{*}P \textless 0.05 relative to the uninduced control. \textsuperscript{+}P \textless 0.05 relative to the uninduced RDG control strain. (I) RDG blocks RusA HJ endonuclease in living cells. RusA produced from an IPTG-inducible plasmid reduced RDG spontaneous HJ foci (foci described in Fig. 2) when produced before (right) but not after RDG (left). Left bar in each panel, no IPTG induction. \textsuperscript{*}P \textless 0.05, two-tailed paired t test.
In biochemical experiments, purified RecQ both promotes RecA-mediated strand exchange, leading to HJs (16), and disentangles model double HJs (17). Although RecQ promotes the net accumulation of HJs in living cells (18) and allows the degradation of DNA at stalled replication forks (19, 20), whether it acts before or after HJs in cells is unknown (18), as are its main roles in cells. Of the five human orthologs, WRN (and yeast Sgs1) acts nonredundantly to reduce HJ levels in cells (21, 22). Sgs1 also works redundantly in DSB resection/HJ promotion (23–25), and RECQL4 is implicated in this role (26). BLM is implicated in HJ-level reduction (15). RECQL5 may act mainly on RNA (27). RECQL1, RECQL5, and RECQL4 also prevent genome instability and cancer in humans and mice (27–29), but whether via HR, and at what stage(s), is unknown.

We present engineered protein derivatives of *E. coli* RuvC four-way DNA junction (HJ)–specific endonuclease (Fig. 1B) (30–32) that trap HJs, inhibit their further chemistry with both purified proteins in solution, and in cells, label and quantify HJs as fluorescent foci in single living cells, and map sites of HJs in genomes via chromatin immunoprecipitation sequencing (ChIP-seq). HJs can form both as intermediates in HR and HR independently when replication forks stall and regress (33). Although RecQ promotes the net accumulation of HJs in living cells (18), we describe here a novel “junction-guardian” role of RecQ, both promoting the formation of HR-HJs and preventing the formation of non-HR–HJs. By mining human cancer RNA data, we implicate the RecQ orthologs BLM and RECQL4 in similar roles in many human cancers.

**RESULTS**

**A HJ trap is engineered from RuvC**

We engineered endonuclease-defective, fluorescent protein fusions of four-way DNA junction–specific RuvC by substituting bases encoding catalytic amino acids (described in fig. S1 and text S1 for four-way junction specificity) (30–32). We also built an identical C-terminal green fluorescent protein (GFP) fusion to functional RuvC. RuvCDegFP (RDG), RuvCDemCherry (RDM), and RuvCGFP are encoded (separately) as doxycycline-inducible transgenes in a non-germic specific between the *mntH* and *nupC* genes in the *E. coli* chromosome (Fig. 1, C and D), in cells that also have either the wild-type (WT) or deleted *ruvC* gene at the native locus, as indicated. We purified RDG and RuvCGFP proteins and confirmed that both bind model HJs in solution (fig. S2A, A and B). RuvCGFP cleaves a model HJ, apparently uninhibited by the GFP tag (fig. S2C). RDG does not cleave the model HJ (fig. S2C), indicating that, as designed, RDG binds but does not cleave HJs in solution.

**Purified RDG inhibits action of other proteins on HJs in solution**

Two assays show that RDG inhibits the activities of other proteins at HJs, that is, “traps” HJs in solution. First, prebinding of either RDG or RuvCGFP to a model HJ with an Eco RI recognition sequence near the junction (fig. S2B) slowed cleavage by Eco RI endonuclease of HJ DNA (Fig. 1E) but not linear DNA (fig. S2D), indicating that both retard Eco RI specifically at a HJ. Second, we performed competition assays between RDG and Flp high-affinity site-specific recombinase/HJ resolvase (36), reported to have roughly similar HJ affinity to RuvC (32). More than half (55 ± 2%; mean ± SEM, three experiments) of the RDG bound to a model HJ containing the Flp recognition sequence resisted displacement by Flp (fig. S2, E to G). We conclude that RDG has HJ-trap activity in solution with affinity similar to Flp.

**RDG inhibits action of other proteins on HJs in living cells**

Three assays demonstrate that RDG inhibits HJ processing by other proteins in *E. coli*. First, RDG protects against RuvC, causing a “dominant-negative” sensitivity to ultraviolet (UV) light. *ruvC* cells are UV-sensitive (Fig. 1F, *ΔruvC* compared with *ruvC*+) (32) and became resistant with RuvCGFP production from the chromosomal transgene (Fig. 1, C and F, *ΔruvC P_{N25tetO}–RuvCGFP*), showing that RuvCGFP substituted for RuvC. Thus, RuvCGFP was functional in cells as it was in solution (fig. S2C). By contrast, production of RDG caused a UV sensitivity similar to that of *ΔruvC* cells even in cells that also carry the native WT *ruvC*+ (Fig. 1F, *ruvC*+ *P_{N25tetO–RDG}*, fig. S3, additional controls), implying that RDG blocked the activity of RuvC on HJs. Because RuvC functions as a dimer (Fig. 1B) (32), we constructed regulatable chromosomal cassettes for both RuvC and RDG to vary their ratios. We found that RDG prevents RuvC action, causing a dominant-negative UV sensitivity, even when the molar ratios were adjusted to produce a predicted 36% active-form RuvC homodimers (Fig. 1G, black line) and 16% RuvC homodimers (48% heterodimers), assuming unbiased association of the RuvC and RuvC monomer subunits. Because the Western blots measure denatured proteins (Fig. 1G and fig. S4B), this is predicted rather than measured directly. When RuvC homodimers were predicted to outnumber RDG at 50% RuvC to 8% RDG (41 heterodimers), cells became resistant to UV (fig. S4, A and B; 10−4 mM isopropyl-β-d-thiogalactopyranoside [IPTG]). The data imply that RDG traps HJs in living *E. coli*, inhibiting RuvC action, even when RDG homodimers are only about half as numerous as functional RuvC homodimers (Fig. 1G), but not when RuvC homodimers exceed RDG homodimers by a factor of ≥6 (fig. S4, A and B). In experiments detailed below, we produced RDG homodimers in ≥50-fold excess of native RuvC homodimers (fig. S4C) to capture most or all HJs.

Second, RDG also inhibited recombination of linear DNA with the *E. coli* chromosome [transdutional HR; per Magner et al. (18)], implying inhibition of the pro-HR activities of HJ-processing proteins, including RuvC and RecG. In transductional HR, RuvC and RecG partially substitute for each other such that cells deleted for *ruvC* or *recG* are somewhat HR-deficient, and *ruvC recG* double mutants are far more HR-deficient (Fig. 1H, gray bars; RDG not induced, compare RDG with its *ΔruvC* and *ΔrecG* derivatives and both with *ΔruvC ΔrecG*) (32). We found that induction of RDG (Fig. 1H, green symbols) reduced transductional HR by 6.6 ± 0.01 times in the presence of endogenous RuvC and RecG proteins (Fig. 1H, RDG green bar, induced, compared with RDG gray bar, not induced), regardless of native RuvC (Fig. 1H, RDG *ruvC* green bar), and somewhat more in *ΔrecG* or *ΔruvC ΔrecG* cells (Fig. 1H, green). The data imply that RDG blocks both RuvC and part of RecG HR-promoting action on DNA in living cells.

Last, RDG blocks the action of the *RusA* HJ and three-way junction endonuclease [reviewed by Mahdai et al. (37)] in living *E. coli*. In the following section, we show that RDG fluorescent foci correspond with HJs. Here, we used timed production experiments with regulatable *RusA* and RDG to show that the numbers of spontaneous foci of RDG are reduced if *RusA* is produced before RDG but are not reduced if RDG
is produced first (Fig. 1I). The data imply that when RuvA is produced first, it reduces cellular levels of spontaneous HJs (Fig. 1I, right), the remainder of which become RDG foci when RDG is produced. Further, when RDG was produced first, RuvA had no significant effect on levels of spontaneous RDG foci (Fig. 1I, left). The data imply that RDG blocks the nuclease activity of RuvA on HJs, seen as foci in living E. coli cells.

