Human Cell Assays for Synthesis-Dependent Strand Annealing and Crossing over During Double-Strand Break Repair

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ABSTRACT DNA double-strand breaks (DSBs) are one of the most deleterious types of lesions to the genome. Synthesis-dependent strand annealing (SDSA) is thought to be a major pathway of DSB repair, but direct tests of this model have only been conducted in budding yeast and Drosophila. To better understand this pathway, we developed an SDSA assay for use in human cells. Our results support the hypothesis that SDSA is an important DSB repair mechanism in human cells. We used siRNA knockdown to assess the roles of a number of helicases suggested to promote SDSA. None of the helicase knockdowns reduced SDSA, but knocking down BLM or RTEL1 increased SDSA. Molecular analysis of repair products suggests that these helicases may prevent long-tract repair synthesis. Since the major alternative to SDSA (repair involving a double-Holliday junction intermediate) can lead to crossovers, we also developed a fluorescent assay that detects crossovers generated during DSB repair. Together, these assays will be useful in investigating features and mechanisms of SDSA and crossover pathways in human cells.

KEYWORDS double-strand break repair crossing over synthesis-dependent strand annealing

Double-strand breaks (DSBs) are considered to be one of the most detrimental types of DNA damage. There are numerous mechanisms for repairing DSBs, broadly classified into end joining and homology-directed recombination (HDR). Among the latter, the double-strand break repair (DSBR) (Figure 1) model has been popular since it was proposed 30 yr ago (Sztok et al. 1983). A hallmark of this model is the double-Holliday junction (dHJ) intermediate, which has two of the four-stranded junctions originally hypothesized by Holliday (1964). In DSBR, as in Holliday’s model, specialized nucleases resolve Holliday junctions (HJs) by introducing symmetric nicks; independent resolution of the two HJs results in 50% of repair events having a reciprocal crossover. It has also been proposed that dHJs can be processed without the action of a nuclease if a helicase and topoisomerase migrate the two HJs toward one another and then decatenate the remaining link (Figure 1) (Thaler et al. 1987); this process has been called dissolution to distinguish it from endonucleolytic resolution (Wu and Hickson 2003).

In studies of DNA DSB repair resulting from transposable element excision in Drosophila, Nassif et al. (1994) noted that crossovers were infrequent and the two ends of a single DSB could use different repair templates. To explain these results, they proposed the synthesis-dependent strand annealing (SDSA) model (Figure 1). In addition to continued use of Drosophila gap-repair assays (e.g., Kurkulos et al. 1994; Adams et al. 2003), other types of evidence have been interpreted as support for the SDSA model. In Saccharomyces cerevisiae meiotic recombination, gel-based separation and quantification of intermediates and products showed that noncrossovers are generated by SDSA (Allers and Lichten 2001). In vegetatively growing S. cerevisiae, Mitchel et al. (2010) studied repair of a small gap DSB in cells defective in mismatch repair. Based on the high frequency with which heteroduplex DNA tracts (regions that contain one template strand and one recipient strand) in noncrossover products were restricted to one side of the DSB, they concluded that most noncrossover repair occurred through SDSA. Miura et al. (2012) used an S. cerevisiae assay designed specifically to detect SDSA. A plasmid with a DSB was introduced into cells in which templates homologous
to the two sides of the DSBs were on different chromosomes, eliminating the possibility of a dHJ intermediate. Based on results of these various assays, many researchers now believe SDSA to be the most common mechanism of mitotic DSB repair by HDR (reviewed in Andersen and Sekelsky 2010; Verma and Greenberg 2016).

