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Homologous recombination repair intermediates promote efficient de novo telomere addition at DNA double-strand breaks

Anoushka Dave¹,², Chen-Chun Pai¹, Samuel C. Durley¹,², Lydia Hulme¹, Sovan Sarkar¹, Boon-Yu Wee¹, John Prudden¹, Helen Tinline-Purvis¹, Jason K. Cullen¹,³, Carol Walker¹, Adam Watson², Antony M. Carr², Johanne M. Murray² and Timothy C. Humphrey¹,*

¹CRUK/MRC Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, Oxford OX3 7DQ, UK, ²Genome Damage and Stability Centre, School of Life Sciences, University of Sussex, Sussex BN1 9RQ, UK and ³QIMR Berghofer Medical Research Institute, Brisbane 4006, Australia

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ABSTRACT

The healing of broken chromosomes by de novo telomere addition, while a normal developmental process in some organisms, has the potential to cause extensive loss of heterozygosity, genetic disease, or cell death. However, it is unclear how de novo telomere addition (dnTA) is regulated at DNA double-strand breaks (DSBs). Here, using a non-essential minichromosome in fission yeast, we identify roles for the HR factors Rqh1 helicase, in concert with Rad55, in suppressing dnTA at or near a DSB. We find the frequency of dnTA in rqh1Δ rad55Δ cells is reduced following loss of Exo1, Swi5 or Rad51. Strikingly, in the absence of the distal homologous chromosome arm dnTA is further increased, with nearly half of the breaks being healed in rqh1Δ rad55Δ or rqh1Δ exo1Δ cells. These findings provide new insights into the genetic context of highly efficient dnTA within HR intermediates, and how such events are normally suppressed to maintain genome stability.

INTRODUCTION

DNA double-strand breaks (DSBs) are potentially lethal lesions if unrepaired, and their misrepair can give rise to chromosomal rearrangements, a hallmark of cancer cells (1,2). To maintain both viability and genome stability in response to such lesions cells have evolved two types of DSB repair pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). During classic non-homologous end joining (C-NHEJ), the broken ends are bound by the Ku70/Ku80 heterodimer, and following the removal of damaged bases, are ligated together through the activity of Ligase 4 (Lig4) (reviewed in (3)). During HR repair, homologous sequences within a chromatid or chromosome are used as a template for accurate repair. HR repair is initiated by nucleolytic resection of the 5′ end to generate a 3′ single-stranded DNA (ssDNA) overhang. Resection is a two-step process, which is initiated by the MRN complex (comprised of Mre11–Rad50–Nbs1 in Schizosaccharomyces pombe (S) and in Homo sapiens (Hs)), and CtIP resulting in partly resected intermediates. During the second step, Exo1 together with Rqh1 (BLMΔ) facilitates extensive resection (4–6) (reviewed in (7)). The 3′ ssDNA overhang is bound by Replication Protein A (RPA), which facilitates binding of the mediator Rad52 (8,9), together with the auxiliary heterodimers Rad55Δ–Rad55Δ or Swi5Δ–Swi5Δ to mediate the loading of the RecA homologue, Rad51Δ onto the ssDNA overhang to create a Rad51 nucleoprotein filament. This structure facilitates a homology search and strand exchange between the broken end and the homologous sequence to form a displacement-loop (D-loop) structure (10–13). Following DNA synthesis the invading strand can be expelled by BLM and RecQL5 in mammalian cells, thus facilitating synthesis-dependent strand annealing (SDSA). Alternatively, second-end capture and ligation can result in a double-Holliday junction structure, which can be resolved with or without crossovers, a process involving Yen1, Mus81Δ–Eme1Δ, or dissolved through the activities of BLM-Top3 (reviewed in (14)).

Consistent with multiple roles in HR-dependent DSB repair, the RecQ family of DNA helicases plays a key role in maintaining genome stability in all organisms (15). A hallmark of BLM mutations in human cells is increased levels of sister chromatid and inter-homolog exchanges (16). In fission yeast, loss of the BLM orthologue, Rqh1, results in increased genome instability and sensitivity to DNA damaging agents (17,18). Rqh1 is an ATP-dependent 3′ to 5′ he-
licase, in which the N-terminus interacts with Top3 (19,20). Rqh1 has been implicated in a variety of processes including HR, both before and after Rad51 filament formation (19,21,22); suppressing mitotic crossovers and promoting meiotic crossovers (23–25); suppressing inappropriate recombination following S phase arrest (17,18); facilitating the repair of collapsed replication forks (26–28); intra-S checkpoint function (29); efficient chromosome segregation (30) and telomere maintenance (31,32). A role for Rqh1 has also been identified in regulating HR-dependent Alternative Lengthening of Telomeres (ALT) pathway in the absence of Taz1 in fission yeast (33).

