Distinct genetic control of homologous recombination repair of Cas9-induced double-strand breaks, nicks and paired nicks

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ABSTRACT

DNA double-strand breaks (DSBs) are known to be powerful inducers of homologous recombination (HR), but single-strand breaks (nicks) have also been shown to trigger HR. Both DSB- and nick-induced HR (nixHR) are exploited in advanced genome-engineering approaches based on the bacterial RNA-guided nuclease Cas9. However, the mechanisms of nixHR are largely unexplored. Here, we applied Cas9 nickases to study nixHR in mammalian cells. We find that nixHR is unaffected by inhibition of major damage signaling kinases and that it is not suppressed by nonhomologous end-joining (NHEJ) components, arguing that nick processing does not require a DSB intermediate to trigger HR. Relative to a single nick, nicking both strands enhances HR, consistent with a DSB intermediate, even when nicks are induced up to ~1kb apart. Accordingly, HR and NHEJ compete for repair of these paired nicks, but, surprisingly, only when 5' overhangs or blunt ends can be generated. Our study advances the understanding of molecular mechanisms driving nick and paired-nick repair in mammalian cells and clarify phenomena associated with Cas9-mediated genome editing.

INTRODUCTION

The reactive byproducts of endogenous metabolic activities as well as exogenous agents, such as ionizing radiation, threaten the integrity of mammalian genome. In response, cells have evolved complex protein networks that can detect and repair DNA damage (1). Although the different types of DNA lesions are typically repaired by dedicated pathways, a functional overlap between these pathways has also been observed. One interesting example of such overlap has been reported for the repair of DNA single-strand breaks (SSBs or nicks). These abundant lesions are typically repaired by specialized SSB repair mechanisms (2), but replication can convert nicks to the more dangerous double-strand breaks (DSBs) (3,4). Multiple mechanisms compete to repair DSBs in mammalian cells, including homologous recombination (HR) and non-homologous end joining (NHEJ) (5,6). Intriguingly, nicks also may trigger HR directly (7–11), even though this pathway is not commonly associated with nick repair. Two recent studies suggested that nick-induced HR (nixHR) utilizes factors involved in classical DSB-induced HR (DSBHR) (12,13).

Recent advances in the field of genome engineering provide a powerful approach to investigate nick and DSB repair mechanisms. The programmed induction of a DSB at a genomic locus has long been known to trigger HR which can precisely edit the genomic sequence when provided with the appropriate repair template (14). Alternatively, the DSB may be processed by error-prone NHEJ to generate random insertions or deletions – a desirable outcome when the targeted gene is to be inactivated. Various tools have been developed to induce DNA damage in a controlled and targeted manner, including homing endonucleases, zinc finger nucleases and TAL effector nucleases (15–17). Recently, the bacterial RNA-guided endonuclease Cas9 has been adapted for application in eukaryotic cells, providing a versatile method to induce SSBs and DSBs at a chosen genomic locus (18,19) and enable efficient HR- or NHEJ-mediated genome manipulation in multiple model organisms (20). Cas9 recognizes a 19 bp target sequence based on its complementarity to a short guide RNA (gRNA) and the presence of a downstream NGG protospacer adjacent motif (PAM). Upon DNA binding, the two catalytic domains of wild-type Cas9 cleave both DNA strands ~3 bp upstream from the PAM to generate a DSB (18).
Mutation of either catalytic domain can convert Cas9 into a nickase, e.g. Cas9ΔD10A or Cas9H840A (18). Because Cas9 tolerates multiple nucleotide mismatches in the 5’ part of the targeted sequence, off-target NHEJ events are not uncommon (21), such that Cas9-mediated NHEJ events and thus have a much lower mutagenic potential (11,13,22–26). However, it is not well understood how nickHR is regulated, how similar it is to classical HR, and whether NHEJ factors participate in this process. Conversely, paired nicks in both DNA strands can result in a DSB to induce either HR or NHEJ, but with increased specificity since binding of two gRNAs in sufficient proximity is required (27–31).

Here, we applied the RNA-guided Cas9 nickases, derived from the Streptococcus pyogenes CRISPR-Cas9 system, to study the mechanisms of nickHR. Our results show that nickHR is mildly reduced by inhibition of ATM or ATR signaling and that it is stimulated by replication if the lagging template strand is nicked. NickHR is partly overlapping with DSBHR in that it is dependent on the known HR factors BRCA1 and RAD51 (13), but, in contrast to DSBHR, it is not affected by NHEJ deficiency, suggesting that it does not typically proceed through a DSB intermediate. We then focused on the scenario when HR is triggered by nicking of both DNA strands, i.e. paired-nick HR (PNHR). With paired nicks, we detected an increased stimulation of HR as compared to single nicks, which is consistent with a DSB intermediate. Both 3’- and 5’-overhangs resulting from paired nicks were found to stimulate HR, even with nicks separated by up to 940 bp. Surprisingly, however, only HR induced by paired nicks which would result in a 5’ overhang was suppressed by the NHEJ factor Ku. These results advance our understanding of the mechanism behind nickHR and PNHR.

MATERIALS AND METHODS

Plasmids and cloning

The following plasmids were previously described: pDRGFP (32) hprtDR-GFP (33), pPC-BaSe (I-SceI expression vector) (34), pCD2E (empty vector), pCD2E-XH (XRCC1 vector) (35), EBNA1 expression vector pCEP4 (36), pCAGGS (empty vector) and pCAGGS expression vectors with BARD1-hB202, BRC3 and KU70 (33,37–41).

Cas9WT, Cas9ΔD and empty gRNA expression vectors were purchased from Addgene (ID 41815, 41816 and 41824) (24). The generation of Cas9ΔH nickase and the catalytically dead Cas9ΔD was described earlier (42). Cas9N863A expression vector was generated by PCR mutagenesis using the Cas9WT vector as template and primers Cas9-N863A-F (5’-GCTAGAGGGAGATGGATAACGTCCTCC-3’) and Cas9-N863A-R (5’-ACTCTTCCCTTAGGTTATCG GATCTTGTCAACATTTATTC-3’). Subsequently, the PCR product was circularized using In-Fusion kit (Clontech). The cloning of targeting gRNA expression vectors is described in Supplementary experimental procedures. The enhanced versions of the gRNAs with adjustments in the stem-loop of the gRNA were described earlier (43) and generated by PCR mutagenesis as detailed in Supplementary experimental procedures. hprtDR-GFP (33) was the basis for generation of the pDRDR-GFP reporter plasmids (Supplementary experimental procedures). To generate the DR-OriP-GFP reporter plasmid, the TR-OriP-GFP (36) and DR-OriP-GFP plasmids were digested using Accl and MfeI (New England Biolabs). Subsequently, the restriction fragment containing the OriP cassette from the TR-OriP-GFP was ligated with the fragment of the DR-OriP-GFP containing SceGFP and iGFP using the DNA Dephos & Ligation Kit (Roche).