In mammalian cells, the direct-repeat GFP (DR-GFP) assay (Pierce et al. 1999) has been an instrumental tool for studying DSB repair by HDR. In this assay, an upstream GFP gene (SceGFP) is disrupted by insertion of an I-SceI site, and a downstream GFP fragment (iGFP) serves as a template for repair. Gene conversion replaces the region surrounding the I-SceI site in SceGFP, generating an intact GFP gene. This gene conversion has been suggested to arise through SDSA, but it is not possible to distinguish between SDSA and other noncrossover DSBR repair in this assay (see Figure 1). Xu et al. (2012) developed a novel human cell assay in which gene conversion could be detected simultaneously at the DSBS site and at another site >1 kbp away. They found that the two were often independent and concluded that SDSA is a major mechanism for DSB repair in human cells, but they also could not exclude DSBR as a possible source.

Development of the CRISPR/Cas9 system for genome engineering (Cong et al. 2013; Mali et al. 2013) provides additional emphasis on the importance of understanding SDSA mechanisms in human cells, as it has been suggested that replacement of multi-kilobase pair fragments after Cas9 cleavage, and probably other HDR events, occurs through SDSA (Byrne et al. 2015). We therefore designed an assay to detect DSB repair by SDSA in human cells. Here, we describe this assay and show that, as hypothesized, SDSA appears to be an important pathway for HDR in human cells. We report the effects of knocking down various proteins proposed to function during SDSA. We also describe a fluorescence-based assay for detecting crossovers generated during DSB repair. Use of these assays should help to further our understanding of DSB repair pathways used in human cells.

**MATERIALS AND METHODS**

**Construction of assay plasmids**

The SDSA assay construct, pGZ-DSB-SDSA, was based on pEF1α-mCherry-C1 vector (catalog no. 631972; Clonetech). A fragment of mCherry was removed by cutting with NheI and HindIII and inserting annealed oligonucleotides containing an I-SceI recognition sequence and a part of the mCherry sequence. The product, pEF1α-mCherry-I, had 350 bp of mCherry deleted and replaced with an I-SceI recognition sequence. In parallel, 5’ and 3’ mCherry fragments, overlapping by 350 bp, were PCR-amplified and cloned into a vector containing a fragment of the copia retrotransposon from D. melanogaster. A fragment of HPRT was cloned out of the DR-GFP construct. This entire module (5’ mCherry–copia–3’ mCherry–HPRT) was PCR-amplified and cloned into the pEF1α-mCherry-I to produce pGZ-DSB-SDSA. The full sequence was deposited in GenBank under accession KY447299.

The crossover assay construct, pGZ-DSB-CO, was based on pHPRT-DRGFP (Pierce et al. 2001) and the intron-containing mCherry gene from pDN-D2irC6kwh (Nevozhay et al. 2013). The iGFP fragment was expanded to include the entire 3’ end of the transcribed region, and this was cloned into the mCherry intron of pDN-D2irC6kwh. This module (mCherry with an intron containing 3’ GFP) was cloned into the pHPRT-DRGFP vector cut with HindIII, so that it replaced the iGFP fragment and was in reverse orientation relative to the SceGFP gene. The full sequence of pGZ-DSB-CO was deposited in GenBank under accession KY447298.

**Generation of stably-transfected cell lines**

U2OS and HeLa cells were cultured under normal conditions (DMEM + 10% FBS + pen/strep) for 24 hr until they reached 80% confluence before transfection with either SDSA or crossover assay constructs using a Nucleofector 2b Device (catalog no. AAB-1001; Lonza) and Cell Line Nucleofector Kit V (catalog no. VCA-1003; Lonza). At 1 wk post-transfection, appropriate antibiotics were added to select for the cells with a stably-integrated construct. pGZ-DSB-SDSA assay has a gene for neomycin resistance; cells receiving this construct were treated with 700 µg/ml G418 (catalog no. A1720; Sigma) for 1 wk and then single-cell clones were derived. pGZ-DSB-CO contains a PKG1 gene that confers resistance to puromycin; cells receiving this construct were treated with 10 µg/ml puromycin (catalog no. P8833; Sigma) for 1 wk and then single-cell clones were derived. Initial attempts to determine copy number by Southern blot were unsuccessful; however, the analyses described below strongly suggested that the lines we characterized each carried a single insertion or possibly a single tandem array.