**RDG binds and labels HJs in single living cells**

We show that RDG forms fluorescent foci that are correlated with HJs from HR-DSB repair in E. coli cells (for example, Fig. 1A, HR repair model), as follows. We induced low levels of chromosomally encoded I–Sce I double-strand endonuclease (38) in proliferating E. coli with the I–Sce I cleavage site either near to or far from the replication origin (ori) (I-sites, red arrows, DSB; Fig. 2A) to create more or fewer reparable DSBs, respectively, in the more and fewer copies of those two chromosomal regions caused by replication (diagrammed in Fig. 2A). We validated the differential numbers of DSBs per cell as fluorescent foci of GamGFP, a DSB-specific trap protein (Fig. 2B), per Shee et al. (39).

We find that DSB repair induces RDG foci; we found 11 ± 3 and 9 ± 3 times more cells with RDG foci (means ± SEM) with I–Sce I cleavage than in uncleaved control cells (cut site, no enzyme; Fig. 2, C and D; values are for ori-proximal and ori-distal cleavage, respectively). Because of the extra DNA copies near the ori caused by replication (Fig. 2A), both more DSBs (Fig. 2B) and more opportunities for repair with a potential uncleaved sister chromosome are expected for ori-proximal than ori-distal DSBs (Fig. 2A). We found that ori-proximal cleavage of the chromosome produced 10 ± 3 times more cells with >1 RDG focus than did ori-distal cleavage (Fig. 2D). These data correlate the number of RDG foci with DSBs expected to be undergoing HR repair via HJs. The data also indicate that multiple events can be visualized as >1 focus per cell—the foci do not coalesce into a single spot.

Further, RecA and RecB proteins, which are required for HJ formation during HR-DSB repair (Fig. 1A) (40, 41), were required for I–Sce I–induced RDG focus formation (Fig. 2E). This supports the interpretation that DSB-induced RDG foci indicate HR-HJs. RecF loads RecA at non-DSB-instigated HR events (40–43) and is not required for I–Sce I–induced RDG foci, as expected (Fig. 2E).

RDG foci are also correlated with numbers of HR-reparable DSBs produced by gamma rays (Fig. 2F and text S2). In text S2, these and other data are used to estimate an efficiency of RDG detection of HJs of about 50%.

Four additional lines of evidence support the conclusion that the RDG foci represent HJs. (i) I–Sce I–induced (Fig. 2E) and spontaneous (Fig. 3A) RDG focus formation requires RuvB, which stabilizes purified RuvC binding to HJs in solution (32), and does not require RuvA, which is not required for RuvC HJ binding biochemically (44). Purified RuvC can recruit RuvB to model HJs in the absence of RuvA protein in solution (45). Because of the specificity of RuvC for HJs (text S1) (30) and the ability of RuvB to stabilize RuvC at HJs (45), our data imply that in living cells, RuvC/RDG can also bind HJs without RuvA and can recruit and be stabilized by RuvB, implying that RDG foci indicate HJs.

Next, (ii) production of HJ endonuclease RuvA before RDG reduced the number of spontaneous RDG foci, implying that the foci indicate HJs (Fig. 1I). (iii) The mCherry-tagged RDM forms spontaneous foci that colocalize with foci of a partial-function mutant RecA-GFP fusion protein (fig. S5) (46), but not with GFP alone, placing RDM foci in the cellular vicinity of DNA damage or repair (fig. S5). Foci of proteins bound to defined DNA sites are easily distinguishable at sites separated by 55 kb (39) [13 kb for Wang and Sherratt (47)], and are separated at ≥80 kb (39, 47), such that the colocalization here puts RDG roughly near RecA-bound DNA. (iv) Using ChIP-seq in the following section, we demonstrate that RDG binds sites of HR-DSB repair. We conclude that RDG foci indicate four-way DNA junctions in single living cells.

**RDG ChIP-seq of HR-HJs at repairing DSBs shows genomic directionality of DSB repair**

We mapped the genomic repair landscape of RDG at sites of DSB repair induced by I–Sce I cleavage by ChIP-seq using an antibody against RuvC. Figure 2G shows significant enrichment of DNA sequences near I-site L (red line), near the chromosomal replication origin, oriC (black line), and downstream of I-site L in the unidirectional replication path (fig. S6, additional controls). RDG enrichment extends 63 to 73 kb ori-proximally and 169 to 173 kb ori-distally from the cut site (change point analysis; two independent experiments). Surprisingly, a smaller enrichment occurred near the replication terminus (Fig. 2G), possibly from break-induced repair replication HJs that continue with the replication fork to the terminus (see Discussion). Both areas of enriched reads are DSB-dependent and are not observed in cells without I–Sce I cleavage (DSB–, Fig. 2G). Therefore, the enrichment reflects binding of RDG to DNA associated with DSB repair.

In Fig. 2H, with cleavage about halfway between the replication ori and terminus, reads were again enriched at the I-site and preferentially downstream in the replication path (143 to 145 kb ori-proximally and 207 to 207 kb ori-distally, change point analysis; two experiments). The RDG-DNA binding required the strand-exchange (HJ-producing) protein RecA and its DSB-specific loader (48) RecB (Fig. 2H), indicating HR-DSB repair specificity, and was independent of RecF [RecA loader at single-stranded DNA (ssDNA) gaps (42, 43) and RuvA (Fig. 2H), which is not required for RuvC binding biochemically (44)]. We conclude that the ChIP-seq maps of RDG binding show the genomic locations of HJs formed by HR-DSB repair. These first glimpses of the genomic footprints of HJs during DSB repair demonstrate a directionality of DSB repair along the chromosome not observed previously, with more HJs ori-distally than ori-proximally (see Discussion).