While normally repaired by the NHEJ or HR pathways, broken chromosome ends can sometimes be ‘healed’ as a result of telomeric capture or de novo telomere addition (deTA). While deTA is part of the normal developmental process in unicellular ciliates, chromosome healing in mammalian cells is associated with terminal deletions and genetic disease (34,35). Indeed, chromosome healing of a break within the body of a chromosome would be expected to result in extensive loss of heterozygosity (LOH) or potentially cell death through loss of genetic material centromeric to the break site. Accordingly, deTA is not normally observed in response to ionizing radiation (IR) or enzyme-induced DSBs in yeasts or mammals (36–38), and may reflect the absence of telomeric seed sequences close to the break site, low levels or inhibition of telomerase, or competition with DSB repair pathways (39–41).

Here, we have investigated the relationship between DSB repair and loss of heterozygosity arising through deTA. By introducing a site-specific DSB into a non-essential minichromosome, Ch16, we have uncovered a critical role for Rqh1 helicase, together with Rad55 in suppressing chromosome healing through deTA at break sites. Further analysis suggests that stabilized HR intermediates are efficient substrates for deTA.

MATERIALS AND METHODS

Yeast strains, media and genetic methods

All S. pombe strains were cultured, manipulated, and stored as previously described (42). A list of strain genotypes can be found in Supplementary Table S1.

DSB assay

The DSB assay using Ch16-MGH was performed as previously described (41,43). The minichromosome (Ch16) is a mitotically and meiotically stable 530 kb chromosomal element derived from ChIII (44). The DSB assay was performed at 25°C for strains containing the cold-sensitive mutant pfh1-R20cs (45) and appropriate comparison strains as indicated in the tables. The colony percentage undergoing NHEJ/SCR (ade+ G418R his−), GC (ade+ G418S his−), Ch16-MGH loss (ade+ G418R his−), or LOH (ade+ G418S his−) were calculated. LOH in this context refers to events which retain the ade+ marker but have lost the G418S marker. It is not possible to distinguish genetically between minichromosome loss and other rearrangements resulting in ade− G418S his− colonies, such as isochromosomosome formation, using Ch16-MGH so this population is collectively termed here ‘Ch16 loss’. Each experiment was performed three times using independently derived strains for all mutants tested. More than 1000 colonies were scored for each time point. Mean ± SEM values were obtained from triplicate strains. Differences were deemed significant if P-values obtained using Student’s t test were ≤0.05.

Pulsed field gel electrophoresis (PFGE)

The PFGE protocols used in this study have been previously described (42). For higher resolution separation of Ch16-MGH, a 1.2% chromosomal grade agarose gel was used under the following conditions: 4 V/cm 112° angle with a switch time of 1 min. Samples were separated for 48 h in 1 × Tris–acetate–EDTA at 14°C.

PCR assay for de novo telomere addition

Up to 20 randomly chosen ade+ G418R his− (LOH) colonies from each genetic background indicated were screened for telomeric sequence distal to the MATA break site as described. PCR amplification with primers targeted to the rad21 gene (5′-GATTTAACCTGGATTTGGGC-3′) and telomeric repeats (5′-CTGTAACCGTAAACGTAAC-3′) was performed, followed by digestion with MfeI, yielding a distinct 300 bp band in telomere-positive strains.

Rapid DSB-induction

Strains encoding urg1::hph were generated and urg1::HO containing strains subsequently generated by cassette exchange as previously described (46). Strains were grown at 32°C in 500 ml of pombe minimal glutamate media (PMG) containing G418 (200 µg/ml), leucine and arginine (100 µg/ml) but lacking adenine, uracil and histidine (47). To induce urg1::HO expression, cultures were grown to an OD605nm of 0.3–0.5. Cells were harvested, washed with water and suspended in PMG containing leucine, adenine, histidine, arginine (100 µg/ml) and uracil (250 µg/ml). 50 ml samples were harvested, washed in water with 0.5% sodium azide then stored at −80°C.

Gene targeting

Plasmid pJK148 (48) was linearized with NdeI restriction, and transformed into the strains indicated using Lithium Acetate protocol (47), and the number of Leu+ transformants determined for each strain. The gene targeting efficiency was adjusted according to the relative transformation efficiencies of each strain, as determined using a circular pREP81X (49) as a control.