**DNA repair assays and flow cytometry**

U2OS cells with pGZ-DSB-SDSA integrated were cultured in 10-cm dishes containing 10 ml of DMEM medium with high glucose (Corning) until split onto six-well plates at a concentration of 5 × 10^4 cells/ml using 0.05% trypsin 0.53 mM EDTA solution (Corning). Upon reaching ~60% confluence, the cells were treated with an siRNA reaction mixture (90 nmol siRNA and 8 µl lipofectamine 2000 reagent per well; Invitrogen). At 24 hr after transfection, the siRNA reaction mixture was replaced with the fresh culture medium. After 12 hr the cells were split so that knockdown could assessed in one half (see qPCR evaluation of the siRNA knockdown efficiency). The other half was treated with 100 µl I-SceI–expressing adenovirus (Anglana and Bacchetti 1999) (previously titrated to a nonlethal concentration). After another 24 hr, the medium was replaced and thus the adenovirus removed. After another 72 hr, the cells were harvested and resuspended in 1× PBS (Corning) supplemented with 2% fetal bovine serum (FBS) and 5 mM EDTA, for flow cytometry acquisition on a BD LSRFortessa, using 488 and 561 nm lasers to detect the mCherry fluorescence.

U2OS cells with pGZ-DSB-CO integrated were cultured and treated under the same conditions. Flow cytometry acquisition was conducted on a BD FACSARiaII, using 388 and 532 nm lasers to detect GFP and mCherry fluorescence.

**U2OS genomic DNA isolation**

Cells were cultured in a 15-cm dish until they reached 100% confluency, then rinsed with 1× PBS and harvested in 0.05% trypsin, 0.53 mM EDTA, by centrifuging for 3 min at 2000 rpm. Cells were washed with PBS and transferred to 1.5-ml microfuge tubes and spun for 10 sec to pellet. PBS was removed and cells were resuspended in TSM (10 mM Tris-HCl, pH 7.4; 140 mM NaCl; 1.5 mM MgCl2) with 0.5% NP-40 and incubated on ice for 2–3 min. After pelleting, cells were resuspended in 1 ml nuclei dropping buffer (0.075 M NaCl, 0.024 M EDTA, pH 8.0). The suspension was transferred to a 15-ml tube containing 4 ml nuclei dropping buffer with 1 mg Proteinase K (final Proteinase K concentration = 0.2 mg/ml), and 0.5% SDS. The cells were lysed overnight at 37°. The next day, an equal volume of phenol was added and mixed on an orbital shaker for 2 hr followed by a 5-min spin at 2000 rpm. The aqueous phase was transferred to a clean tube, an equal volume of chloroform was added, and the mix was incubated for 30 min on an orbital shaker. After spinning, the aqueous phase was transferred to a new tube and 0.1 vol 3 M NaOAc was added, followed by 1 vol isopropanol. The DNA was spooled out using a glass Pasteur pipette and resuspended overnight in 1 ml TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The next day, the DNA was precipitated using 0.5 vol 7.5 M NH4OAc and 2 vol ethanol. DNA was spooled out and...
PCR analysis of the repair events
DNA from the BRCA2 knockdown repair events was isolated according to the protocol described in U2OS genomic DNA isolation, and used in a PCR reaction to amplify a desired DNA fragment for sequencing or fragment length characterization. A total of 1.5 µl DNA was added to each PCR mixture containing primer sets according to Supplemental Material, Table S1 in File S1, ReAgro polymerase (catalog no. 424264; BioRad) and buffer. PCR amplification reaction program was 33 cycles of the following: 20 sec at 98°, 20 sec at 64°, and 20–150 sec at 72°. Products were run on 1–1.5% agarose gels with ethidium bromide before being imaged.