**Most spontaneous RDG foci result from HR DNA repair**

With no DNA damage induced, RDG foci appeared spontaneously (figs. S7 and S8, A to C), most, we show, as a result of spontaneous DNA damage and HR repair. Appearance of most spontaneous RDG foci required the RAD51-orthologous strand-exchange HR repair protein RecA and its loader at ssDNA gaps (42, 43), RecF (Fig. 3, A and B, and fig. S8A), which is analogous with RAD52, the human RAD51 loading preparation protein (49). Purified RecA aids formation of relaxed forks (RFs) in solution (50), but RecA is not required for the formation of, or RuvABC action on, RFs in living E. coli (33) and is required for HR repair (40, 41). About 75% of spontaneous RDG foci required RecA, RecF, and RuvB (Fig. 3A), supporting their origin as spontaneous HR repair events. The RuvB dependence supports focus occurrence at HJs. The different numbers of foci in these strains do not result from different growth rates/numbers of replication forks, as shown in fig. S9. Mutants that lack RecA and also RuvB or RecF show no further decrease in spontaneous RDG foci than recA single mutants, indicating that those proteins promote spontaneous RDG foci independently (Fig. 3B). The origin of the 25% RecA-independent spontaneous foci will be addressed in a separate study.
Fig. 2. RDG foci represent HJs in living cells. (A) Strategy for E. coli chromosome cleavage with chromosomally encoded I–Sce I endonuclease at engineered cut sites (red arrows, DSB). Because of DNA replication, proliferating cells have more copies of oriC-proximal than oriC-distal DNA and thus will have more DSBs (and HR repair) per cell when cleaved by I–Sce I oriC-proximally than oriC-distally. (B) DSBs quantified as GamGFP focus, per Shee et al. (39), show that most of the cells with the oriC-proximal cut site have >1 GamGFP/DSB focus, and those with the oriC-distal cut site have mostly 1 focus per cell, as previously described by Shee et al. (39). (C) Representative images of RDG foci after I–Sce I cleavage (top row) or spontaneous foci (bottom row). (D) I–Sce I–induced RDG foci are positively correlated with numbers of DSBs (and HJs) (quantification of images as in (B)), similarly to GamGFP foci in both oriC-proximal and oriC-distal sites. RDG foci increase with DSBs (P = 0.0001 for each locus, two-tailed unpaired t test), and more cells with >1 focus with oriC-proximal than oriC-distal cleavage (P = 0.0001, two-tailed unpaired t test). (E) HR protein dependence of I–Sce I/DSB–induced RDG foci. I–Sce I/DSB–induced RDG foci are reduced by a significant 26.1 ± 0.1 times in recA and 4.3 ± 0.1 (means ± SEM) times in recB null mutants indicating RecAB dependence, supporting their interpretation as HR-dependent foci formed during DSB repair. The dependence of RDG focus formation on RuvB supports their formation at four-way junctions. The independence of ruvA implies that RDG binds directly to DNA four-way junctions, as shown biochemically (44). **P < 0.01, ***P < 0.001, relative to DSB control, two-tailed unpaired t test. (F) RDG foci are positively correlated with dose of DSB-inducing γ radiation (R^2 = 0.99; P < 0.0001, Pearson’s correlation analysis). (G) RDG ChIP-seq shows that RDG localized DNA near a reparable I–Sce I–induced DSB (vertical red line) in the E. coli chromosome. I-site L, I–Sce I endonuclease site L. The large peaks disappear in DSB− cells (cells carrying the cut site but no I–Sce I enzyme), RDG ChIP-seq reads are normalized to the total reads in each sample and further normalized to the input genomic DNA. (H) RDG ChIP-seq shows enrichment at a different chromosomal I–Sce I cleavage site: I-site J, roughly halfway between the replication origin and terminus in the E. coli “right” replichore. The I–Sce I–induced DSB ChIP-seq peak is RecA- and RecB-dependent (indicating HR at a DSB), RecF-independent (indicating formation not at single-strand gaps), and RuvA-independent (supporting direct binding of RDG to four-way junctions, as for purified RuvC) (44). Figure S6B shows the DSB− (enzyme no cut site) controls. WGS, whole-genome sequencing.
Fig. 3. Spontaneous HR repair HJs are replication-dependent and instigated by mostly non-DSB DNA damage. (A) Non-DSB damage provokes most spontaneous HJs in vegetative, growing E. coli: RecB independence indicates that most spontaneous DNA damage repaired is not DSBs. RecF dependence implies ssDNA gaps (42, 43). RuvB dependence: four-way junction–specific. recG mutant has increased spontaneous RDG foci, indicating that RecG reduces the steady-state level of spontaneous HJs, as predicted by Whitby et al. (91). (B) RecA dependence of the spontaneous HR-HJ foci in the various mutants. (C and D) DSB independence of most spontaneous HJ foci. Gam, DSB-specific DNA end–binding protein (92, 93) blocks I-Sce I–induced HJ foci (C), but does not block most spontaneous HJ/HR repair foci (D), n.s., not significant (control, fig. S13). (E) Replication dependence of most spontaneous RDG HJ foci. Spontaneous HR-HJ foci are reduced in dnaATS cells relative to WT at nonpermissive temperature (42°C), at which replication initiation is blocked. P = 0.03, two-tailed unpaired t test. (F to I) Microfluidic time-lapse imaging shows the birth, generation dependence, and persistence of spontaneous RDG HJ foci. (F) Representative images of microcolony with RDG foci (blue arrows) in WT strain. (G) Quantification of fates of spontaneous RDG HR-HJ foci and cells that acquire them: most spontaneous RDG foci persist over hours after formation; most cells with RDG foci cease to divide, indicating that the birth of each spontaneous focus reports on a new HJ event. (H) Quantification of generation dependence of the birth of spontaneous RDG foci. Rapid growth in glucose was followed by washing cells in the same medium lacking glucose to slow and halt cell divisions in the WT strain. Blue circles, number of cell divisions; green circles, cumulative number of spontaneous foci that appear in each microfluidic microcolony. (I) Rates of spontaneous RDG focus formation in rapid and stationary growth phases shown in (F).
Replication-dependent non-DSB DNA damage underlies most HR-HJs in vegetative *E. coli*

Surprisingly, most HR protein–dependent RDG/HJ foci do not result from DSB repair, as shown in two ways. *E. coli* HR-DSB repair requires RecBC [RecA loader at DSBs (S1)], analogous with human BRCA2, which loads RAD51 at DSBs (S2–S4), and not RecF, RecJ, or RecQ (40–43). In agreement, we see RecB dependence and RecF independence of I–Sce I–induced RDG foci (Fig. 2E, DSB+). By contrast, spontaneous RecA/HR-dependent RDG/HJ foci form independently of RecB (Fig. 3A), and were promoted by RecF (Fig. 3A), indicating repair of non-DSB DNA damage. Further, production of the phage Mu DSB end–binding protein Gam inhibits DSB repair (39) and I–Sce I–induced RDG foci (Fig. 3C) but did not affect spontaneous HR-dependent RDG foci (Fig. 3D and Fig. 59, additional controls). We conclude that the main use of HJ-mediated HR in vegetative (nonsexual) *E. coli* is repair of DNA damage other than DSBs. The RecF dependence implicates ssDNA gaps, a substrate at which RecF loads RecA (42, 43).

The spontaneous DNA damage that necessitates HR-repair HJs is replication-dependent. We blocked replication initiation at the *E. coli* origin, oriC, using a dnaATS allele, which allows replication at 30°C but blocks oriC use at 42°C (55). We found 30.8 ± 0.2 times fewer spontaneous RDG foci per amount of DNA (fig. S8G) in *dnaATS* than the WT control strain at the 42°C restrictive temperature (Fig. 3E, quantified spontaneous HJ/RDG foci via microscopy, and found a nearly constant spontaneous RDG focus frequency per replication fork: 5.0 × 10⁻³ (±0.3 × 10⁻³) and 4.2 × 10⁻³ (±0.6 × 10⁻³) in rich and minimal medium, respectively (Fig. S8F). Most cells with RDG foci had one focus per cell (70.6 ± 0.4%), with the minority having more than one (Fig. S8B). Because multiple HR repair events appear as multiple foci per cell (Fig. 2, A to D), we infer that most spontaneous repair HJs result from one or few DNA lesions per cell, rather than genome-wide catastrophe. The data support DNA replication as the origin of most spontaneous DNA damage repaired by HJ-associated HR.

RecQ and RecJ promote spontaneous HR-HJ formation

RecQ promotes both formation and dissolution of HJs biochemically (16, 17, 57) and promotes net accumulation of HR-HJs in cells (18), however, whether RecQ promoted HR formation, aiding HR, or inhibited HJ resolution, reducing spontaneous HR in cells, was unknown (18). We show that both RecQ DNA helicase and its partner RecJ ssDNA-dependent exonuclease promote spontaneous HR-HJ formation and spontaneous HR in vegetative *E. coli* cells.

We found that spontaneous RDG foci were reduced by deletion of recJ or recQ, or recJ and recQ genes (Fig. 4A and figs. S9 and S10). We performed timed expression studies to trap HJs with RDG before the production of RecQ. HJ formation proteins are expected to increase HJ levels independently of whether RDG is present first to trap the HJs, whereas proteins that act after HJ formation (on HJs), such as RusA, are stopped by the production of RDG first (Fig. 1I). We found that RecQ increased RDG/HJ focus levels whether it was produced before or after RDG (Fig. 4B). These data support RecQ and RecJ as promoters of HJ formation.

Further supporting this conclusion, the following data suggest that RecQ and RecJ act before RecA. We observed that deletion of recQ or recJ from recA cells did not reduce spontaneous HJs further than in recA cells (Fig. 4, A and C), indicating their action in the same pathway as RecA.

The human RecA ortholog is repair of DNA damage other than DSBs. The RecF +RecQ act upstream of RecA in their pathway. We also saw that spontaneous foci of a mutant RecA-GFP fusion protein (46) were reduced in *RecQ* or *RecQ* single mutants (Fig. 4C) argues [per Avery and Wasserman (58)] that RecQ and RecJ act upstream of RecA in their pathway. We also saw that spontaneous foci of a mutant RecA-GFP fusion protein (46) were reduced in *RecQ* or *RecQ* strains (Fig. 4, D and E). These data imply that RecQ and RecJ act before RecA (strand exchange) in HR and may promote RecA loading on ssDNA. RecF is the RecA loader at ssDNA gaps (42, 43), and 46 ± 11% of the spontaneous RecA-GFP foci were RecF-dependent (Fig. 4E), implying that these spontaneous RecA-GFP foci represent RecA on DNA at ssDNA gaps. We discuss a possible pre-RecA role of RecQ and RecJ in postreplication ssDNA gap repair in the Discussion.