Cell lines and cell culture

U2OS cells harboring a single, genomically integrated copy of the DR-GFP reporter were described previously (36). In addition, mouse J1 (wild-type), E14 (wild-type), Ku70−/−, Xrcc4−/− and DNA-PK−/− ES cells with a single Hprt-targeted DR-GFP reporter were used, as were mouse J1 (wild-type) ES cells with a single Pim1-integrated DR-GFP reporter (33,37–39). XRCC1−/− CHO cells EM9-V (complemented with an empty vector) and EM9-XH (complemented with the human wild-type XRCC1) as well as empty and XRCC1 expression plasmids were kindly provided by Dr K.W. Caldecott (35). U2OS, HEK293T and EM9 cells were cultured in high glucose DMEM with 10% fetal bovine serum (FBS), the medium for culturing the mouse ES cells was described earlier (44). All cells were incubated at 37°C in an atmosphere containing 5% CO2. The various pnDR-GFP plasmids were stably integrated at the Hprt locus of wild-type (E14) and Ku70−/− ES cells (33). To this end, 2 × 106 cells were transfected with pnDR-GFP plasmids linearized with KpnI/SacI, or PvuII in case of pnDR-GFP-940 bp, (New England Biolabs) using Amaxa nucleofector II (Lonza), program X-005. Selection of clones was performed as described previously (44). Genomic DNA was extracted using Salt-X and digested with PstI (New England Biolabs). Southern blot analysis of the digested DNA, using iGFP fragment from the hprtDR-GFP plasmid as a probe, confirmed correct targeting (Supplementary Figures S3A and S4).

Transfections, inhibitors and repair assays

A total of 2 × 106 cells were transfected using the Amaxa nucleofector II (Lonza) and home-made nucleofection solution (45). The cell lines with corresponding nucleofector programs and expression vector concentrations are described in supplementary experimental procedures. Cas9 WT with empty gRNA expression vector at the ratio described in Supplementary experimental procedures were used as a negative control. For paired nicking, the total amount of gRNA expression vectors was kept constant by using an empty gRNA expression construct. After nucleofection, Dimethyl sulfoxide (DMSO) or 1 μM of olaparib (Organic Synthesis Core Facility, MSKCC) were added for 24 or 48 h, while indicated concentrations of KU55933 (46) (EMD Millipore), VE-821 (47) or NU7441 (Selleck Chemical) (48) were added for 48 h. Flow cytometry data were analyzed with FlowJo (Treestar).
Statistics

Unless stated otherwise, the values in the figure graphs are derived from at least three independent experiments and error bars represent the standard deviation; *P-values are indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

Cell cycle analysis

Cells were pre-treated with DMSO or 500 nM Nocodazole (Sigma-Aldrich) for 16 h before transfection and for an additional 24 h after transfection. Samples for cell cycle and GFP expression analysis were collected immediately and 24 h after transfection. Cells were fixed in ice-cold 70% ethanol for 30 min, then washed twice with phosphate buffered saline (PBS), resuspended in 100 μg/ml RNase (Thermo Scientific) with 50 μg/ml propidium iodide (Sigma-Aldrich) and incubated for 30 min at 37°C. After incubation, cells were directly analyzed by flow cytometry.

Western blotting

Cells were collected using a scraper, washed with PBS and resuspended in lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM EGTA) containing protease and phosphatase inhibitor cocktail (Roche Molecular Biochemicals). The following antibodies with corresponding concentrations were used: anti-PAR (Trevigen, 1:1000), anti-Chk2 (EMD Millipore, 1:1000), anti-pChk2-T68 (Cell Signaling, 1:1000), anti-Chk1 (Santa Cruz, 1:750), anti-pChk1-S345 (Cell Signaling, 1:1000), anti-pKAP1-S824 (Bethyl Laboratories, 1:2000), anti-Cas9 (Santa Cruz, 1:1000), anti-Clathrin Heavy Chain (BD Transduction Laboratories, 1:10 000) and anti-Tubulin (Sigma, 1:10 000).

RESULTS

Cas9-induced nicks stimulate intrachromosomal HR

To investigate nickHR, we exploited the DR-GFP reporter (32) which is commonly used to study DSBHR in mammalian cells. DR-GFP consists of two tandem, inactive copies of the GFP gene, the first copy (SceGFP) containing the 18 bp I-SceI endonuclease recognition site with an in-frame stop codon and the second copy (iGFP) harboring truncations at both ends (Figure 1A). A DSB at the I-SceI site induces HR using the downstream copy as a donor, restoring the GFP open reading frame and leading to GFP positive (GFP+) cells (Figure 1A).

NGG motifs flank the I-SceI cleavage site on both strands such that both strands can be recognized by Cas9 paired with gRNAs that overlap the site (18,19) (Figure 1A). gRNAs were designed to bind either the transcribed (gRNA01) or non-transcribed (gRNA02) strand. We used human codon-optimized versions of wild-type Cas9 (Cas9WT) to induce DSBs, Cas9DH/ΔCas9 (Cas9H) and Cas9D/ΔCas9 (Cas9D) nickases to induce SSBs, and catalytically inactive Cas9 (Cas9DH) as a control (18,24,42). Cas9D nicked the strand complementary to the gRNA, while Cas9H nicked the strand identical to the gRNA sequence (18,19) (Figure 1A).

Human osteosarcoma (U2OS-DR-GFP) cells and mouse embryonic stem (ES-DR-GFP) cells harboring a single, genomically integrated copy of the DR-GFP reporter were transiently transfected with Cas9 and gRNA01 expression vectors. To measure the relative efficiency of HR, cells were analyzed by flow cytometry 48 h after transfection. In U2OS-DR-GFP cells, Cas9WT and both nickases stimulated HR; Cas9WT was more efficient than either nickase, and Cas9D was more efficient than Cas9H (Figure 1B). The nuclease-dead Cas9DH failed to induce HR, as did Cas9WT in the absence of a targeting gRNA. Similar results were obtained in U2OS cells transiently transfected with the DR-GFP reporter plasmid (Supplementary Figure S1A) and in mouse ES-DR-GFP cells, although the difference between Cas9D and Cas9WT was less pronounced (Figure 1D).

Our results suggest that, although somewhat variable, the efficiency of nickHR is generally lower than that of DSBHR, although we cannot rule out that SSB formation by Cas9 nickases is less efficient than DSB formation by Cas9WT. Overall, the level of DSBHR induced by Cas9WT was similar to that induced by I-SceI (Figure 1B and Supplementary Figure S1A–C). However, nickHR induced by Cas9D was much more efficient than nickHR induced by I-SceI nickases (unpublished results; (49)).