RESULTS

Rqh1 suppresses loss of heterozygosity in rad55Δ

To investigate the role of Rqh1 in genome stability, we examined the relationship between Rqh1 and other DNA recombination genes in the cellular response to DSBs. We found that deletion of rqh1Δ in a rad55Δ background...
Figure 1. Rqh1 suppresses break-induced LOH in a rad55Δ background. (A) Spot dilutions of wild-type (TH1900) rad55Δ (TH1760) rqh1Δ (TH1807) and rqh1Δ rad55Δ strains (TH2136) strains grown on YES plates following exposure to 0 Gy, or 100 Gy IR, as indicated. (B) Schematic of the Ch16-MGH strain and ChIII as previously described. The loci of the centromeres (black oval), ade6-M216 and ade6-M210 alleles (white boxes), MATa target site (black box), kanMX6 gene (gray) and his3+ gene (striped) are as indicated. pREP81X-HO generates a DSB at the MATa target site (scissors). The expected marker loss profiles associated with different repair outcomes are indicated. (C) Site-specific DSB repair profile of wild-type (TH1900), rqh1Δ, rad55Δ and rad55Δ rqh1Δ strains following HO-endonuclease induction for 48 h. Data are derived from Table 1.
resulted in a significant increase in the IR-sensitivity of rad55Δ (Figure 1A). To investigate this further, we examined the relationship between rad55Δ and rqh1Δ deletion mutants using a site-specific DSB assay. Using this assay, different repair and misrepair events can be quantified by determining genetic marker loss following HO endonuclease induction of a site-specific DSB at the MATa site inserted within a non-essential minichromosome, ChΔMGH, derived from chromosome III (Figure 1B). ChΔMGH carries an ade6-M216 heterolelele which complements the ade6-M210 heterolelele on ChIII to confer an ade6 phenotype through intragenic complementation (50). Following HO endonuclease expression from a thiamine-repressible nmt promoter (rep81X-HO) DSB induction can result in a variety of outcomes: DSB repair through NHEJ or sister chromatid recombination (SCR) in which a broken chromatid uses its unbroken sister chromatid as a repair template; failed DSB repair with loss of the minichromosome; gene conversion using the homology of chromosome III; extensive loss of heterozygosity, resulting through break-induced non-reciprocal translocations or partial loss of heterozygosity (Figure 1B) (41).

Surprisingly, HO endonuclease-induced cleavage at the MATa site in an rqh1Δ rad55Δ double mutant resulted in a striking increase in LOH (27.3%, P < 0.001) compared to wild type (1.7%). This increase in LOH was associated with significantly increased NHEJ/SCR (46.1%, P < 0.001) and significantly reduced GC (3.3%, P < 0.001) compared to wild type, while Ch16 loss (18.6%) was similar to both single mutant and wild-type backgrounds (Figure 1C; Table 1). These findings indicate that Rqh1 suppresses LOH in a rad55Δ background. No loss of viability was observed in these strains following DSB induction (Supplementary Figure S1).

Rqh1 suppresses de novo telomere addition in a rad55Δ background

To identify the mechanism of break-induced LOH, the chromosomes of 21 LOH colonies from an rqh1Δ rad55Δ background were examined by pulsed-field gel electrophoresis (PFGE). While endogenous chromosomes I and II derived from these LOH colonies remained unchanged, crossovers were sometimes observed (9.5% of LOH colonies) between ChIII and the homologous minichromosome, Ch16 (Figure 2A, lane 4). Importantly, minichromosomes from the remaining 90.5% of the LOH colonies appeared truncated, as determined by high-resolution separation of chromosomal DNA by PFGE (Figure 2B). As break-induced LOH retained the ade6 marker ∼25kb centromeric proximal to the break site (Figure 1A), this raised the possibility that Ch16 truncations resulted from dTIA, as was previously observed in a rad55Δ background (41). This possibility was examined by colony PCR amplification using primers annealing to rad21Δ (centromeric proximal to the MATa break site) and a telomere specific primer. A PCR product of 300 bp following digestion with MfeI (a restriction site just upstream of the MATa site) was scored positive for dTIA. Sequence analysis of the PCR products indicated the presence of ∼300 bp of G2.5TTACA0.1 repeats, consistent with dTIA at, or very close to, the break site in 13 of the LOH colonies tested (Figure 2C). In 6 of the remaining colonies, telomeres were added ~9–19 kb centromeric proximal to the break site. In full, 24.7% of colonies underwent dTIA in rqh1Δ rad55Δ strains, which equated to a 1450-fold increase in dTIA compared to wild type (0.017%) (Figure 2D; Table 1).