We examined spontaneous HR events themselves to verify these conclusions. We found that most spontaneous HR that recombined close chromosomal direct repeat sequences required RecF, RecQ, and RecJ (in addition to RecA) (Fig. 5, A and B), showing a pro-HR role for RecQ, compatible with HJ formation and incompatible with inhibition of HJ resolution—the two ways that RecQ could have promoted net accumulation of HR-HJs in cells (18). The RecF dependence of most spontaneous RDG foci (Fig. 3, A and B) and HR events (Fig. 5B) supports recombinational ssDNA gap repair as the origin of most spontaneous HJ foci in vegetative, growing *E. coli* cells (model, Discussion). The data demonstrate that RecQ and RecJ promote HR-HJ formation in living cells, and imply that they act before RecA during replication-induced ssDNA gap repair, and that this is a primary role normally in growing cells.

RecQ and RecJ prevent non-HR-HJs in a cancer-state model

The human RecA ortholog RAD51 is overexpressed in a wide range of tumors with p53 defects (11), and is correlated with poor prognosis (9, 10); but how increased RAD51 supports the cancer state is unknown. We modeled RAD51 overexpression in cancers by overproducing RecA in *E. coli* and discovered that RecA overproduction increased HJ focus levels by a significant 2.09 ± 0.02 times [Fig. 4F, pVector versus pRecA (blue bars)].

The following data indicate that the increased RDG/HJ foci caused by RecA overproduction are not HR-HJs but rather are non-HR–HJs,
such as regressed replication forks. Although RecA overproduction caused more HJ foci (Fig. 4F), it did not increase intrachromosomal HR events in close direct repeat sequences (Fig. 5C), showing a lack of correlation between HR and the extra HJ foci observed.

In addition, the increased RDG foci caused by RecA overproduction were formed independently of RecA loader proteins that promote HR repair of DSBs, RecB (51), and ssDNA gaps, RecF (Fig. 4F) (42, 43). Note that spontaneous RDG foci are RecF-dependent (Fig. 4F, no IPTG induction). If these are subtracted from the additional RDG foci added by RecA overproduction (Fig. 4F, pRecA − pVector), then we see that all of the RecA overproduction-induced RDG foci are RecF-independent (Fig. 4F, pRecA − pVector). RecB and RecF are analogous with human BRCA2 (51–54, 59–61) and RAD52 (49). Because the RecF-dependent component of spontaneous HR-HJ/RDG foci (Fig. 4F, pVector) remained present when RecA was overproduced (Fig. 4F, pRecA), and only the additional RDG/HJ foci are unaffected by RecF (Fig. 4F, pRecA − pVector), we can rule out the possibility that overproduction caused RecA to become RecF-independent for HR. Therefore, RecA overproduction did not cause HR to become independent of the HR RecA loader. Rather, the extra HJs during RecA overproduction appear to arise by a different, non-HR process. We infer that the increased HJs in this RAD51-overexpressing cancer model result from non-HR events (uncorrelated with HR as illustrated in Fig. 5C, and independent of RecA loaders as shown in Fig. 4, F and G): we suggest from replication fork stalling and regression, which occurs independently of RecF (35) and RecB (Fig. 6A) (33), and, biochemically, is promoted by excessive RecA independently of loader proteins (35).

Surprisingly, we found that RecA overproduction resulted in RecQ and RecJ roles opposite to their roles in spontaneous HR (Fig. 5B) and HR-RDG focus formation (Fig. 4, A to E); RecQ and RecJ opposed

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**Fig. 4.** RecQ and RecJ promote formation of spontaneous HR repair HJs and prevent non-HR–HJs caused by RecA overproduction in a model of RAD51-overexpressing cancers. (A) RecQ and RecJ promote spontaneous HJ accumulation acting in the same pathway. P = 0.002, P = 0.002, and P = 0.006, two-tailed unpaired t test for recJ, recQ, and recJ recQ, respectively, compared with WT. (B) RecQ produced from an IPTG-inducible plasmid increased RDG spontaneous HJ foci when produced before (right) or after (left) RDG. Left bar in each panel, no IPTG induction. The data indicate that the RecQ promotion of RDG foci results from RecQ-promoted HJ formation, not RecQ-inhibiting HJ removal, which is blocked by RDG. (C) RecQ and RecJ promote spontaneous HJ/RDG foci via the RecA-dependent (HR repair) pathway, seen as no further reduction of HJ/RDG foci in recA recQ or recA recJ double mutants beyond that in the recA single mutant. (D and E) RecQ and RecJ promote RecA-GFP focus formation, suggesting that they act before RecA. (D) Representative images. Blue arrows indicate foci. (E) Quantification. Spontaneous RecA4155-GFP foci (WT) are reduced in recJ, recF, and recQ null mutant strains. P = 0.001, P = 0.004, and P = 0.02, two-tailed unpaired t test (means ± SEM of three experiments, >1000 total cells scored in each experiment). (F) RecA overproduction causes increased apparent non-HR–HJs, opposed by RecQ and RecJ. RecA overproduction increased RDG foci 2.09 ± 0.02 times (P = 0.01, two-tailed unpaired t test, pVector compared with pRecA; blue bars), independently of RecF or RecB, implying a non-HR origin. There is no RecF dependence when the spontaneous HR-HJs are subtracted out (pRecA − pVector). Moreover, RecA overproduction increased RDG foci by 11- and 9-fold in recQ- or recJ null mutant strains, respectively (P = 0.001 and P = 0.0001, two-tailed unpaired t test). (G) The great increase in RDG foci caused by RecA overproduction in ΔrecQ cells is both RecF- and RecJ-dependent, implying that RecQ prevents non-HR–HJs. We suggest that HJs are regressed replication forks caused by overproduced RecA (Fig. 6B).
accumulation of the extra non-HR–HJs caused by overproduced RecA (Fig. 4F). Cells that lack RecQ or RecJ (DrecQ or DrecJ) showed 11.1 ± 0.2 and 8.8 ± 0.1 times more RDG foci/HJs caused by RecA overproduction, respectively (Fig. 4F). These data support the model shown in Fig. 8. C) Spontaneous HR events are not increased by RecA overproduction. This finding suggests that the increased RDG/HJ foci seen with overproduced RecA (Fig. 4F and G) are not increased HR–HJs, and supports the interpretation that they reflect regressed replication forks (illustrated in Fig. 6Biii). The small decrease in spontaneous HR with overproduced RecA compared with the pVector control (P = 0.02, unpaired t test) could be caused by titration of HJ resolution capacity in the cells via their increased non-HR–HJ levels (Fig. 4F). We hypothesize that the RecQ and RecJ independence of the spontaneous HR events in RecA-overproducing cells may indicate that DSB-instigated HR may dominate spontaneous events during RecA overproduction. This is expected because the presence of increased non-HR–HJs (probable RFs; Fig. 6Biii) is cleaved by RuvABC in living cells producing DSBs (33) and thus may cause DSB-instigated HR, which is RecQ- and RecJ-independent, to dominate.

Cancer transcriptome data show correlation of expression of human RecQ orthologs and HJ resolution proteins with RAD51 in the eight most common human cancers

We looked for RNA data correlations that might indicate human RecQ orthologs with potential similar junction-guardian roles in RAD51-overexpressing cancers. We hypothesized that if the RecQ junction-guardian function improves the fitness/growth of RecA-overproducing cells, then RAD51-overexpressing cancers might require proteins that play RecQ-like roles in preventing or reducing excess HJs caused by RAD51 overproduction. Thus, the genes encoding proteins with RecQ-like or HJ resolvase functions might be overexpressed in RAD51-overexpressing cancers. We found that the human RecQ orthologs BLM and RECQL4, as well as a HJ endonuclease gene, EME1, are frequently co-overexpressed with RAD51 in the eight most common cancers for BLM and in four of the eight most common cancers for RECQL4 (Spearman’s correlation analysis shown in Fig 7, A to E, and table S1).
studies considered to be highly indicative to definitive. For example, CDH1 and ZEB1 (62), and KLF4 and KLF5 (63), showed $R^2$ values of 0.15 and 0.08, both in only a single cancer type, compared with the $R^2$ values for BLM with RAD51 (0.29 to 0.62) in the eight most common cancer types, and for RECQL4 with RAD51 (0.26 to 0.41) in four of the eight most common cancer types (Fig. 7, A to E). Thus, these are extremely robust correlations. Negative control and other genes did not show such correlations (for example, ACTB encoding a subunit of actin; Fig. 7D).