The higher efficiency of Cas9D relative to Cas9H may be due to an inherently higher nickase activity or may be related to which strand is nicked. To attempt to distinguish between these possibilities, a second gRNA (gRNA02) was used which binds the opposite strand (Figure 1A). Overall, gRNA02 was less efficient at inducing HR than gRNA01, suggesting that this gRNA targets Cas9 less well. As with gRNA01, Cas9D+gRNA02 induced a higher level of nickHR than Cas9H+gRNA02, indicating that Cas9D is a more efficient nickase than Cas9H (Figure 1C). The transcribed strand is nicked by Cas9D+gRNA01 and Cas9H+gRNA02 (Figure 1B and C), while the non-transcribed strand is nicked by Cas9D+gRNA02 and Cas9H+gRNA01 (N, Figure 1B and C). The induction of nickHR by Cas9D was higher than Cas9H with gRNA01, but the relative level of induction with Cas9D was even greater with gRNA02. Thus, these results suggest that the transcribed-strand bias recently reported for nick-induced gene targeting events (13) may not be recapitulated with intrachromosomal HR. NickHR induced by Cas9H in ES cells was very low under the transfection conditions that we used (Supplementary Figure S1C). To improve the efficiency, a redesigned gRNA backbone was used which has been reported to enhance gRNA transcription and stability (43). The enhanced gRNA (gRNA01 enh) substantially improved the induction of both DSBHR and nickHR in ES-DR-GFP cells (Figure 1D) as well as in U2OS-DR-GFP cells, especially for the nickases (Supplementary Figure S1B). With the enhanced gRNA design we compared the kinetics of nickHR and DSBHR and found that their overall kinetics were similar (Supplementary Figure S1D).

It has been reported that the Cas9N863A (Cas9N) mutant, like Cas9H, also nicks the strand identical to the gRNA sequence (50). We compared the efficiency of Cas9N with Cas9H at inducing nickHR and found that they were similar (Supplementary Figure S1B). Importantly, differences in...
HR induction by the different Cas9 variants were not due to varying expression levels (Supplementary Figure S1B). However, we observed significantly increased nickHR, but not DSBHR, in cells deficient for XRCC1, a molecular scaffold protein essential in later stages of SSB repair (51) (Supplementary Figure S1G and H). These results suggest that Cas9-induced nicks can be processed by SSB repair pathways which thus may indirectly modulate nickHR frequencies.

HR repair of nicks on the lagging-strand template is promoted by replication

DSBHR has been reported to be reduced outside of S phase (52). To determine whether cell cycle phase influences
nickHR, U2OS DR-GFP cells were arrested at G2/M by 16 h incubation in the presence of nocodazole, a microtubule depolymerizing agent (53). The arrested cells were transfected with Cas9 and gRNA expression vectors, and nocodazole treatment was continued for another 24 h, at which time cells were collected for flow cytometry. Approximately 85–90% of nocodazole-treated cells were in G2/M, compared with 35% of DMSO-treated control cells (Figure 1E), and the nocodazole-induced arrest persisted until GFP expression was analyzed 24 h later (Supplementary Figure S1I). In agreement with previous results (32), a 4-fold reduction in DSB HR was observed in the arrested cells (Figure 1F). We detected a similar 4-fold reduction in nickHR, regardless of which DNA strand was nicked (Figure 1F), suggesting that nickHR is not active in all phases of the cell cycle. Although we cannot rule out that the absolute levels of break formation were affected, nocodazole treatment had little effect on transfection efficiencies (NZE-GFP, Supplementary Figure S1I).

To explore the effect of DNA replication on nickHR, we used the DR-OriP-GFP reporter (36,54) which has the Epstein–Barr virus origin of replication (OriP) and can replicate in the presence of the EBNA1 protein (36,54) (Figure 1G). Replication from OriP at the dyad symmetry (DS) element is essentially unidirectional, given that EBNA1 binding to the family of repeat (FR) elements acts as a replication fork barrier (55). U2OS cells were transiently transfected with DR-OriP-GFP and an EBNA1 expression vector, together with Cas9 and gRNA01 expression vectors, and the frequency of GFP+ cells was determined 48 h later. In agreement with previous results (36), a modest (~1.3-fold) increase in the number of GFP+ cells was observed in the presence of the EBNA1 expression vector (Figure 1H), suggesting that DSB HR is weakly stimulated by replication. Alternatively, the modest increase in DSB HR in the presence of EBNA1 could be at least partly explained by enhanced GFP expression due to replication, which is supported by ~3-fold increase in the mean fluorescence intensity of GFP+ cells (data not shown) and sustained GFP expression at 96 h post-transfection (Supplementary Figure S1J). As with Cas9WT, the fraction of GFP+ cells was only marginally increased with Cas9D with EBNA1 expression (Figure 1H). By contrast, however, the fraction of GFP+ cells was considerably higher with Cas9H when the plasmid could replicate (~1.8-fold). The much larger increase with Cas9H cannot be explained by enhanced GFP expression due to replication, as the mean fluorescence intensity of GFP+ cells in samples transfected with Cas9D or Cas9H was only ~30% higher for both nickases (data not shown).

In these experiments performed with gRNA01, Cas9D nicked the leading-strand template, whereas Cas9H nicked the lagging-strand template (Figure 1G). To distinguish whether the apparent difference in the stimulation of nickHR by the two nickases is due to the nicked strand or due to the Cas9 nickase variant, we repeated this experiment using gRNA02, which binds to the opposite strand. In contrast to results with gRNA01, with gRNA02 nickHR was only marginally increased with Cas9H when the plasmid could replicate, but was considerably higher with Cas9D (~2.1-fold; Figure 1I). These results suggest that replication promotes HR at a nick on the lagging-strand template.

NickHR requires established HR factors but is not suppressed by NHEJ components

Among the early DNA damage responders are ATM and ATR, specialized kinases that typically respond to DSBs and replication stress, respectively (56,57). To test their involvement in nickHR, we applied chemical inhibitors KU55933 (ATM) and VE-821 (ATR) to transfected U2OS-DR-GFP cells. Inhibition of ATM led to a small (up to 25%) decrease in DSB HR induced by either Cas9WT or I-SceI at relatively low inhibitor concentrations (1–5 μM, Figure 2A), analogous to earlier results obtained using I-SceI in mouse cells (58). A similarly moderate (up to 25%) reduction in nickHR was observed when nicks were induced by either Cas9D or Cas9H. Conversely, inhibition of the ATR kinase significantly reduced DSB HR triggered by either Cas9WT or I-SceI; e.g. at concentrations which do not considerably influence the cell cycle (0.5 and 1 μM, (59)), DSB HR was decreased up to ~3-fold (Figure 2B). In contrast, at these concentrations nickHR induced by either Cas9D or Cas9H was only decreased by ~40%. The effectiveness and specificity of both inhibitors was confirmed by Western blot analysis of kinase substrates (Supplementary Figure S2A). Thus, neither ATM nor ATR signaling appear to be essential for nickHR, although both kinases may play a role in this pathway.