Suppression of de novo telomere addition requires Rqh1 helicase activity

To determine whether Rqh1 required its helicase activity to suppress dTIA, we introduced a helicase-dead mutation rqh1-K547A (19,51) into a rad55Δ background. In this strain, levels of break-induced LOH and dTIA resembled those observed in the rqh1Δ rad55Δ strain (Figure 3A; Table 1) suggesting Rqh1 helicase activity is required to suppress dTIA in a rad55Δ background.

Rqh1 has been shown to function in a complex with Top3 (19,20,52). As the top3Δ strain is non-viable (19,52), we tested the requirement of the Top3 interaction in suppressing dTIA using an rqh1ΔΔN1–322 mutant which has lost the Top3 binding domain (20) and is expressed at similar levels to the wild-type Rqh1 (20). DSB-induced LOH in the rad55ΔΔrqh1ΔΔN1–322 mutant was significantly higher (11.9%, P < 0.001) than that observed in rad55ΔΔ (1.8%), but less than the rqh1Δ rad55Δ strain (27.3%; Figure 3A; Table 1). This effect could be attributed to a requirement for Rqh1-Top3 interaction in suppressing break-induced LOH in a rad55Δ background or to partial loss of Rqh1 helicase activity in the rad55ΔΔN1–322 mutant.

To determine whether other helicases could function similarly to Rqh1, we introduced a deletion of srs2+ or a cold-sensitive allele of pfh1+, pfh1-R20 (pfh1Δ) (45) into a rad55Δ background and examined levels of dTIA. Srs2 is implicated in regulation of HR where it antagonizes the activity of the Rad55–Rad57 heterodimer (53–55). In contrast to the rqh1Δ mutant, the srs2Δ mutant failed to significantly increase levels of break-induced LOH in a rad55Δ background (Figure 3A; Table 1). The S. cerevisiae Pfh1 homologue, Pif1 has been identified as a suppressor of dTIA and gross chromosomal rearrangements (56,57). The rad55ΔΔ pfh1Δ strain showed a modest yet significant increase in LOH (6.4%) compared to wild-type background at semi-permissive temperature (0.8%, P = 0.013), and a significant increase in comparison to rad55Δ ΔLOH levels (P = 0.027), at semi-permissive temperature (Figure 3A; Table 1). Therefore, Pfh1 can suppress LOH arising predominantly from dTIA, in accordance with the described role in S. cerevisiae. However, in our assay, Rqh1 helicase clearly plays a more prominent role in suppressing dTIA than Srs2 or Pfh1.

Rqh1 functions with early acting HR proteins to suppress dTIA

Next we wished to examine the potential role of other HR factors in suppressing dTIA in an rqh1Δ background. As Exo1 functions early in HR during DSB resection, we examined the relationship between Rqh1 and Exo1 (5,6,58). Deletion of exo1Δ did not significantly alter levels of break-induced LOH compared to wild type (41). However, a striking increase in levels of break-induced LOH (20.7%) was
Figure 2. *De novo* telomere addition causes LOH in an *rqh1*Δ *rad55*Δ background. (A) Pulsed Field Gel Electrophoresis (PFGE) of LOH colonies obtained after HO induction in the *rqh1*Δ *rad55*Δ background. (B) High resolution PFGE of LOH colonies. (C) The sequence of the HO endonuclease cleavage site within *MATα* is shown, together with representative genomic DNA sequence data of the region surrounding the *MATα* site from five individually isolated ade* G418* LOH colonies with truncated minichromosomes, indicating the presence of *de novo* telomeres. (D) Graph depicting mechanisms of LOH in wild type (WT, TH1900), *rqh1*Δ, *rad55*Δ and *rqh1*Δ *rad55*Δ backgrounds following DSB induction in Ch16-MGH. Data are derived from Table 1.
observed in an rqh1Δ exolΔ double mutant, 60% (12 of 20 examined colonies) of which was due to dnTA (Figure 3B, Table 1). Break-induced marker loss after deletion of exol+ in an rqh1Δ or rqh1Δ rad55Δ background was also determined (Table 1). Break-induced LOH was significantly reduced in the rqh1Δ rad55Δ exolΔ mutant compared to the rqh1ΔΔ rad55Δ mutant ($P = 0.001$) and no dnTA products were obtained upon further analysis (Table 1). This requirement for exol+ in facilitating dnTA in the rqh1Δ rad55Δ background is in accordance with Exol-dependent end-resection facilitating dnTA, as previously proposed (41). We were unable to test the role of Rad52 in suppressing dnTA as the rqh1Δ rad52Δ strain was extremely sick, consistent with previously reported findings (19).