Western blot of BLM protein in siRNA-treated cells
Cells treated with siRNA as described in DNA repair assays and flow cytometry were harvested on the third day post-transfection using 0.05% Trypsin, 0.53% mM EDTA solution (Corning). After washing with 1× PBS, the cells were resuspended in a protein sample buffer (Tris–HCl; SDS; glycerol; bromophenol blue; 150 mM DTT) and boiled. A total of 20 µl of the protein sample was loaded on a 7.5% SDS–PAGE gel and the gel was run for 1–2 hr at 100 V. Protein was transferred to a PVDF membrane using a wet transfer method (1.5 hr at 90 V in 4°). The membrane was blocked in PBS with 5% powdered milk and incubated in PBS plus 0.1% Triton-X plus primary antibodies [rabbit anti-BLM (catalog no. 2179; Abcam) at 1:2000 and mouse anti-α Tubulin (catalog no. T9026; Sigma) at 1:8000] overnight at 4° on a rocker. The membrane was then washed three times in PBS-T solution. HDRP-conjugated secondary antibodies were added (goat anti-rabbit at 1:5000 and goat anti-mouse at 1:100,000) and the blot was incubated on a rocker. The membrane was then washed three times in PBS-T solution and then incubated in an ECL solution (Thermo Fisher Scientific) for chemiluminescence for 2 min. The Western blot image was taken using a BioRad Molecular Imager (ChemiDoc XRS+) or the X-ray film was developed using a developer.

qPCR evaluation of the siRNA knockdown efficiency
Cells treated with siRNA as described in DNA repair assays and flow cytometry were harvested on the third day post-transfection using 0.05% Trypsin 0.53% mM EDTA solution (Corning). RNA was extracted using the manufacturer’s protocol for ReliaPrep RNA Cell Miniprep System (Promega). Purified RNA was used as a template to generate the cDNA library with QuantiTect Reverse Transcription Kit (catalog no. 205310; Qiagen). The qPCR mix contained gene-specific DNA primers, RT primers, and QuantiTect SYBR Green PCR kit (catalog no. A 204141; Qiagen). Amplification and quantification was conducted on a RealTime PCR machine (QuantiStudio 6 flex Real Time PCR System).

Statistical analysis
Statistical comparisons were performed on the raw data (Tables S2 and S3 in File S1) using GraphPad Prism version 6.07 for Windows (GraphPad Software Inc., La Jolla, CA). In the case of BLM knockdown in the SDSA assay, one value (271% of control) was found to be a significant outlier based on the Grubb’s test using GraphPad QuickCalculc online (https://graphpad.com/quickcalc/Grubbs2/), and was excluded from further analysis.

RESULTS AND DISCUSSION
An SDSA assay for human cells
To study SDSA in human cells, we used an approach conceptually similar to the P[w+] assay used in Drosophila (Adams et al. 2003; McVey et al. 2004). In this assay, if both ends of the DSB generated by P element excision are extended by synthesis from the sister chromatid, the nascent strands can anneal at repeats inside the P[w+] element, generating a product that is unique to SDSA and easily distinguishable by phenotype. To mimic this situation in human cells, we built a construct (Figure 2) that has an mCherry gene in which a 350-bp segment was replaced with the 18 bp I-SceI recognition sequence, rendering the gene nonfunctional. When a DSB is generated by I-SceI (Figure 2A), HDR can be completed using a downstream repair template. The repair template is split: each half has 800 bp of homology adjacent to the break site plus the 350 bp of deleted mCherry sequence. The two halves are separated by a 3-kbp spacer of unique sequence. Since the 350-bp sequence is on both sides of the spacer, it constitutes a direct repeat. We hypothesized that both ends of the I-SceI-induced break will invade the side of the template to which they are homologous, either simultaneously or sequentially (Figure 2B). If synthesis on both sides extends through or far enough into the 350-bp repeat before the nascent strands are dissociated from the template, the overlapping regions can anneal to one another (Figure 2B). Completion of SDSA restores a functional mCherry gene at the upstream location.