DSB HR involves multiple steps, including end resection, RAD51 filament formation on the resulting 3’ overhang, strand invasion into the homologous template and repair DNA synthesis (60) (Figure 2C). BRCA1, together with its heterodimeric partner BARD1, is implicated in end resection and recruitment of other HR proteins to DNA damage sites. BRCA2 plays a critical downstream role in HR by binding RAD51 and promoting RAD51 filament formation (6). To test the involvement of BRCA1 in nickHR, we expressed a truncated form of BARD1 (hB202), which inhibits BRCA1 function by preventing its interaction with full-length BARD1 (37,40). Similar to the reduction observed in DSB HR, inhibition of BRCA1 function reduced the frequency of nickHR by about 2-fold in both U2OS-DR-GFP and ES-DR-GFP cells (Figure 2D, Supplementary Figure S2B). We then inhibited the interaction between BRCA2 and RAD51 by expressing the BRC3 repeat fragment of BRCA2 which disrupts RAD51 filament formation (37,41). BRC3 expression reduced nickHR even more substantially than hB202 in both U2OS-DR-GFP and ES-DR-GFP cells, as it did DSB HR (Figure 2D, Supplementary Figure S2B). These results suggest that nickHR proceeds, at least in part, via a pathway similar to classical DSB HR.

NHEJ is the other major pathway of DSB repair in mammalian cells (61). The canonical NHEJ pathway involves the rejoicing of broken DNA ends through the activity of the Ku70/Ku80 and LIG4/XRCC4 complexes and the protein kinase DNA-PKcs, as well as a number of other proteins. As NHEJ is active throughout the cell cycle, including S phase when HR is also active, it can ‘compete’ with HR for the repair of broken DNA ends, such that DSB HR is elevated in NHEJ mutants (62) (Figure 2E). We thus
Figure 2. nick HR requires established HR factors but is not suppressed by NHEJ components. (A and B) U2OS-DR-GFP cells transfected with the indicated Cas9, I-SceI and gRNA expression vectors were incubated for 48 h in the presence of the various concentrations of ATM inhibitor KU55933 (A) or ATR inhibitor VE-821 (B). (C) Schematic representation of HR and its steps that are inhibited by the BRCA1-interacting peptide hB202 (from BARD1) and the RAD51-interacting peptide BRC3 (from BRCA2). (D) U2OS-DR-GFP cells were transfected with the indicated Cas9 and gRNA or I-SceI expression vectors and either with the empty expression vector (pCAGGS) or with the vectors encoding the hB202 and BRC3 peptides. (E) Canonical NHEJ is known to suppress DSB HR, but its effect on nick HR is uncertain. (F) Wild-type (J1), Ku70<sup>−/−</sup> or Xrcc4<sup>−/−</sup> ES-DR-GFP cells were transfected with the indicated expression vectors. (G) Ku70<sup>−/−</sup> ES-DR-GFP cells were transfected with the indicated expression vectors and either the empty (+ Vector) or KU70 (+ KU70) expression vector. (H) Wild-type (E14) or Dna-Pk<sup>−/−</sup> ES-DR-GFP cells were transfected with the indicated expression vectors. (I) U2OS-DR-GFP cells were transfected and analyzed as in (A and B), but incubated in the presence of various concentrations of a DNA-PK inhibitor (NU7441).
asked whether loss of canonical NHEJ components would affect nickHR, as it does DSBHR. As previously observed, loss of canonical NHEJ components would affect nickHR, as it does DSBHR. As previously observed with single nicks (Supplementary Figure S1), such that P̂\text{N}\text{HR} with the largest nick offsets approached background levels (i.e. without targeting gRNA).

To determine if P̂\text{N}\text{HR} can occur between chromosomal sequences, we targeted the pnDR-GFP reporters to the Hprt locus in ES cells (33) and confirmed correct, single-copy integration by Southern blotting (Supplementary Figure S3A). With Cas9D, HR was significantly higher when two gRNAs were expressed in reporters with nick offsets \(\geq 50\) bp (Figure 3F). The fold increase, relative to HR induced by a single nick, was not as high as observed with the plasmid reporters in U2OS cells, indicating either that paired nick is more efficiently converted to DSBs in plasmid substrates or that single nicks can be inducers of HR in the chromosomal setting, even when heterology is present adjacent to the nick. With Cas9H, higher levels of HR were also typically observed with expression of both gRNAs compared with either single gRNA, although this was significantly higher only at nick offset of 50 bp (Figure 3G), indicating that P̂\text{N}\text{HR} can also occur at DNA ends with 3' overhangs in the chromosomal context.

Efficiency of P̂\text{N}\text{HR} was considerably decreased at larger nick offsets, suggesting that the additional heterology may inhibit HR. Removal of the long heterology should, therefore, stimulate HR. To test this, we overexpressed exonuclease I (EXO1 (63)), which can cleave 5' overhangs and resect a nicked strand in a 5' to 3' direction (64), potentially generating a blunt-ended DSB. Surprisingly, however, overexpression of EXO1 in mES-pnDR-GFP-940 bp cells reduced both DSBHR and nickHR (Supplementary Figure S3C).

With Cas9WT, paired DSBs could be introduced into the pnDR-GFP reporters when both gRNAs were expressed (Figure 3B). Unlike in many of the paired nick configurations, however, paired DSBs did not substantially increase HR compared with a single DSB in either the plasmid or chromosome setting (Figure 3E and H).

An additional reporter was tested in which paired nick could give rise to blunt ends, should both gRNA/Cas9 complexes bind (pnDR-GFP-0 bp, gRNA05 and gRNA06; Figure 3I). For the plasmid reporter in U2OS cells, HR induced by Cas9D or Cas9H and both gRNAs was only \(\sim 3\)-fold higher than with either single gRNA and the absolute levels were much lower than with Cas9WT (Figure 3J), suggesting that paired nick was not efficiently induced, possibly as a result of occlusion of the target site by the binding of a single gRNA/Cas9 complex. For the chromosome reporter in ES cells, HR induced by either nickase was similar regardless of whether one or both gRNAs were expressed, also consistent with cleavage site occlusion preventing DSB formation. However, in this case, interestingly, Cas9D induced HR to nearly the same extent as Cas9WT (Figure 3K). While single nick are generally less efficient at inducing HR than DSBs, at least one study has suggested an equivalence between nicks and DSBs (24), although the underlying mechanisms remain unclear.
Figure 3. Paired nicks induce intrachromosomal HR. (A) Schematic of paired nicks with the potential to generate 5’ or 3’ overhangs to be tested for induction of PNHR. (B) Reporters for measuring PNHR between repeats. The left panel shows a schematic of the pnDR-GFP20-940 bp reporters. The inset shows the relative binding positions of the gRNAs (marked by the green or black horizontal bars) and Cas9 cleavage sites (dotted vertical lines). The lengths of the 5’ or 3’ nick offset as well as the heterology (orange bars) are indicated. The right panel shows expected cleavage positions in the pnDR-GFP reporter with the indicated gRNA/Cas9 combinations. (C-E) U2OS cells were transiently transfected with the indicated pnDR-GFP reporter and Cas9/gRNA expression vectors required to generate single or paired nicks or DSBs (schematically drawn under each bar). (F-H) ES cells with the various pnDR-GFP reporters integrated at the Hprt locus were transfected with the indicated Cas9 and gRNA expression vectors to generate single or paired nicks or DSBs (schematically drawn under each bar). (I) Schematic representation of the pnDR-GFP0 bp reporter, analogous to (B). (J) U2OS cells were transfected with the pnDR-GFP0 bp reporter and the indicated Cas9 and gRNA expression vectors. (K) ES cells with pnDR-GFP0 bp integrated at the Hprt locus were transfected with the indicated Cas9 and gRNA expression vectors.
NHEJ components suppress paired nick HR, depending on overhang type and length