As Rad57 forms a heterodimer with Rad55 (59), we examined gene marker loss in a rad57Δ background. The resultant marker loss profile was similar to that of rad55Δ strains (Table 1). Following DSB induction in an rqh1Δ rad57Δ background, 7% of colonies exhibited extensive LOH ($P = 0.011$ compared to a rad57Δ single mutant), out of which 50% of the double mutant had undergone dnTA (Table 1). Thus, Rqh1 can suppress LOH in a rad57Δ background, albeit not to the same extent as in a rad55Δ background.

DSB induction within Ch16 in a rad51Δ background has previously been shown to result in a higher proportion of minichromosome loss, demonstrating a failure to repair the DSB (41,42). DSB induction in a rhp55Δ rad51Δ background resulted in reduced levels (0.39% of LOH colonies compared to wild type ($1.7%; P = 0.007$) and $rhp55Δ (1.5%; $P = 0.051$), indicating that, in contrast to a rad55Δ background, Rqh1 does not suppress break-induced LOH significantly in a rad51Δ background (Figure 3B; Table 1). Instead, a significant increase in NHEJ was observed in an rhp55Δ rhp55ΔΔ background compared to that of rad51Δ Δ (35.9%, (41)), indicating that DSBs in an rhp55Δ rad51Δ double mutant are still competent for HR-independent repair, even though HR is severely impaired. These observations are consistent with an early role for Rqh1 in HR, as described for Sgs1 and BLM in budding yeast and human cells, respectively (4–6,58).

We have previously shown that LOH is significantly reduced in mus81Δ (0.2%) compared to rad55Δ strain (1.8%, $P = 0.014$) (41). In a mus81Δ rad55Δ strain, Ch16 loss dramatically increased (60.5%) compared to the mus81Δ Δ (38.1%) or rad55Δ Δ (30.5%) single mutants (41). As expected, GC is dramatically reduced in mus81Δ rad55Δ Δ (5.1%) compared to mus81Δ Δ (29.0%) as Rad55 acts upstream of Mus81 in HR. Consistent with the late role of Mus81 in HR, SCR in mus81Δ rad55Δ Δ (23.4%) is similar to mus81Δ Δ (28.1%) in comparison to rad55Δ Δ (62.8%) (41). Although LOH was not measured in mus81Δ Δ rad55Δ Δ, the high levels of Ch16 loss in mus81Δ Δ rad55Δ Δ and the low levels of LOH in mus81Δ Δ suggest that Mus81 does not suppress dnTA in an rhp55Δ Δ background.