We hypothesize that the increased expression of the RecQ orthologs BLM and RECQL4 and the HJ resolvase EME1 may also prevent (or remove) excess HJs. The most coexpressed HJ resolvases and RecQ ortholog with BLM are EME1, GEN1, and RECQL4 (Spearman’s correlation analysis shown in fig. S12 and table S1). These correlations may indicate that EME1, GEN1, and RECQL4 may work together with BLM to reduce or prevent excess HJs caused by RAD51 overexpression, a common event in many and varied cancers. Further work is needed to test these hypotheses.

DISCUSSION

Engineered HJ-trap proteins

We showed that RuvC-derived, catalytically inactive fluorescent protein fusions (RDG, green; RDM, red) specifically bind, trap, and label four-way DNA junctions (HJs), allowing their quantification as fluorescent foci in single living cells (Figs. 1I, 2, A to F, and 3 and figs. S7 and S8) and their mapping in genomes via ChiP-seq (Fig. 2, G and H, and fig. S6). RDG binds and protects HJs from the action of other proteins, that is, traps HJs, when purified in solution (Fig. 1E and fig. S2) and in living cells (Figs. 1, F to I, and 2). The estimated efficiency of detection of HJs (or double HJs) as individual fluorescent foci is roughly 50% (text S2).

Like the engineered DSB-trap proteins created previously (39), RDG and RDM are useful tools for studying DNA reaction intermediates in single living cells and in genomes.

A limitation of RuvC-based HJ traps is their dependence on E. coli RuvB (Figs. 2E and 3, A and B), probably via their specific protein-protein interactions (32). We are currently exploring whether this will limit the effectiveness of these tools in other organisms.

The main use of HJ-associated HR is repair of replication-instigated single-strand gaps

Although DSB repair mechanisms are the most studied HR reactions, we find that the main use of HJ-associated HR in vegetative (nonsexual) E. coli cells is not DSB repair. Surprisingly, spontaneous HJs are (i) 75% HR-based (Fig. 3, A and B), (ii) DSBR-independent (Fig. 3, C and D), (iii) mostly RecFJQ-dependent (Figs. 3, A and B, and 4, A to C), (iv) replication-dependent (Fig. 3E), (v) correlated with replication fork numbers (Fig. 3, F to H, and fig. S8), and (vi) about 100 times less frequent than the once per E. coli genome replication estimated previously (Fig. 3, F to H; fig. S8; and text S2) (14, 40). Collectively, our data imply that repair of replication-induced ssDNA gaps (illustrated in Fig. 8) is the commonest use of HR and source of HJ intermediates in growing E. coli cells. Endogenous DNA damage is the primary instigator of repair-based genomic changes that drive cancer, genetic diseases, and microbial evolution (see Introduction). We suggest that single-strand lesions constitute the primary instigator of these changes. Other environmental conditions might produce other results.

Genomic footprints of DSB repair HJs show genome-scale coordination and directionality of repair

When DSBs do occur, as when induced by I–Sce I endonuclease, their repair shows evidence of two modes of whole-genome control and
Fig. 7. Increased BLM and RECLQ4 mRNA in RAD51-overexpressing human cancers of the eight, and four of the eight, most common cancer types, respectively. Spearman’s rank correlation analyses of data from cBioPortal (88, 89), per Materials and Methods. Each data point represents the mRNA level in one patient sample relative to the reference population (Z score; Materials and Methods). Data from 129 to 1100 patient samples were analyzed per cancer type (table S1). (A) Increased BLM mRNA levels (y axis, Z scores) correlated with increased RAD51 mRNA (x axis, Z scores) in eight of eight of the most common cancer types ($R^2 > 0.25; P \leq 6.23 \times 10^{-21}$, Spearman’s rank correlation analysis). (B) Increased RECLQ4 mRNA levels (y axis, Z scores) correlated with increased RAD51 mRNA (x axis, Z scores) in four of eight of the most common cancer types ($R^2 > 0.25; P \leq 2.07 \times 10^{-8}$, Spearman’s rank correlation analysis). (C) Summary: correlation of increased RAD51 mRNA levels with increased levels of BLM and RECLQ4 RecQ orthologs, EME1 HJ resolution protein, and other RecQ orthologs and resolution proteins in patient tumor data of the eight most common cancers (numbers in parentheses, number of common cancers of the eight most common types with correlation). mRNA Spearman’s correlation coefficient calculated between these and other human RecQ orthologs and other HJ resolvases with RAD51 and with each other among the eight most common cancers is summarized in table S1 ($R^2 > 0.25$ indicates moderate correlation; see table S1 for details). (D) Summary of Spearman’s $R^2$ values for cancer RNAs of genes correlated with RAD51 overexpression ($R^2 > 0.25$ for moderate correlation) and control genes poorly correlated or uncorrelated with RAD51 overexpression (for example, ACTB, which encodes a subunit of actin).
largely dependent on the major replicative DNA polymerase, Pol III terminus (Fig. 9E), which is a new discovery. DSB repair in and imply that HR-initiated replication bubbles can sometimes drag (side) but also at the chromosomal replication terminus region, mega-

continues from the DSB to the replication terminus (Fig. 9) (initiated by HR-DSB repair [break-induced replication (BIR)] primed by HR strand-exchange intermediates (replication restart at a bases away (Fig. 2, G and H, and fig. S6). These data support a rep-

lication model for HR-DSB repair (Fig. 4C); (ii) that RecQ/J act before HJ formation, shown by their promotion of HJs even after RDG is produced (Fig. 4B); and (iii) that RecQ/J promote apparent RecA loading in assays of spontaneous RecA-GFP foci (Fig. 4, D and E) thought to reflect RecA-DNA nucleoprotein filaments (46). Model: During vegetative growth and DNA replication, (A) when a synthesis tract of lagging strand DNA (an Okazaki fragment, arrowheads, 3′ ends) encounters a replication-blocking lesion (star) in its template strand, (B) the adjacent Okazaki fragment can be unwound by RecQ DNA helicase, which translocates on DNA in the 3′ to 5′ direction as shown by Hishida et al. (69), exposing a 5′-ssDNA end, which is then degraded by RecJ 5′-ssDNA–dependent exonuclease. This creates a single-stranded gap, which can be coated by SSB (E. coli single-stranded DNA binding protein, like human replication protein A (96)). (C) RecF is guided to a 5′ end of a ssDNA-dsDNA junction (43) and loads RecA onto SSB-coated ssDNA (42). The RecA-DNA nucleoprotein complex then promotes strand exchange (40, 41) so that the blocked 3′ Okazaki fragment end can displace the identical sequence (black line) in the sister chromosome, and prime continued DNA synthesis (dashed red line) using the new strand of the sister as a template. Lines, strands of DNA; red lines, new DNA strands; black lines, old strands; dashed red line, new DNA synthesis after strand exchange. (D) Branch migration of the HJ rightward, past the lesion, places newly synthesized DNA across from the lesion. Further branch rightward returns the newly synthesized strand to its original template (E) and returns the 3′ end to the original duplex (F), removing the HJ and D loop. The lesion is bypassed and, once it is part of dsDNA, can be repaired by excision repair pathways, which can act only when the damaged base or nucleotide is present in dsDNA [base excision repair and nucleotide excision repair; reviewed by Friedberg et al. (97)]. An alternative mode of resolution of the HJ is endonuclease cleavage, for example, by RuvC (not shown).

directionality along the chromosome (Fig. 2, G and H, and fig. S6). First, I-Sce I–induced, RecA- and RecB-dependent HR-HJs occurred not only at the repairing DSB sites (between 60 and 200 kb on either side) but also at the chromosomal replication terminus region, megabases away (Fig. 2, G and H, and fig. S6). These data support a replicative model for HR-DSB repair (64), in which replication forks are primed by HR strand-exchange intermediates (replication restart at a DSB) that contain a HJ (Fig. 9C). The data suggest that the replication initiated by HR-DSB repair [break-induced replication (BIR)] continues from the DSB to the replication terminus (Fig. 9) (65), and imply that HR-initiated replication bubbles can sometimes drag their associated HJs from the DSB (65) all the way to the replication terminus (Fig. 9E), which is a new discovery. DSB repair in E. coli is largely dependent on the major replicative DNA polymerase, Pol III (65), and produces conservatively segregated new strands of DNA (65), supporting the model in Fig. 9, modified from the model suggested by Motamed et al. (65). In yeast, BIR bubble migration was observed and thought to D loops without HJs (similar to Fig. 1A) (66), but we suggest that it might also, at least sometimes, include unresolved HJs, as we have observed here (Fig. 2, G and H, and fig. S6). These data support both the replicative nature of much of DSB repair and the frequent asymmetry (one-endedness) of the events, which allows extended genomic replication (for example, in contrast with two-ended mechanisms such as shown in Fig. 1A). Alternatively, the DSB repair–instigated HJs at the replication terminus might result from site-specific HJ-dependent resolution of chromosome dimers caused by crossing-over during HR-DSB repair, which is performed by the XerCD site-specific recombinase at the replication terminus dif site [re-

viewed by Lesterlin et al. (67)]. This seems less likely because the en-

richment of terminus-proximal reads in ChiP-seq was far broader and larger than the 29–base pair (bp) dif site, to which XerCD site-specific HJs are confined.