Our results demonstrating that paired nicks are more potent inducers of HR than single nicks are consistent with the notion that paired nicks are converted to DSBs. This interpretation is supported by previous work which showed that paired nicks induce mutagenic NHEJ events, while NHEJ is rarely detected at single nicks (27,28). Given that HR and NHEJ can compete for the repair of a DSB induced by Cas9WT or I-SceI (Figure 2F–I) (33), we asked whether and NHEJ can compete for the repair of a DSB induced is rarely detected at single nicks (27,28). Given that HR and NHEJ can compete for the repair of a DSB induced by paired nicks and whether the overhang type influences this process (Figure 4A). To test this, we first focused on the pnDR-GFP-50 bp reporter, since PNHR was significantly higher than nickHR for both overhang types in ES-pnDR-GFP-50 bp cells (Figure 3F and G). The reporter was targeted to the Hprt locus of Ku70−/− ES cells (Supplementary Figure S4) and HR frequencies were compared in the absence or presence of exogenous KU70 expression 48 h after transfection.

As expected, DBSHR was significantly reduced with KU70 expression when either single or paired DSBs were induced by Cas9WT (Figure 4B). A significant reduction in Cas9D-induced PNHR was also observed with KU70 expression, indicating that NHEJ components can suppress HR involving DSBs with 5′ overhangs (Figure 4B). Surprisingly, however, Cas9H-induced PNHR was not suppressed, suggesting that NHEJ cannot compete with HR at 3′ overhangs. As observed with DR-GFP (Figure 2G), levels of nickHR were not influenced by KU70 expression, regardless of the Cas9/gRNA combination used to induce the single nicks.

To examine whether the effect of NHEJ deficiency on PNHR is influenced by nick offset, we generated a panel of Ku70−/− ES cell lines harboring the remaining pnDR-GFP reporters at the Hprt locus (Supplementary Figure S4). Cells were transfected with a Cas9 expression vector and the appropriate gRNA pairs. In each case, DBSHR induced by paired DSBs was considerably reduced (~4-fold) by KU70 expression (Figure 4D). Similarly, KU70 expression significantly reduced PNHR at Cas9D-generated nicks offset by 20 to 100 bp (Figure 4E), suggesting that paired nicks at these distances are converted to DSBs. However, KU70 expression did not affect PNHR at nick offsets of 500 or 940 bp, raising the possibility that paired nicks are not converted to DSBs at these distances or that DSBs with long 5′ overhangs cannot be processed by NHEJ. In contrast, HR of paired nicks generated by Cas9H was not affected by KU70 expression, regardless of the distance between nicks (Figure 4F). However, given that PNHR induced by Cas9H was significantly higher than nickHR only at a nick offset of 50 bp in wild-type ES cells (Figure 3G), we cannot exclude that the lack of an effect with KU70 expression at other distances is due to the lack of robust PNHR.

Interestingly, at nick offset of 0 bp, KU70 overexpression reduced frequency of HR when we used Cas9H but not Cas9D (Figure 4C). In the case of Cas9D, HR was equivalently high in wild-type ES cells with a single nick as with two nicks (Figure 3K), such that the bulk of the events may be attributable to nickHR. However, in the case of Cas9H, HR was somewhat higher with two nicks than with a single nick (Figure 3K), such that a low level of HR may be attributable to DSB formation which is suppressed by KU70.

These results suggest that although short 5′ overhangs of 20–100 bp generated by paired nicking can be processed by NHEJ as well as HR, neither longer 5′ overhangs nor 3′ overhangs of any length can engage the NHEJ machinery.

DISCUSSION

Despite the recent development of genome engineering techniques relying on the CRISPR-Cas9 system for the induction of single or paired nicks, the mechanisms of (nick)HR and PNHR and their importance in vivo remain largely unexplored. Here, we utilize the HR reporter DR-GFP and Cas9 nickases to study HR induced by either single or paired nicks in comparison to Cas9-induced DSBs in mammalian cells and determine that there is distinct genetic control of HR triggered by these different lesions.

NickHR requires core HR proteins and is not suppressed by NHEJ factors

BRCA1, BRCA2 and RAD51 are established core HR factors for the repair of DSBs in mammalian cells (65–67). The importance of RAD51 and BRCA2 in nickHR has been reported recently (13) and our results confirm that nickHR is reduced upon disruption of RAD51 function and extend these observations to BRCA1. Thus, nickHR requires the same core factors as DBSHR and proceeds via RAD51/BRA2-mediated strand invasion (6). BRCA1 may be required only at this invasion step, possibly to promote the recruitment of PALB2-BRCA2, although we cannot rule out its involvement in an earlier step (6).

NHEJ is well established to compete with HR for the repair of DSBs (62). Our results with Cas9-induced DSBs concur with these earlier findings. Notably, nickHR is not increased in the absence of canonical NHEJ factors, suggesting that it does not necessarily rely on DSB intermediates. In agreement, single Cas9-generated nicks fail to induce oncosomatic translocations, which require DSB formation (68). However, nicks may reportedly become NHEJ substrates in some contexts, e.g. when nickHR is impaired by reduction of BRCA2 activity (12,13).

Two master kinases, ATM and ATR, control the cellular response to DNA damage (57). Inhibition of ATM results in a limited reduction in DBSHR (58), possibly by impairing Mre11-mediated end resection (69). Our results show a similarly limited reduction in nickHR upon ATM inhibition. Since ATM is not likely activated by DNA nicks or gaps (70), the small reduction in nickHR might be attributable to an involvement in later steps of HR (71) or to its role in activation of ATR (69,70). In cells treated with concentrations of an ATR inhibitor that are not likely to affect the cell cycle, we observe up to a ~3-fold reduction of DBSHR but only a small and non-significant reduction of nickHR, more comparable to that observed after ATM inhibition. This suggests that, in contrast to DBSHR, involvement of ATR in nickHR is also limited. Since a robust activation of ATR by gapped DNA has been reported (72), either nicks are not converted to gaps or activation of ATR is not critical for
The NHEJ component Ku suppresses DSB and PNHR resulting from 5' nicks with the potential to form 5' overhangs. While the previous studies used Cas9D with different gRNA pairs to create both types of overhangs (27,28), our study uses Cas9D and Cas9H with a single set of gRNAs to generate 5' and 3' overhangs, respectively. In principle, the PNHR we observe with 3' overhangs could be due to how Cas9H cleaves DNA: Cas9D and Cas9H use distinct catalytic domains, and cleavage by Cas9H has been reported to be accompanied by exonuclease activity at the 3' ends (18). However, this minimal exonuclease activity is unlikely to promote HR as it would be directed at the long overhangs. Thus, it seems more likely that contrasting results with 3' overhangs are due to other differences in experimental design, e.g. nicking of the donor homology region (28), which has been shown to affect HR (13).