### Table 1. Suppression of LOH by chromosome healing in HR mutant backgrounds

| Ch16-MGH in genetic background (strain No.) | % ade+ G418r/ HygR his+ (GC) | % ade+ G418r/ HygR his+ (NHEJ/SCR) | % ade+ G418r/ HygR his+ (Ch16 loss) | % ade+ G418r/ HygR his+ (LOH) | % ade+ G418r/ HygR his+ (dnTA) | P-value (LOH relative to wild type) |
|-------------------------------------------|-------------------------------|-----------------------------------|------------------------------------|-------------------------------|---------------------------------|-----------------------------------|
| **Wild type** | 49.7 ± 2.6 | 25.0 ± 1.4 | 20.5 ± 2.6 | 1.7 ± 0.3 | 0.0% (0/22) | 1.000 |
| rad53AΔ | 2.9 ± 0.7 | 62.0 ± 9.8 | 50.5 ± 10.9 | 1.8 ± 1.1 | 1.4% (4/20) | 0.936 |
| rhp55Δ | 22.8 ± 1.4 | 53.3 ± 0.4 | 18.6 ± 2.7 | 1.5 ± 0.3 | 0.3% (4/20) | 0.615 |
| rad55Δ rhp1Δ | 3.3 ± 0.7 | 46.1 ± 1.3 | 18.6 ± 1.6 | 27.3 ± 2.1 | 24.7% (19/21) | 3.3 E–06 |
| rad57Δ | 3.8 ± 0.9 | 73.1 ± 6.0 | 21.4 ± 5.7 | 1.8 ± 0.7 | 0.9% (10/20) | 0.955 |
| rad57Δ rad57Δ | 2.0 ± 0.7 | 76.8 ± 2.1 | 13.5 ± 3.3 | 7.7 ± 0.17 | 3.9% (10/20) | 9.75E–06 |
| rad55Δ rhp1ΔK547A | 3.2 ± 0.7 | 46.7 ± 7.0 | 22.9 ± 7.6 | 22.5 ± 5.0 | 20.3% (18/20) | 0.001 |
| rad55Δ rhp1ΔΔ-322 | 2.1 ± 0.4 | 68.6 ± 3.3 | 17.4 ± 1.8 | 11.9 ± 1.1 | 6.0% (10/20) | 0.008 |
| Wild type (25°C) | 28.5 ± 1.3 | 50.9 ± 1.6 | 10.2 ± 0.8 | 0.8 ± 0.2 | 0.0% (0/6) | 0.567 |
| rad55Δ (25°C) | 6.6 ± 3.7 | 47.4 ± 11.2 | 43.9 ± 11.3 | 0.6 ± 1.1 | 0.5% (15/20) | 0.867 |
| pfh1A (25°C) | 16.5 ± 1.7 | 63.0 ± 10.5 | 6.5 ± 2.0 | 0.6 ± 0.3 | 0.1% (1/9) | 0.609 |
| rad55Δ pfh1A (25°C) | 0.9 ± 0.7 | 72.7 ± 4.2 | 20.0 ± 2.6 | 6.4 ± 1.3 | 4.2% (13/20) | 0.013 |
| srs2Δ | 26.1 ± 2.7 | 49.9 ± 3.6 | 12.2 ± 2.5 | 0.2 ± 0.1 | 0.0% (0/18) | 0.006 |
| rad55Δ srs2Δ | 7.2 ± 1.3 | 43.0 ± 9.8 | 36.4 ± 12.1 | 2.1 ± 0.4 | 1.7% (16/20) | 0.402 |
| exolΔ* | 52.7 ± 1.0 | 33.1 ± 0.7 | 13.0 ± 0.8 | 1.1 ± 0.5 | 0.5% (10/22) | 0.233 |
| rhp1Δ exolΔ | 3.7 ± 1.7 | 54.3 ± 5.8 | 14.4 ± 6.6 | 20.7 ± 5.1 | 12.4% (12/20) | 0.004 |
| rhp1Δ rad57Δ | 7.5 ± 5.5 | 58.7 ± 13.5 | 28.1 ± 18.3 | 2.7 ± 0.8 | 0.0% (0/20) | 0.209 |
| exolΔΔ | 1.0 ± 0.5 | 35.9 ± 2.9 | 57.0 ± 2.9 | 0.8 ± 0.3 | 0.6% (20/25) | 0.058 |
| rhp1Δ rad51Δ | 3.1 ± 0.2 | 76.8 ± 3.4 | 19.5 ± 3.3 | 0.39 ± 0.07 | 0.3% (15/20) | 0.007 |

The mean ± SE from at least three independent experiments with three individual strains are shown. * denotes values as previously described (Cullen et al., 2007), shown here for comparison.
Mechanisms of LOH observed when Rqh1 helicase activity requires Swi5 or Rad51, thus further indicating a role for Rad51-loading being required for efficient telomere addition in a rad51Δ background (Figure 3D; Table 2). However, whilst rad51Δ rad55Δ rqh1Δ background resulted in significantly increased NHEJ/SCR colonies (57%, \( P < 0.001 \)) compared to wild type (25%), resembling levels observed in a rad55Δ background (63%). GC (22% \( P < 0.001 \)) was significantly reduced in a swi5Δ background compared to wild type (50%), but levels of Ch16 loss (20.5%) and LOH (0.8%) were similar to wild type (Table 2).

We also tested the rad55Δ swi5Δ double mutant. Marker loss in a rad55Δ swi5Δ strain was very similar to that in a rad51Δ strain, resulting in high levels of Ch16 loss (66%), consistent with failed Rad51 loading. Levels of NHEJ/SCR colonies were also reduced in the rad55Δ swi5Δ background (30%) compared to rad55Δ (62%) or swi5Δ (57%) single mutants, consistent with this population arising through HR-dependent SCR. Further, levels of LOH through dnTA (1.9%) in rad55Δ swi5Δ strain were equivalent to that of rad55Δ strain alone (1.4%; \( P = 0.743 \); Table 2).

Introducing a helicase-dead rqh1 mutant into a rad55Δ swi5Δ background (rad55Δ swi5Δ rqh1-K547A) resulted in a striking increase in break-induced LOH (17.9%, \( P < 0.001 \)) compared to wild type. However, further analysis indicated that only 25% of these were a result of dnTA (4.5%; Figure 3C; Table 2). Interestingly, dnTA levels were reduced 4.5-fold in rqh1-K547A rad55Δ swi5Δ background compared to an rqh1-K547A rad55Δ background (20.5%; Table 1). Therefore efficient dnTA in rad55Δ strains in the absence of Rqh1 helicase activity requires Swi5 or Rad51, thus further indicating a role for Rad51-loading being required for efficient dnTA.