Second, the data show skewed distributions of HJs around re-

pairing two-ended DSBs in the E. coli chromosome with more HJs on the terminus-proximal side of the DSB than on the ori-proximal side of the DSB. That is, there are more HJs downstream than upstream of the DSB in the chromosomal replication paths (Fig. 2, G and H, and fig. S6) or "replichores." For two different I-Sce I cleavage sites, there were more RecA- and RecB-dependent HJs terminus-proximally than ori-proxi-

mally (Fig. 2, G and H, and fig. S6). These data support the Kuz-

minov model (68) of asymmetrical DNA degradation and repair at DSBs in the E. coli chromosome (illustrated in Fig. 9, A to C) controlled by the asymmetrical distribution of chromosomal Chi sites. Chi sites attenuate RecBCD dsDNA exonuclease activity to allow HR end repair (40, 41) and fall asymmetrically in the E. coli genome with more active Chi sites upstream than downstream from any point in the replichores. Therefore, at any DSB in the genome, the ori-proximal DNA end is better protected from degradation by RecBCD exonuclease (more active Chi’s) and is more likely to be preserved, whereas the terminus-proximal DSB end is more likely to be degraded (few active Chi’s), shown in Fig. 9B (68). Preservation of the ori-proximal end and loss of the ter-proximal end (Fig. 9B) were proposed to cause one-ended DSB repair replication (BIR), with the direction of the repair replication preferentially from ori (the preserved end that initiates HR-

mediated replication restart; Fig. 9, B and C) toward the terminus (Fig. 9, B to E). This model is compelling but untested. Our observation of more
A junction-guardian role of RecQ and its implication for BLM and RECQL4 human orthologs in RAD51-overexpressing cancers

We discovered a novel junction-guardian role for E. coli RecQ DNA helicase, ortholog of five human cancer prevention proteins (15), in promoting and preventing HJs. First, RecQ appears to act upstream of RecA loading in living cells (Fig. 4, D and E) and is required for the formation of most RecA-dependent spontaneous chromosomal HR-HJs (Fig. 4, A to C) and also for most spontaneous intrachromosomal HR events (Fig. 5B). Our data indicate that RecQ promotes HR-HJs during replication-instigated ssDNA gap repair (model illustrated in Fig. 8). Second, we also discovered that RecQ and RecJ prevent non-HR–HJs induced by RecA overproduction (Figs. 4, F and G, and 5C; model illustrated in Fig. 6B), an E. coli model of many p53-defective cancers with RAD51 overexpression (11). Our results are partly similar to previous observation of UV light–induced accumulation of plasmid DNA structures identified by bulk two-dimensional gel electrophoresis as either double-Y junctions or HJs (20), which, like our non-HR–HJs, were dependent on RecF (20). Whereas those authors suggested a post-HJ role of RecQ and RecJ in removing regressed replication fork HJs, our data imply that RecQ and RecJ prevent the formation of (RecF-independent) RF HJs. That these non-HR–HJs are induced by overproduction of RecA models many p53-defective cancers with RAD51 overexpression (11).

RAD51 overexpression supports breast cancer metastases (9) and is correlated with decreased survival of lung cancer patients (10). We discovered that RecA overproduction significantly increased HR protein–independent HJs and that RecQ/J play a novel junction-guardian role in DSB-induced repair HJs terminus-proximally to DSBs (Fig. 2, G and H, and fig. S6) is predicted by the Kuzminov one-ended repair model if the HJs that accompany the strand exchange that primes BIR sometimes remain with the replication bubble, unresolved, and travel with the bubble toward the terminus (Fig. 9, C to E). The bias toward HJs ori-distally from DSBs might alternatively reflect two-ended DSB repair HJs (not shown in Fig. 9, but similar to that in Fig. 1A, iii and iv) following asymmetrical degradation (Fig. 9B). Other Chi-independent asymmetrical chromosomal features might underlie the HJ asymmetry observed (replication forks in progress, dominant directions of transcription). Additional work is needed to distinguish specific models. Regardless of the specific mechanism, the data demonstrate genome-scale control and directionality of DSB repair events along the E. coli genome.

Fig. 9. Model: One-ended DSB-induced repair (“BIR”) replisomes drag HJs to the E. coli replication terminus. (A) to (C) incorporate ideas from Kuzminov (68), and (D) and (E) illustrate a model of Motamedi et al. (65). (A) Chi sites attenuate RecBCD double-strand exonuclease activity (40, 41) and are situated asymmetrically in the genome such that if a two-ended DSB were to occur, (B) the DNA on the replication terminus–proximal side would be likely to be degraded extensively (few active Chi’s in the ori-to-ter direction), whereas the DNA on the ori-proximal side would suffer less degradation (many active Chi’s in the ter-to-ori direction) (68). (C) Thus, the non-degraded DSB end would initiate HR repair by strand exchange that would prime a replication fork that would run in the chromosome’s natural ori-to-ter direction toward the terminus. (D and E) The model parts illustrated in (C) to (E) were offered previously by Motamedi et al. (65) in support of our observations that most DSB repair via HR requires the major replicative DNA polymerase (Pol III) and that the new strands are segregated conservatively (65), as observed subsequently in yeast BIR (98, 99). We suggested that the replication bubble proceeds toward the terminus, dragging an unresolved HJ behind it, which forces the new DNA strands out of the bubble, causing the observed conservative segregation of newly synthesized DNA strands in DSB repair replication (65). (E) With their trailing HJs, forks that begin 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 site(s) (red, triangular sides stop oncoming forks) on the opposite side of the chromosome center point from which the bubble began. This pattern of HJ accumulation is seen in the ChIP-seq data shown in Fig. 2 (G and H).
preventing these (Fig. 4, F and G; model, Fig. 6B). RecA overproduction did not cause RecF independence of HR (Fig. 4F), and HR was not correlated with increased RecA-induced HJs (Fig. 5C), such that most of the RecA overproduction–induced extra HJs (Fig. 4, F and G) are implicated to arise HR-independently. We suggest that these non-HR–HJs are regressed replication forks (Fig. 6Biii). We hypothesize that excess RecA/RAD51 causes fork regression spuriously on undamaged DNA (illustrated in Fig. 6B). RecQ and RecJ could prevent the HJ stage of fork regression by unwinding and digesting the lagging strand, as shown (Fig. 6Biv) and implicated by RecQ and RecJ biochemistry (69) and DNA degradation in cells (19, 70, 71). This dual, junction-guardian role (promoting HR–HJs, preventing RF–HJs) could be shared by one or more human RecQ orthologs. Supporting this hypothesis, we found that BLM and RECQL4 are co-overexpressed with RAD51 in the eight and in four of the eight most common cancer types, respectively (Fig. 7, fig. S12, and table S1). Moreover, the known human HJ resolution protein genes EME1 and GEN1 (72) are co–up-regulated with BLM and RAD51 (Fig. 7, fig. S12, and table S1). We suggest that at least one consequence of overexpressed RAD51 in tumors could be increased RFs, which are genome-disturbing and cause genome evolution that could drive the cancer state (73), and that BLM and RECQL4 may prevent RF–HJs, whereas EME1 and GEN1 may cleave the RFs, creating DSB ends that cycle through repair and replication. Purified BLM can promote fork regression in solution (74), and RECQL4 promotes HR–HJs (26). Perhaps they also prevent RF–HJs in cells. Failure to remove RFs would block replication and chromosome segregation, so their removal would be expected to be selected in cancers. Further work is needed to test these hypotheses. We are working toward human cell–compatible HJ traps that may aid exploration of these and many other ideas concerning HJs and genome instability in human cancers.