Even though it seems surprising that directional DNA unwinding or resection can occur from nicks separated by tens or hundreds of nucleotides, paired nicks with the potential to form 5' overhangs appear to be processed to DSBs because they can give rise to NHEJ-mediated insertions and deletions (27,28,68). Supporting the generation of a DSB intermediate also for HR, the level of HR induced by paired nicks is higher than by single nicks, although the overall levels were reduced compared to more closely-spaced nicks. PNHR at these offsets was not, however, suppressed by Ku. Even at 100 bp, the suppression by Ku was...
much weaker than at shorter offsets, raising the possibility that not all paired nicks are converted to DSBs. Alternatively, the long overhngs may not be recognized by Ku as DSB ends, but rather as ssDNA, which is a poor substrate for Ku binding (74). Since nicks are the primary DNA lesion induced by ionizing radiation (75), the ability of distant paired nicks to generate a DSB advances our understanding of DSB induction by ionizing radiation and other agents or processes that result in DNA nicks.

In contrast to 5′ overhangs, HR was not affected by loss of Ku when we used Cas9H which should generate 3′ overhangs. For example, at the 50 bp nick offset which gave the highest level of PNHR, no significant difference was observed in the presence or absence of Ku. One possibility is that DNA unwinding (or resection) required to generate DSBs at paired nicks can only occur efficiently in a single direction (i.e. 5′ > 3′). An alternative explanation is that DSBs with long 3′ overhangs are formed but are not processed by NHEJ, such that the HR and NHEJ machineries are not competing for this type of DNA end. The poor recovery of NHEJ events from DSBs with 3′ overhangs in human cells (generated in vivo by Cas9D; (27,28)) and in yeast cells (generated by an exonuclease in vitro; (76)) supports this interpretation. Mechanistically, weaker binding of Ku to 3′ overhangs in vitro as compared with 5′ overhangs (~20 nt; (77)) would support a greater role for Ku at 5′ overhangs; further, one or more polymerases could fill in 5′ overhangs (68,78) to make them more readily bound by Ku. Because 3′ overhangs are generated during HR-associated DSB end resection, inefficient Ku binding could be important for channeling these intermediates into HR.

Paired nicks induced directly opposite each other should, in principle, result in a blunt-ended DSB. However, Cas9-gRNA complexes may remain tightly bound to DNA after the induction of the initial nick (79) which would hamper the nicking of the second strand. Consistent with this, HR is only slightly increased by gRNA pairs that would have the potential to produce blunt ends, suggesting a low level of DSB formation. Accordingly, Ku suppresses PNHR when blunt ends are generated by Cas9H, which also indicates that the modulation of PNHR by Ku is not limited to lesions generated by Cas9D.

Influence of replication on nickHR

Our experiments confirm previous reports that nicks trigger HR (8,9,11–13,27,49,80). nickHR is generally lower than DSBHR; nicks may thus be inherently less recombinogenic, or, alternatively, they may be preferentially repaired by dedicated SSB repair pathways, which could limit the availability of nicked DNA for nickHR. Indeed, it has been reported that inhibition of PARPs, some of which are involved in early steps of SSB repair (81), increases nickHR repair of I-AniI-induced nicks (12). Although in our system nickHR was not clearly affected by PARP inhibition, we did detect increased nickHR in cells lacking the key SSB repair factor XRCC1 (51). Our results suggest, therefore, that Cas9-induced nicks are legitimate substrates for SSB repair pathways and that SSB repair and HR can compete for repair of nicked DNA, analogous to the competition between NHEJ and HR for a DSB (62).

Lesions that arise during DNA replication have been proposed to trigger HR (82). We indeed observe that replication stimulates nickHR, but only when the lagging-strand template was nicked. While further experiments will need to be performed to determine the generality of this result, it is interesting to speculate on its potential implications. Since DSBHR is generally more efficient than nickHR, conversion to a DSB could explain the increase in nickHR on a replicating plasmid, as proposed in other studies (4,83–85). Indeed, physical evidence has been provided for conversion of lagging-strand and leading-strand nicks induced by the HO endonuclease to a DSB at the yeast mat1 locus (84). But the question then arises as to why repair of the leading-strand template nick is unaffected by replication in our system. The authors of this previous study suggested that a nick on the lagging-strand template would be converted to a two-ended DSB by replication upstream of the nick, however, a similar conversion of the nick on the leading-strand template would require a second replication fork arriving from the opposite direction (84). In the absence of the second fork, the leading-strand template nick could only be converted to a one-ended DSB. Since in our experimental system forks should arrive from a single direction dictated by the orientation of the OriP cassette (Figure 1G), only the lagging-strand template nick would produce a two-ended DSB. Such a DSB might lead to a stronger stimulation of HR than a one-ended DSB generated by a leading-strand template nick. An interesting alternative explanation is that incomplete replication of the lagging-strand template results in an inherently recombinogenic 3′ overhang, while replication to the nick on the leading-strand template generates a blunt-ended DSB that would require additional processing prior to engaging the HR machinery. In either case, these results indicate the power of the Cas9 nickase system for probing the relationship between replication and HR. It should be noted, however, that gRNA binding causes DNA unwinding which could potentially influence repair of single or paired nicks. While other nuclease platforms (e.g. zinc-finger nucleases, (22,23) or I-AniI (9,13)) lack the flexibility of Cas9, they could thus further our understanding of various aspects of nickHR.

The data presented in this study begin to unravel the complex relationships between nick and DSB repair pathways during processing of nicked DNA in mammalian cells and may advance the genome engineering capabilities that are essential in developing effective and safe gene therapies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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**REFERENCES**

1. Ciccia, A. and Elledge, S.J. (2010) The DNA damage response: making it safe to play with knives. *Mol. Cell.*, **40**, 179–204.

2. Caldecott, K.W. (2014) DNA single-strand break repair. *Exp. Cell Res.*, **329**, 2–8.

3. Haber, J.E. (1999) DNA recombination: the replication connection. *Trends Biochem. Sci.*, **24**, 271–275.

4. Cox, M.M., Goodman, M.F., Kreuzer, K.N., Sherratt, D.J., Sandler, S.J. and Marians, K.J. (2000) The importance of repairing stalled replication forks. *Nature*, **404**, 1235–1250.

5. Gulli, A. and Scollay, R.H. (1998) Effects of DNA double-strand and single-strand breaks on intrachromosomal recombination events in cell-cycle-arrested yeast cells. *Genetics*, **149**, 1235–1250.