We have previously demonstrated that Rad51 overexpression (OPrad51) reduced levels of dnTA in a rad55Δ background (41), consistent with competition between the Rad51 recombinase and telomerase for resected ends. We therefore tested whether Rad51 overexpression could similarly reduce levels of dnTA observed in an rqh1Δ rad55Δ background by introducing pIRT3-rad51 (60). OPrad51 resulted in significantly increased levels of GC (3.3%, \( P = 0.05 \)), and SCR (88.3%, \( P = 0.03 \)), and significantly reduced levels of Ch16 loss (3.9%, \( P = 0.04 \)) and LOH (4.45%, \( P = 0.04 \)), and therefore reduced levels of dnTA, in an rqh1Δ rad55Δ background compared to vector alone (Figure 3D; Table 2). However, whilst rad55Δ, rqh1Δ and rad51Δ rqh1Δ are exquisitely sensitive to radiation in a rad51Δ background (Supplementary Figure S2), overexpression of Rad51 does not significantly rescue radiation sensitivity in these mutants (Supplementary Figure S3). Together, these data identify a critical role for Rad51 recombinase levels in facilitating dnTA in an rqh1Δ rad55Δ background.
The above findings indicate that efficient dsTA is associated with HR intermediates. To test this further, we asked whether dsTA would be further increased under circumstances in which post-synaptic second-end capture was abrogated. To address this, the (130 kb) homologous arm centromere-distal to the break site was replaced by a construct containing the 1.8 kb MatA target sequence/G418-resistant marker and a 1 kb synthetic telomere, TASTel fragment containing 700 bp of subtelomeric DNA (TAS) and 300 bp of telomeric DNA (Tel) (Figure 4A) (61), in which there is no distal homologous chromosome arm (Ch16-MGTASTel). Following break-induction, DSB repair by NHEJ/SCR in Ch16-MGTASTel resulted in cells that retain the ade^− G418^R phenotype. Cells that fail to repair the DSB lose the minichromosome and become ade^+ G418^S, while those which undergo LOH become ade^+ G418^R. Following DSB induction in Ch16-MGTASTel cells, homologous recombination, strand invasion and DNA synthesis steps should still be possible for the broken centromere-proximal arm, while the later HR stages of second-end capture or strand annealing are obviated. In contrast to DSB induction in the Ch16-MGH strain, it is not possible for Ch16-MGTASTel cells to undergo GC and become ade^+ G418^S since GC requires the participation of two homologous DSB arms (62).

We found DSB induction in Ch16-MGTASTel in a wild-type background resulted in 79.3% of the colonies becoming ade^− G418^S, consistent with very high levels of unrepaired breaks leading to chromosome loss or other undetectable rearrangements; 17.4% remained ade^+ G418^R, consistent with NHEJ or SCR; and 3.3% became ade^+ G418^S, having undergone LOH (Table 4). Further PCR analysis of 20 individually isolated ade^+ G418^S colonies failed to detect dsTA (Figure 4B; Table 4). Deletion of rqh1^+, exo1^+ or rad55^+ each resulted in increased levels of NHEJ/SCR and reduced Ch16 loss, as was observed in Ch16-MGH. This was associated with modest increases in LOH and dsTA with 13% dsTA noted in a rad55Δ background (Figure 4B; Table 4). Remarkably, DSB induction in an rqh1^Δ rad55Δ background resulted in 53% of the colonies becoming ade^+ G418^S, corresponding to 45.2% dsTA (Figure 4B; Table 4). Similarly, following DSB induction in an rqh1Δ exo1Δ background, 51% of the colonies became ade^+ G418^S which corresponded to 41.4% dsTA.