The scenario above requires two-ended SDSA, but sequential one-ended SDSA is also possible (Figure 2C). If only one end of the break invades the downstream template, is extended by repair synthesis, and if the other end is

Figure 1 Models of DSB repair by homologous recombination. (A) Blue lines represent two strands of a DNA duplex that has experienced a DSB. HDR begins with resection to expose single-stranded DNA with 3’ ends (arrows). One of these can undergo strand invasion into a homologous duplex (red) to generate a D-loop; the 3’ invading end is then extended by synthesis. (B) In SDSA, the nascent strand is dissociated and anneals to the other resected end of the DSB. Completion of SDSA may result in noncrossover gene conversion (red patch, shown after repair of any mismatches). (C) An alternative to SDSA is annealing of the strand displaced by synthesis to the other resected end of the DSB. Additional synthesis can lead to a dHJ intermediate. (D) In DSBR, the dHJ is resolved by cutting to generate either crossover or noncrossover products (one of two possible outcomes for each case is shown). (E) The dHJ can also be dissolved by a helicase-topoisomerase complex to generate noncrossover products.

were deposited in GenBank under accession numbers KY447298 (pGZ-DSB-CO) and KY447299 (pGZ-DSB-SDSA).
is then dissociated from the template, the nascent strand will not be complementary to the other resected end; however, this nascent strand will have homology to the repeat on the other side of the template. A second cycle of strand exchange and repair synthesis using the other repeat could lead to addition of sequences complementary to the other resected DSB end. This would also restore a functional mCherry gene by SDSA.

A functional mCherry gene might also be generated through a combination of SDSA and DSBR. In the sequential SDSA scenario, the second strand exchange event could be processed into a dHJ. The product of dissolution or noncrossover resolution of such a dHJ will be identical to that of SDSA (Figure 2B), but if the dHJ is resolved as a crossover, generation of a functional mCherry gene will be accompanied by a deletion of all sequences between the upstream mCherry and the downstream template. Dissolution or noncrossover dHJ resolution in this scenario cannot be distinguished from SDSA, but it should be noted that formation of such a dHJ intermediate still requires at least one cycle of D-loop disassembly—a key step that separates SDSA from DSBR (Figure 1).

Other types of repair that do not generate a functional mCherry are possible. A dHJ can be generated if synthesis extends through one mCherry 350-bp repeat, the entire spacer, and the other 350-bp repeat (Figure 2D). Processing of this dHJ would give a product in which the entire template, including the duplicated 350-bp sequences and the spacer, was copied into the upstream mCherry gene. Dissolution or noncrossover resolution would result in two copies of the template (Figure 2D, middle), whereas crossover resolution would delete intervening sequences (Figure 2D, bottom). Nonhomologous end joining (NHEJ) can restore or disrupt the I-SceI recognition sequence, depending on whether it is precise or imprecise (not depicted). Hybrid repair, in which repair is initiated by HDR but completed by end joining instead of annealing, can give rise to an mCherry in which the 350-bp gap is not completely filled or, if synthesis extends into the spacer, in which part of the spacer is copied into the upstream mCherry gene (Figure 2E).

To generate cell lines with the SDSA repair construct, we transfected both U2OS and HeLa cells with linearized SDSA construct and used G418 to select stably-transfected lines. To induce DSBs, we
infected cells with an adenovirus expressing I-SceI expression after I-SceI infection. (B) Flow cytometry of cells after I-SceI expression. In this example, 10,000 single cells were assayed and 200 were gated as exhibiting red fluorescence. (C) Effects of siRNA knockdown on acquisition of mCherry expression (see Materials and Methods). Fluorescence frequencies from flow cytometry were normalized to NT; raw data are given in Table S2 in File S1. Error bars indicate SD. The ratio paired t-test was used to compare raw values for each siRNA target to its NT control and each double-knockdown to the single knockdown of the corresponding helicase. For helicase single knockdowns, P values were Bonferroni-corrected for multiple (six) comparisons. * P < 0.05, ** P < 0.01, *** P < 0.0001.