6. Lee, G.S., Neiditch, M.B., Salus, S.S. and Roth, D.B. (2004) RAG proteins shepherd double-strand breaks to a specific pathway, suppressing error-prone repair, but RAG initiating homologous recombination. *Cell*, **117**, 171–184.

7. Prakash, R., Zhang, Y., Feng, W. and Jasin, M. (2015) Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins. *Cold Spring Harb. Perspect. Biol.*, **6**, a016600.

8. Galli, A. and Scollay, R.H. (1996) Effects of DNA double-strand and single-strand breaks on intrachromosomal recombination events in cell-cycle-arrested yeast cells. *Genetics*, **149**, 1235–1250.

9. Lee, G.S., Neiditch, M.B., Salus, S.S. and Roth, D.B. (2004) RAG proteins shepherd double-strand breaks to a specific pathway, suppressing error-prone repair, but RAG initiating homologous recombination. *Cell*, **117**, 171–184.

10. McConnell Smith, A., Takeuchi, R., Pellenz, S., Davis, L., Majiels, N., Monnat, R.J. Jr and Stoddard, B.L. (2009) Generation of a nicking enzyme that stimulates site-specific gene conversion from the I-Anil LAGLIDADG homing endonuclease. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 5099–5104.

11. Nakahara, M., Sonoda, E., Niojima, K., Sale, J.E., Takenaka, K., Kikutki, K., Taniguchi, Y., Nakamura, K., Sumitomo, Y., Bree, R.T. et al. (2009) Genetic evidence for single-strand lesions initiating Nbs1-dependent homologous recombination in diversification of Ig v in chicken B lymphocytes. *PLoS Genet.*, **5**, e1000356.

12. Metzger, M.J., McConnell-Smith, A., Stoddard, B.L. and Miller, A.D. (2011) Single-strand nicks induce homologous recombination with less toxicity than double-strand breaks using an AAV vector template. *Nucleic Acids Res.*, **39**, 926–935.

13. Metzger, M.J., Stoddard, B.L. and Monnat, R.J. Jr (2013) PARP-mediated repair, homologous recombination, and back-up non-homologous end joining-like repair of single-strand nicks. *DNA Repair*, **12**, 529–534.

14. Davis, L. and Maijels, N. (2014) Homology-directed repair of DNA nicks via pathways distinct from canonical double-strand break repair. *Proc. Natl. Acad. Sci. U.S.A.*, **111**, E924–E932.

15. Rouet, P., Smith, F. and Jasin, M. (1994) Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 6064–6068.

16. Ueno, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S. and Gregory, P.D. (2010) Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.*, **11**, 636–646.

17. Stoddard, B.L. (2011) Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. *Structure*, **19**, 7–15.

18. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, **337**, 816–821.

19. Gasanias, G., Barrangou, R., Horvath, P. and Siksnys, V. (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, E2579–E2586.

20. Hsu, P.D., Lander, E.S. and Zhang, F. (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, **157**, 1262–1278.

21. Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K. and Sander, J.D. (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.*, **31**, 822–826.

22. Wang, J., Friedman, G., Doyon, Y., Wang, N.S., Li, C.J., Miller, J.C., Hua, K.L., Yan, J.J., Babiarz, J.E., Gregory, P.D. et al. (2012) Targeted gene addition to a predetermined site in the human genome using a ZFN-based nicking enzyme. *Genome Res.*, **22**, 1316–1326.

23. Ran, F.A., Hsu, P.D., Lin, C.-Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S., Zhang, Y. et al. (2013) Double nicking by RNA-guided CRISPR-Cas9 for enhanced genome editing specificity. *Cell*, **154**, 1380–1389.

24. Mali, P., Aach, J., Stranges, P.B., Esvelt, K.M., Moosburner, M., Kosuri, S., Yang, L. and Church, G.M. (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol.*, **31**, 833–838.

25. Fujii, W., Onuma, A., Sugira, K. and Naito, K. (2014) Efficient generation of genome-modified mice via offset-nicking by CRISPR/Cas system. *Biochem. Biophys. Res. Commun.*, **445**, 791–794.

26. Rong, Z., Zhu, S., Xu, Y. and Fu, X. (2014) Homologous recombination in human embryonic stem cells using CRISPR/Cas9 nickase and a long DNA donor template. *Protein Cell*, **5**, 258–260.

27. Duda, K., Lonowski, L.A., Koloed-Nielsen, M., Ibarra, A., Delay, C.M., Kang, Q., Yang, Z., Pruett-Miller, S.M., Bennett, E.P., Wandall, H.H. et al. (2014) High-efficiency genome editing via 2A-coupled co-expression of fluorescent proteins and zinc-finger nucleases or CRISPR/Cas9 nickase pairs. *Nucleic Acids Res.*, **42**, e84.

28. Pierce, A.J., Johnson, R.D., Thompson, L.H. and Jasim, M. (1999) XRC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes Dev.*, **13**, 2633–2638.

29. Pierce, A.J., Hu, P., Han, M., Ellis, N. and Jasim, M. (2001) Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. *Genes Dev.*, **15**, 3237–3242.

30. Richardson, C., Moynahen, M.E. and Jasim, M. (1998) Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. *Genes Dev.*, **12**, 3831–3842.

31. Taylor, R.M., Moore, D.J., Whitehouse, J., Johnson, P. and Caldecott, K.W. (2000) A cell cycle-specific requirement for the XRC31 BRCT II domain during mammalian DNA strand break repair. *Mol. Cell. Biol.*, **20**, 735–740.

32. Nakashiki, K., Cavello, F., Perrouault, L., Giovannangeli, C., Moynahen, M.E., Barchi, M., Brunet, E. and Jasim, M. (2011) Homology-directed Fanconi anemia pathway cross-link repair is dependent on DNA replication. *Nat. Struct. Mol. Biol.*, **18**, 500–503.

33. Stark, J.M., Pierce, A.J., Oh, J., Pastink, A. and Jasim, M. (2004) Genetic steps of mammalian homologous repair with distinct mutagenic consequences. *Mol. Cell. Biol.*, **24**, 9305–9316.

34. Weinstock, D.M., Richardson, C.A., Elliott, B. and Jasim, M. (2006) Modeling oncogenic translocations: distinct roles for double-strand break repair pathways in translocation formation in mammalian cells. *DNA Repair*, **5**, 1065–1074.

35. Elliott, B. and Jasim, M. (2001) Repair of double-strand breaks by homologous recombination in mismatch repair-defective mammalian cells. *Mol. Cell. Biol.*, **21**, 2671–2682.
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40. Westerman, U.K., Reyngold, M., Olshen, A.B., Baer, R., Jasin, M. and Moynahan, M.E. (2003) BAR1 participates with BRCA1 in homology-directed repair of chromosome breaks. Mol. Cell. Biol., 23, 7926–7936.