**MATERIALS AND METHODS**

**Strains, media, and growth**

Strains used in this study are given in table S2. Bacteria were grown in LBH rich medium or M9 minimal medium (75) supplemented with thiamine (10 μg/ml; vitamin B1) and 0.1% glucose or glycerol as a carbon source. Other additives were used at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), and sodium citrate (20 mM). P1 transductions were performed as described by Thomason et al. (76). Genotypes were verified by antibiotic resistance, polymerase chain reaction (PCR), and, when relevant, UV sensitivity and sequencing.

**Cloning and chromosomal expression cassettes encoding RuvCGFP, RDG, and RDM**

The *ruvC* gene (without stop codon) and *gfpmut3* (77) (or mCherry) (39) gene were fused with a linking sequence (5′-GCTATGCAG-GAAAACAAACAGAAAGCGTTGGCGGCAGCA-3′) using the protocol described by Heckman and Pease (78) and ligated into the pET28a vector. The two mutations (D7N and E66D) of *ruvC* (or *ruvC*Defgfp or *ruvCDefmCherry*) were introduced into the chromosome of SMR19152 by Red-mediated recombinase forming primers P1 and P2 (table S4) and then transduced into the strains listed in table S2. SMR19152 encodes constitutively produced TetR protein that represses the P*ruvC*Def promoter in the absence of the inducer doxycycline (80). All constructs were verified by PCR, antibiotic resistance, and sequencing.

**Protein purification**

The *ruvC*Gfp and *ruvCDefgfp* genes were subcloned into pET28a expression vectors and then transformed into the BL21 derivative strain (T7 Express lyS7/F7 Competent E. coli, New England Biolabs). After induction with 0.1 mM IPTG, the proteins with an N-terminal His6-tag were produced at 20°C for 16 hours and pelleted by centrifugation at 7000 rpm for 30 min. The cell pellet was resuspended in lysis buffer [50 mM Hepes (pH 8.0), 300 mM NaCl, 5 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride] and disrupted by sonication (10 times for 30 s, on/off at output 5; XL-2000, Misonix). The total cell lysate was centrifuged at 30,000g for 40 min, and the soluble recombinant protein was purified by immobilized metal ion chromatography with a Ni-NTA column (Qiagen). After washing with wash buffer (lysis buffer with 50 mM imidazole added), the protein was eluted with elution buffer (lysis buffer with 500 mM imidazole) and further desalted against the storage buffer [20 mM Hepes (pH 8.0) and 20 mM NaCl]. The protein was concentrated to about 2 mg/ml and stored at −80°C. The Flp protein was purified to near homogeneity using DNA affinity enrichment as the final step (81).

**Western blot**

Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane according to the manufacturer’s instructions (Amersham, GE Healthcare). The membranes were blocked with ECL Prime blocking agent (GE Healthcare) and probed with primary mouse anti-RuvC monoclonal immunoglobulin G (IgG) antibodies (Santa Cruz Biotechnology). The membrane was further probed with secondary polyclonal goat anti-IgG antibody (Bethyl Laboratories) and visualized by scanning in a multicolor imager Typhoon detection system (GE Healthcare).

**UV sensitivity test**

Saturated overnight, LBH cultures were diluted 100-fold in LBH medium and grown at 37°C for 1.5 hours, at which time doxycycline (100 ng/ml) was added to induce protein production. After 1 hour of induction, cells were plated on LBH solid medium containing doxycycline (10 ng/ml), and the plated cells were irradiated with different doses of UV light using a Stratalinker 1800 UV crosslinker (Stratagene) and then incubated in the dark overnight at 37°C for colony quantification. Control cultures without doxycycline induction were treated identically.

**Assembly of synthetic HJs**

The oligonucleotides used for the assembly of HJs (HJa-HJc) that served as substrates in binding and cleavage assays (Fig. 1E and fig. S2, A to C) are listed in table S4. For HJa, assembled from oligonucleotides HJa1 to HJa4, the bases that form a 2-bp homology core in
the central region of HJ for RuvC cleavage are shown in bold. For HJb, composed of oligonucleotides HJb1 to HJb4, the bases that form the recognition sequence for Flp are shown in bold. HJc, constructed from oligonucleotides HJc1 to HJc4, was the substrate for restriction enzyme digestion assays. The sequences that form the recognition sites for restriction enzymes in HJc are shown in bold. The four arms of HJb were equal in length, as were those of HJc, and surrounded an immobile branch point. To assemble a given junction, equimolar amounts of the four requisite oligonucleotides were combined in 50 mM tris-HCl (pH 8.0), 5 mM EDTA, and 1 mM dithiothreitol (DTT), and the mixture was placed for 5 min in a water bath maintained at 80°C. Heat was turned off, and the bath was allowed to cool slowly to room temperature on the bench top. Nearly quantitative assembly of the junction was verified by gel electrophoresis.

**Biochemical HJ-binding assays**

The binding and competition assays were performed in 50 mM tris-HCl (pH 8.0), 5 mM EDTA, 1 mM DTT, 5% glycerol, and bovine serum albumin (100 mg/ml), containing 2 pmol of the HJb per incubation mixture. RuvCGFP or RDG gave complete binding of the junction at a molar ratio of 1:10 (junction to protein). For the standard binding assays, incubations were carried out on ice for 15 min after protein addition. For the competition assays, the junction was preincubated with RDG on ice for 15 min (1:10, junction to protein) before addition of Flp (1:4 or 1:8, junction to protein) and incubated for an additional 15 min. The binding mixtures were fractionated by electrophoresis in a 3% agarose gel in 6.7 mM tris-HCl (pH 8.0), 3.3 mM additional 15 min. The binding mixtures were fractionated by electrophoresis in a 3% agarose gel at 4°C for 75 min (10 V cm⁻¹). DNA was visualized by ethidium bromide staining. Quantification in plots shows means ± SEM from three independent experiments.

**Quantification of DNA band intensities**

DNA bands from gels were analyzed using Quantity One software from Bio-Rad.

**Quantitative P1 transduction and intrachromosomal direct repeat HR assays**

Quantitative P1 transduction assays were as described by Magner et al. (18), with the modification that saturated overnight liquid LBH cultures were diluted 100 times in LBH medium with or without doxycycline (100 ng/ml) to induce RDG and grown at 37°C for 3 hours. Phage P1 grown on strain SMR6263 was used to transduce leu::Tn10 into recipient strains. The transductants were plated on the Tet plates with or without doxycycline to induce RDG. The frequencies of TetR transductants were determined by three P1 transductions with recipient cells in excess (multiplicities of infection of <0.01 phage per recipient cell). The direct repeat HR assay was as described by Cotter et al. (82) with the modification that some of the isogenic strains additionally carry plasmid vector or isogenic RecA-overproducing plasmid and were or were not induced to overproduce RecA by adding 1 mM IPTG.

**Microscopy and focus quantification**

Images were visualized with an inverted DeltaVision Core Image Restoration Microscope (GE Healthcare) with a 100× UPlan S Apochromat (numerical aperture, 1.4) objective lens (Olympus) and a CoolSNAP HQ2 camera (Photometrics). Captured images were chosen randomly under the microscope. The images were taken with Z stacks (0.15-µm intervals) and then deconvoluted (DeltaVision SoftWoRx software) to see the whole cell for precise quantification of foci in each cell. For each independent experiment, >1000 cells (about 3000 cells for ArcA and ArcB strains) were counted using ImageJ software with visual inspection in each of three independent experiments.

**Spontaneous and γ-radiation– and I–Sce I–induced foci**

For the measurement of spontaneous HJ focus formation, saturated overnight cultures were diluted 1:100 in fresh LBH medium or 1:25 dilution in M9-glucose medium and incubated at 37°C for 1.5 hours before adding doxycycline (100 ng/ml). Cultures were incubated for another 4 hours before visualizing under the microscope. Growth curves of different strain backgrounds were similar (fig. S9). For I–Sce I–induced foci, saturated overnight cultures started in medium containing 0.1% glucose were diluted 1:100 or 1:25 in fresh LBH or M9-glycerol medium supplied with proline (50 µg/ml), respectively, and incubated at 37°C for 1.5 hours before adding doxycycline (100 ng/ml). After 1 hour, 0.005% arabinose was added and incubation was continued for another 3 hours. The procedure of γ–radiation–induced focus formation was performed as previously described by Shee et al. (39), except for using different doses of γ–radiation.