Knocking down BLM or RTEL1 elevates SDSA frequency

In the model shown in Figure 1, SDSA diverges from dHJ pathways when a helicase dissociates the nascent strand from the template. Several helicases have been suggested to perform this step. *Drosophila* gap repair assays found roles for BLM and Fancm helicases in SDSA (Adams et al. 2003; Kuo et al. 2014). The *Arabidopsis* orthologs of these enzymes promote noncrossover recombination in meiosis, possibly by SDSA (Crismani et al. 2012; Séguela-Arnaud et al. 2015). Sgs1, the yeast ortholog of BLM, is required for noncrossover recombination in budding yeast meiosis (De Muyt et al. 2012), and Sgs1 and Mph1 (the orthologs of Fancm) have been implicated in SDSA in vegetative cells (Mitchel et al. 2013). In *Schizosaccharomyces pombe*, Fml1, the ortholog of Fancm/Mph1, was suggested to promote SDSA in DSB repair during replication and in meiosis (Sun et al. 2008; Lorenz et al. 2012). Yet another helicase, RTEL-1, was hypothesized to disrupt D-loops in *Caenorhabditis elegans* meiosis (Barber et al. 2008; Youds et al. 2010). In human cells, RECQ5 is proposed to promote SDSA (Palival et al. 2014). Aside from the experiments in *Drosophila* and budding yeast, none of the assays performed could distinguish between SDSA and other pathways. Thus, we used our assay to ask whether the orthologs of any of these or related helicases affect SDSA in human cells.

We did not detect any change in the frequency of red-fluorescing cells after knocking down FANCm, RECQ5, WRN, or FBXO18 (Figure 3C and Table S2 in File S1). Knockdown of BLM or RTEL1 significantly altered the frequency of red-fluorescing cells; however, instead of decreasing SDSA as expected, both knockdowns resulted in an increase in red-fluorescing cells (Figure 3C). BLM has been shown to have several functions in HDR pathways, including in DSB end resection in a pathway redundant with *Exo1* (Zhu et al. 2008) and dHJ dissolution (Wu and Hickson 2003; Wu et al. 2006; Singh et al. 2008). Likewise, RTEL1, which was initially identified as a telomere length regulator and is responsible for T-loop disruption (Ding et al. 2004; Sarek et al. 2015), can...
Knocking down BLM or RTEL1 alters repair outcomes

To further develop our SDSA assay and gain additional insights into the effects of knocking down BLM and RTEL1, we determined the structures of repair events produced in knockdown cells. We analyzed 55 clones derived from single red-fluorescing cells, including 23 from the NT control, 21 from BLM knockdown, 10 from FANCM knockdown, and one from RTEL1 knockdown. All but one had the structure expected of SDSA. The remaining clone, which came from NT siRNA treatment, had lost neo and the template spacer, and therefore may have arisen from SDSA followed by DSBR with crossover resolution (Figure 2B). These results support our conclusion that cells with restored mCherry utilized SDSA to repair the DSB, perhaps occasionally coupled with use of DSBR.

We also analyzed cells that failed to produce mCherry. In the NT control, all 45 lines examined appeared to be identical to the initial construct (Figure 5A). We did not measure cleavage efficiency in our assay, but we titrated adenovirus infection to the highest dose that did not cause detectable cell lethality. Delivery of I-SceI by adenovirus infection of HEK293 cells resulted in 85% of sites being cut (Anglana and Bacchetti 1999), so it is likely that most or all of the cells with intact I-SceI sites are likely to result from cleavage followed by precise NHEJ using the 4 nt complementary overhangs left by I-SceI.