41. Davies, A.A., Masson, Y.J., Melilwraith, M.J., Stasiak, A.Z., Stasiak, A., Venkitaraman, A.R. and West, S.C. (2001) Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. Mol. Cell, 7, 273–282.

42. Via, E.M., Jasin, M. and Krawczyk, P.M. (2014) Assaying break and nick-induced homologous recombination in mammalian cells using the DR-GFP reporter and Cas9 nucleases. In: Doudna, J. (ed.) The Use of CRISPR/Cas9, ZFNs, and TALENs in Generating Site-Specific Genome Alterations. Elsevier, Amsterdam, Vol. 546, pp. 145–191.

43. Chen, B., Gilbert, L.A., Ciminia, B.A., Schnitzbauer, J., Zhang, W., Li, G.-W., Park, J., Blackburn, E.H., Weissman, J.S., Qi, L.S. et al. (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. Cell, 155, 1479–1491.

44. Pierce, A.J. and Jasin, M. (2005) Measuring recombination proficiency in mouse embryonic stem cells. Methods Mol. Biol., 291, 373–384.

45. Zhang, Y., Vanoli, F., LaRoque, J.R., Krawczyk, P.M. and Jasin, M. (2014) Biallelic targeting of expressed genes in mouse embryonic stem cells using the Cas9 system. Methods, 69, 171–178.

46. Hickson, I., Zhao, Y., Richardson, C.J., Green, S.J., Martin, N.M.B., Orr, A.I., Reaper, P.M., Jackson, S.P., Curtin, N.J. and Smith, G.C.M. (2004) Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. Cancer Res., 64, 9152–9159.

47. Reaper, P.M., Griffiths, M.R., Long, J.M., Charriére, D., Maccormick, S., Charlton, P.A., Goel, J.M.C. and Pallard, J.R. (2011) Selective killing of ATM- or p53-deficient cancer cells through inhibition of ATR. Nat. Chem. Biol., 7, 428–430.

48. Leahy, J.J., Golding, B.T., Griffin, R.J., Hardcastle, J.R., Richardson, C., Rigoureau, L. and Smith, G.C.M. (2004) Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromone libraries. Bioorg. Med. Chem. Lett., 14, 6083–6087.

49. Katz, S.S., Gimble, F.S. and Storiaci, F. (2014) To nick or not to nick: comparison of I-SceI single- and double-strand break-induced recombination in yeast and human cells. PLoS One, 9, e88480.

50. Nishimatsu, H., Ran, F.A., Hsu, P.D., Konermann, S., Shehata, S., Dohmae, N., Ishitani, R., Zhang, F. and Nureki, O. (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell, 156, 935–949.

51. Caldecott, K.W. (2008) Single-strand break repair and genetic disease. Nat. Rev. Genet., 9, 619–631.

52. Saleh-Gohari, N. and Helleday, T. (2008) Single-strand break repair and genetic disease. Ann. Rev. Biochem., 79, 181–211.

53. Kass, E.M. and Jasin, M. (2010) Collaboration and competition between DNA double-strand break repair pathways. FEBS Lett., 584, 3703–3708.

54. Bolderson, E., Richard, D.J., Edelman, W. and Khanna, K.K. (2009) Involvement of Exo1 in DNA damage-induced apoptosis. Nucleic Acids Res., 37, 3452–3463.

55. Potenski, C.J., Niu, H., Sung, P. and Klein, H.L. (2014) Avoidance of ribonucleotide-induced mutations by RNRase H2 and Sr2-Exo1 mechanisms. Nature, 511, 251–254.

56. Moynahan, M.E., Chiu, J.W., Koller, B.H. and Jasin, M. (1999) Brca1 controls homology-directed DNA repair. Mol. Cell, 4, 511–518.

57. Moynahan, M.E., Pierce, A.J. and Jasin, M. (2001) BRCA2 is required for homology-directed repair of chromosomal breaks. Mol. Cell, 7, 263–272.

58. Stark, J.M., Hu, P., Pierce, A.J., Moynahan, M.E., Ellis, N. and Jasin, M. (2002) ATM phosphorylation by mammalian RAD51 has a key role during homology-directed DNA repair. J. Biol. Chem., 277, 20185–20194.

59. Ghezraoui, H., Piganeau, M., Renouf, B., Jasin, M. and Smith, G.C.M. (2014) Chromosomal translocations in human cells are generated by canonical nonhomologous end-joining. Mol. Cell, 55, 829–842.

60. Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G.C.M., Lukas, J. and Jackson, S.P. (2006) ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat. Cell Biol., 8, 37–45.

61. Shiotani, B. and Zou, L. (2009) Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. Mol. Cell, 33, 547–558.

62. Bakr, A., Ong, C., Köcher, S., Borgmann, K., Dorrreiter, L., Petersen, C., Dikomey, E. and Mansour, W.Y. (2015) Involvement of ATM in homologous recombination after end resection and RAD51 nucleofilament formation. Nucleic Acids Res., 43, 3154–3166.

63. Vidal-Eychene, S., Décailliet, C., Basbous, J. and Constantinou, A. (2013) DNA structure-specific pruning of ATR activation by DNA-PKcs. J. Cell Biol., 202, 421–429.

64. Ristic, D., Modesti, M., Kanaar, R. and Wyman, C. (2003) Rad52 and Ku bind to different DNA structures produced early in double-strand break repair. Nucleic Acids Res., 31, 5229–5237.

65. Mimori, T. and Hardin, J.A. (1986) Mechanism of interaction between Ku protein and DNA. J. Biol. Chem., 261, 10375–10379.

66. Goodhead, D.T. (1989) The initial physical damage produced by ionizing radiations. Int. J. Radiat. Biol., 56, 623–634.

67. Frank-Vaillant, M. and Marcand, S. (2002) Transient stability of DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination in mammalian cells. J. Mol. Biol., 307, 1235–1245.

68. Fisher, A.E.O., Hochegger, H., Takeda, S. and Caldecott, K.W. (2007) Poly(ADP-ribose) polymerase 1 accelerates single-strand break repair in concert with poly(ADP-ribose) glycohydrolase. Mol. Cell Biol., 27, 5597–5605.

69. Arnaudeau, C., Lundin, C. and Helleday, T. (2001) DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells. J. Mol. Biol., 307, 1235–1245.
83. Saleh-Gohari, N., Bryant, H. E., Schultz, N., Parker, K. M., Cassel, T. N. and Helleday, T. (2005) Spontaneous homologous recombination is induced by collapsed replication forks that are caused by endogenous DNA single-strand breaks. Mol. Cell. Biol., 25, 7158–7169.

84. Cortés-Ledesma, F. and Aguilera, A. (2006) Double-strand breaks arising by replication through a nick are repaired by cohesin-dependent sister-chromatid exchange. EMBO Rep., 7, 919–926.

85. Roseaulin, L., Yamada, Y., Tsutsui, Y., Russell, P., Iwasaki, H. and Arcangioli, B. (2008) Mus81 is essential for sister chromatid recombination at broken replication forks. EMBO J., 27, 1378–1387.