For experiments with temperature-inducible Gam production, cultures were inoculated into rich medium from saturated overnight cultures at 30°C for 1.5 hours before shifting to 37°C (to induce Gam production) and adding doxycycline (100 ng/ml to induce RDG). Spontaneous foci were counted after another 4 hours of incubation.
For Gam production under DSB-inducing conditions, the temperature was shifted and doxycycline was added for 1 hour before adding arabinose to induce I–Sce I to generate DSBs. Foci were counted after another 3 hours of incubation at 37°C. RecA was overproduced by adding 1 mM IPTG. For dnaA/TTS temperature shift experiments, cultures were inoculated into rich medium from saturated overnight cultures at 30°C for 1.5 hours before shifting to 42°C (to inactivate DnaA) and adding doxycycline (100 ng/ml; to induce RDG). Spontaneous foci were counted after another 4 hours of incubation.

Microfluidics and time-lapse fluorescence microscopy
The procedure was as described by Shee et al. (39), except for a slight difference in growth condition. Saturated cultures of SMR19382 were diluted 100-fold in M9 glucose medium supplemented with vitamin B1, 0.5% casamino acid, and doxycycline (10 ng/ml) and grown at 37°C for 1 hour before loading cells into the microfluidic chamber (time 0). For the next 7 hours, cells were bathed with the same medium, but with only doxycycline (2 ng/ml) to allow division, then switched at 7 hours to the same medium without glucose, and bathed until 18 hours. The number of cell divisions and the appearance of RDG foci were captured using time-lapse microscopic photography throughout the experiment.

ChIP-seq library preparation and sequencing
Cell cultures were grown in LBH with 0.1% glucose to saturation overnight, then diluted 100-fold into 100 ml of LBH in 500-ml flasks, and incubated at 37°C for 2.5 hours before the addition of doxycycline (100 ng/ml) to induce RDG production. After 30 min, 0.005% arabinose was added to induce I–Sce I expression. After another 2.5 hours of incubation at 37°C, proteins and DNA were cross-linked by the addition of 1% formaldehyde for 30 min at room temperature. The cross-linking was quenched by the addition of 0.5 M glycine, and cells were harvested by centrifugation and washed once with tris-buffered saline. Cells were lysed as described by Bonocora and Wade (83) with lysis buffer containing lysozyme (4 mg/ml). Lysates were sonicated using the Bio- Ultrasonicator, but with only doxycycline (2 ng/ml) to allow division, then switched at 7 hours to the same medium without glucose, and bathed until 18 hours. The number of cell divisions and the appearance of RDG foci were captured using time-lapse microscopic photography throughout the experiment.

Whole-genome sequencing
Cultures were grown as described in ChIP-seq library preparation, except that before that protocol’s cross-linking step, cells were collected by centrifugation and genomic DNA was extracted and purified using the DNeasy Blood & Tissue Kit (Qiagen). Libraries were prepared using Nextera XT kits (Illumina), and sequencing was performed on an Illumina MiSeq.

Sequencing data analyses and deposit
Before mapping, FASTX-Toolkit (v0.0.14) was used to preprocess the sequences: Adapter sequences were removed, and reads were trimmed and filtered according to quality. The sequence alignment was performed by BWA-MEM (v0.7.12) with –M option to mark shorter split hits as secondary alignments and other parameters as default (84). Depending on the strain background, reads were mapped to either the W3110 genome (National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) Database accession: NC_007779.1) or the MG1655 genome (NCBI RefSeq accession: NC_000913.3). Reads that had multiple primary hits or low mapping quality were discarded. Potential PCR duplicates were removed by retaining only one pair of reads with the highest mapping quality when multiple read pairs were mapped to identical external coordinates (Picard Tool MarkDuplicates). BedGraph files that report the physical genomic coverage (taking into account the unsequenced part between read pairs) in each 2000-bp bin were generated from BAM files using deepTools (85). For ChIP-seq data, the read counts in the bedGraph files were normalized to the median coverage. For whole-genome sequencing data, the read counts in each bin were first normalized against total read counts, and then the log₂ ratios of DSB and no-DSB samples were calculated. Plots were generated by R software. Genomic regions that contain ribosomal RNA gene clusters had very few uniquely mapped reads and were thus eliminated from the plots. All sequencing data are available in the European Nucleotide Archive (ENA) under study accession no. PRJEB14145.

Chromosome copy number determinations
Replication origins were counted to estimate the number of forks per cell by using rifampicin runoff analyses, as described by Joshi et al. (56) and Bremmer and Dennis (86). Saturated overnight cultures were diluted 1:1000 or 1:750 in LBH glucose or M9 glucose medium, respectively, containing doxycycline (100 ng/ml). After reaching OD₅₆₀ (optical density at 600 nm) = 0.1, rifampicin and cephalaxin were added to the culture, which was allowed to complete ongoing rounds of replication (runoff). Cells were fixed and stained with 4’,6-diamidino-2-phenylindole and then analyzed on a BD Biosciences LSRII Fortessa cytometer. Three independent experiments were performed and results were averaged.

Phage λ red gam plaque assay for phage Mu Gam function
The phage λ plaque assay was described by Shee et al. (39), except for induction of Gam protein by shifting temperature. The production of Gam was controlled by phage λ P₈ promoter, next to the λcI857 gene, encoding a temperature-sensitive CI transcriptional repressor protein that represses λ P₈ at ≤33°C and allows transcription from λ P₈ at ≥37°C (39). Saturated tryptone broth (TB) cultures of E. coli grown at 30°C were diluted 1:100 in fresh TB with 0.2% maltose, 5 mM MgSO₄, thymine (10 µg/ml), and vitamin B1 (10 µg/ml) and grown for half an hour at 30°C before shifting temperature to 37°C to induce production of Mu Gam (or were grown at 30°C continuously). After 2.5 hours of induction, an equal volume of 10 mM tris and 10 mM Mg (TM) buffer (pH 7.5) was added, and cells were mixed with an appropriate volume of λ(red gam) Chλ suspension, adsorbed without shaking for 10 min at room temperature, and then plated with the addition of 2.5 ml of molten soft BBL agar [1% BBL trypticase peptone, 0.1 M NaCl, 0.7% agar (pH 7.5)] onto BBL plates (same medium solidified with 1% agar). The plates were incubated overnight at 37°C or for 24 hours at 30°C before observing the size of plaques (fig. S13). The production of Mu Gam allows large plaque formation by λ(red gam) (39) [reviewed by Smith (87)].

Cancer RNA statistical analyses
Cancer patient RNA sequencing correlation analyses were performed using Spearman’s rank correlation on data from cBioPortal (88, 89). RNA levels of specific genes in patient samples for the eight most common cancer types—breast, lung, acute myeloid leukemia, colon, kidney, thyroid, bladder, and prostate—were compared with those in the cBioPortal reference population to generate Z scores, and the Z scores were compared for each gene of interest against the similarly generated Z scores of another gene (for example, BLM mRNA versus...
RAD51 RNA; shown in Fig. 7A). The reference populations for each gene/cancer are all cancer samples that are diploid for the gene in question (by default for mRNA). The returned value indicates the number of SDs away from the mean of expression in the reference population (Z score). This measure is useful for determining whether a gene is up- or down-regulated relative to all other tumor samples. As detailed in table S1, the number of patient sample data sets per cancer type ranged from 129 to 1100 (table S1). Spearman’s correlation coefficient was computed to assess the relationship between mRNA levels among genes of interest.

Other statistical analyses

Wet-bench experiments: All were performed at least three times independently, and a two-tailed unpaired t test was used to determine significant differences unless otherwise specified. Error bars represent 1 SEM except where otherwise indicated. Pearson’s correlation coefficient was computed to assess the relationship between RDG foci/cell and different doses of γ-irradiation. Changepoint analyses were used to detect peaks in ChIP-seq data by detecting the inflection points of the peak intensity. Changes in the number of ChIP-seq reads between conditions are therefore reflected in the number of changepoints. Spearman’s correlation was used to determine the non-linear relationship between the RNA expression and the foci counts in the different cell lines. This measure is useful for determining whether the data are non-normally distributed.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/11/e1601605/DC1

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