The majority of clones from BLM or RTEL1 knockdown cells that did not produce mCherry also had an intact I-SceI site; however, structures indicating other repair processes were observed in 11 out of 34 of these clones from BLM knockdown (P < 0.0001 compared to NT) and 24 out of 133 of these clones from RTEL1 knockdown cells (P = 0.0007). In four of the BLM knockdown clones and 14 of the RTEL1 knockdown clones, the entire 3-kbp spacer sequence was copied from the repair template into the upstream mCherry (Figure 5, B and C). This extensive repair synthesis might occur through multiple cycles of strand exchange, as is believed to occur in Drosophila gap repair by SDSA (McVey et al. 2004), or through a single, continuous synthesis event. Among the 17 examples in which the entire spacer was copied, one

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from each knockdown sample had lost neo, a structure that is most consistent with a dHJ being resolved to give a crossover (Figure 2D and Figure 5C). The other 15 may have arisen by long-tract SDSA or by dissolution or noncrossover resolution of a dHJ (Figure 2D and Figure 5B).

There were additional repair events from knockdown cells that also had evidence of long-tract synthesis. Three events from BLM knockdown and seven from RTEL1 knockdown had a subset of the spacer copied into the upstream mCherry (Figure 2E and Figure 5D). These are most likely hybrid repair that involved long-tract synthesis followed by end joining. There was one event from the BLM knockdown that had an intact I-SceI site but had lost neo (Figure 5E). The source of this event and whether it occurred following I-SceI cleavage is unknown.

Roles of BLM and RTEL1 in SDSA
It was surprising that none of the helicase knockdowns led to decreased SDSA, since orthologs of all of these have been suggested to promote SDSA. One possibility is that there is redundancy among two or more of these proteins. This possibility can be addressed through simultaneous knockdowns or use of doubly mutant cells. In the case of BLM and RTEL1, knockdown led to dramatic increases in HDR, both in the frequency of red-fluorescing cells (Figure 3C) and in the fraction of nonfluorescing cells that had evidence for HDR (zero out of 45 for NT compared to 11 out of 34 for BLM knockdown and 24 out of 133 for RTEL1 knockdown). This is consistent with a reported increase in gene conversion in the DR-GFP assay after siRNA knockdown of BLM (Paliwal et al. 2014).

Some repair products in knockdown cells had synthesis that spanned the entire 3-kbp spacer (e.g., Figure 5B). These could generate dHJ intermediates that would then be resolved or dissolved. In the case of RTEL1 knockdown, only one out of 13 events in which the entire spacer was copied had had the structure expected of dHJ crossover resolution (Figure 5C). If crossover and noncrossover resolution occur at equal frequencies, then either SDSA or dHJ dissolution were likely responsible for most of these events. The BTR complex, which contains the BLM helicase, is believed to be the major or sole dissolvase (Wu and Hickson 2003; Seki et al. 2006; Dayani et al. 2011). Although there was also only a single event in the BLM knockdown suggestive of dHJ crossover resolution (Figure 5C), there were only three other events in which the entire spacer was copied; it is possible that all three of these came from dHJ resolution that had a noncrossover outcome.
In our assay, SDSA may occur if both ends of the DSB engage with the template. This suggests the possibility that a function of BLM and RTEL1 is to ensure that only one end engages with the repair template. Among the 10 repair events in which only part of the spacer was copied into the upstream mCherry (Figure 5D), all of them appeared to have synthesis from the left end only. This is in contrast to the Drosophila P[w<sup>+</sup>] excision assay, where most repair events have several kilobase pairs of synthesis from both ends of the break (Adams et al. 2003). The disparity could arise from the difference between organisms or tissues, different structures of the DSB ends (4 nt 3' overhangs for I-SceI; 17 nt 3' overhangs for P element excision), or distance between the sequence homologous to the left side of the DSB and the sequence homologous to the right side (~3.5 kbp for this assay but >14 kbp for P[w<sup>+</sup>]).

The causes of partial copying of the spacer most likely derive from hybrid repair in which the initial steps of SDSA are executed but dissociation of the nascent strand does not reveal complementary sequences for annealing, so repair is instead completed by DNA polymerase θ-mediated end joining (also called microhomology-mediated end joining) (Chan et al. 2010; Wyatt et al. 2016). As in SDSA, these D-loops must have been disassembled by another helicase than the one knocked down, or by residual helicase present after the knockdown.

Knocking down BLM did lead to elevated crossovers in the crossover assay (Figure 4D). This result was expected, based on phenotypes like elevated sister chromatid exchange (German et al. 1977). However, there was also an overall increase in HDR, as noncrossovers were also elevated. Thus, knocking down BLM resulted in elevated HDR in the DR-GFP assay (Paliwal et al. 2014), our SDSA assay, and our crossover assay. This might be expected if knockdown affects the cell cycle profile, such that more cells are in S or G2 phases and therefore more likely to repair by HDR instead of NHEJ. We conducted cell cycle profiling of cells in which BLM was knocked down, but did not detect any significant differences in the cell cycle profile compared to the NT control (Figure S4 in File S1).

The causes of increased HDR when BLM or RTEL1 is knocked down remain unknown. This is an interesting area for future investigation, as understanding this unexpected effect will no doubt reveal important functions of these proteins.

Concluding remarks
We have demonstrated that our assays efficiently detect DSB repair by SDSA or that lead to crossovers, and that these assays can be used to study the effects of knocking down or knocking out different repair genes. Strengths of the assays include the ease of identifying the SDSA or crossover outcomes and the ability to investigate other types of repair based on structures of repair products. We did not determine whether the lines we used had only a single copy of the assay construct integrated (see Materials and Methods), but analyses of cells exposed to I-SceI strongly argue that there was only one insertion location in both cases. In the SDSA assay, an average of 2% of cells acquired red fluorescence in any given experiment. If there were insertions at two different sites repairing independently, we would expect that in the vast majority of cases SDSA would occur in only one of the two insertions. PCR across the I-SceI site would give two bands, one corresponding to the original construct and a larger band resulting from replacement of the 350-bp fragment. In opposition to this expectation, 55 out of 55 red-fluorescing clones examined had only the larger band. Similarly, crossovers in the crossover assay result in loss of mCherry. If there were several integrations every site would have to lose mCherry simultaneously to be scored as a crossover. It remains possible that one or both constructs integrated in a tandem array in the lines we used. If this happened, then it is likely that all I-SceI sites were cut, leaving some extrachromosomal fragments but only a single chromosomal repair template. It is unknown what effect the extrachromosomal fragments would have on repair of the chromosomal DSB.

These assays can be modified to tailor their use in addressing specific questions. With the development of CRISPR/Cas9 genome editing (Cong et al. 2013; Mali et al. 2013), gene knockouts could be done instead of knockdowns, at least for genes that are not essential in the timeframe of these assays. It may be advantageous to use other cell lines for this approach, as U2OS cells have more than two copies of many genes (Forbes et al. 2015). For some questions, it would be informative to incorporate SNP markers into the template so that gene conversion tract properties could be measured at a higher resolution than reported here. Differences between the two 350-bp repeats in the SDSA assay could be used to identify cases of template switching or two-ended invasions. We did not attempt to develop high-throughput sequencing of repair products, but amplification of the entire SDSA module with single-molecule tagging would make it possible to sequence a large number of independent repair events simultaneously. Finally, various distance parameters, such as changes to the amount of synthesis required to reach the repeats (they are immediately adjacent to the DSB in our assay, but >5 kbp into the gap in the P[w<sup>+</sup>] assay) or length of the repeats (350 bp in this assay, 275 bp in P[w<sup>+</sup>]) could provide insight into the frequency of template switching, the length of a typical synthesis tract, and the ability to repair larger gaps.

Elucidating details of SDSA and crossover repair is important for understanding DSB repair in general, but will also prove vital in future optimization of CRISPR/Cas9 gene replacement or integration strategies that have been hypothesized to occur through SDSA (Byrne et al. 2015). We believe both assays we describe can be useful in achieving these goals.

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