To test whether the increased levels of dsTA resulted from loss of the second homologous chromosome arm, or from proximity to the TASTel synthetic telomere sequence, an additional minichromosome was constructed in which the TASTel sequence was integrated distal to the MatA site of Ch16-MG, (in the same locus as Ch16-MGH), but retaining the distal arm of the minichromosome, to form Ch16-MG(TASTel)Ch (Figure 4C). Surprisingly, DSB induction in a wild-type strain containing Ch16-MG(TASTel)Ch resulted in 76% Ch16 loss or extensive LOH; while 2% of the colonies underwent LOH or GC, and dsTA was not detected (Table 5). Although we cannot distinguish LOH or GC colonies, the levels of ade^+ G418^S colonies (combining LOH and GC) in Ch16-MG(TASTel)Ch were much less than ade^+ G418^S his^− (GC) in Ch16-MGH cells. The
Figure 4. Efficient dnTA occurs at a DSB lacking a homologous distal chromosome arm. (A) Schematic of the Ch16-MGTAS-Tel minichromosome. ChIII as described in Figure 1B. The loci of the centromeres (black oval), ade6-M216 and ade6-M210 alleles (white boxes), MATα target site (black box), KanMX6 gene (gray), and TASTel sequence (gray arrow) as indicated. pREP81X-HO generates a DSB at the MATα target site (scissors). (B) Histogram of percentage break-induced LOH arising through dnTA (gray) or other (white) in wild-type (WT, TH2039), \( \rho qh1Δ \) (TH2254), \( \rho qh1Δexo1Δ \) (TH2420), \( \rho qh1Δrad55Δ \) (TH2253), \( \rho qh1Δexo1Δrad55Δ \) (TH8226) and \( \rho qh1Δrad55Δ \) (TH8708) strains following HO-endonuclease induction for 48 h (Table 1). (C) Schematic of the Ch16-MGTAS-TelCh minichromosome. Minichromosome whose features are described in (A); ChIII as described in Figure 1B. (D) Histogram of percentage break-induced LOH arising through dnTA (gray) or other (white) in wild-type (TH8597), \( \rho qh1Δexo1Δ \) double mutant (TH8598) and \( \rho qh1Δrad55Δ \) double mutant (TH8708) strains following HO-endonuclease induction for 48 h (Table 5).
Further, we find that a DSB lacking a homologous distal Rqh1 case, together with either Rad55 or Exo1 suppresses healing at a break site in fission yeast. We find Rqh1 helicase and Rqh1 paralog, Rad55, in both facilitating homologous recombination and in suppressing chromosome healing and to prevent the formation of double-Holliday junctions, to displace non-allelic recombination.

Our study identifies an independent role for the HR proteins Rqh1, together with Rad55 or Exo1 in suppressing dT in suppressing dTA, with a striking increase in dT being observed in rad1Δ rad55Δ and to a lesser extent rad1Δ exo1Δ background, compared to wild type. As extensive resection requires both Rqh1 and Exo1 (4–6), these findings are consistent with partially resected ends acting as efficient substrates for dTA (37,38). Loss of both Rad55 and Rqh1 may also facilitate presynaptic dTA either through reduced resection or through altering the structure of the Rad51 nucleofilament so that it is more conducive to dTA (53,38). That overexpression of Rad51 in an rad1Δ rad55Δ background led to significantly reduced levels of dTA and significantly elevated levels of both GC and SCR suggests that Rad55 suppresses dTA through facilitating efficient Rad51 assembly. These findings are broadly consistent with a role for HR in preventing dTA through competition for resected ends (41).

However, RecQ helicase activity is also required for branch migration, to displace non-allelic recombination, and to prevent the formation of double-Holliday junctions (15), and loss of these post-synaptic functions may also

### Table 3. DSB-induced marker loss and dTA in rad55Δ and MRN deletion mutants

| Ch16-MGH in genetic background | % ade<sup>*</sup> G418<sup>R</sup>/Hyg<sup>S</sup> (NHEJ/SCR) | % ade<sup>*</sup> G418<sup>R</sup>/Hyg<sup>S</sup> in (Ch16 loss) | % ade<sup>*</sup> G418<sup>R</sup>/Hyg<sup>S</sup> in (LOH) | % ade<sup>*</sup> G418<sup>R</sup>/Hyg<sup>S</sup> in (dT) | P-value (LOH relative to wildtype) |
|---------------------------------|---------------------------------|----------------|----------------|----------------|------------------|
| Wild type                       | 49.7 ± 2.6                      | 20.5 ± 2.6 | 1.7 ± 0.3 | 0.0% (0/20) | 1.000            |
| rad55Δ*                         | 2.9 ± 0.7                       | 30.5 ± 10.9 | 1.8 ± 1.1 | 1.4% (16/22) | 0.396            |
| mre11A*                         | 30.7 ± 2.0                      | 35.7 ± 5.8 | 0.6 ± 0.2 | 0.3% (11/21) | 0.013            |
| mre11A rad55Δ                  | 6.6 ± 0.4                       | 61.7 ± 0.1 | 0.6 ± 0.2 | 4.8% (16/22) | <0.05            |
| rad50Δ*                        | 18.3 ± 1.8                      | 49.7 ± 1.9 | 0.6 ± 0.2 | 0.2% (6/20) | 0.011            |
| rad50Δ rad55Δ                  | 1.0 ± 0.3                       | 53.9 ± 5.4 | 4.9 ± 0.7 | 4.5% (21/23) | 0.088            |
| nbs1 Δ *                       | 15.6 ± 0.7                      | 43.6 ± 3.3 | 1.4 ± 0.2 | 1.7% (17/21) | 0.441            |
| nbs1 Δ rad55Δ                  | 0.1 ± 0.1                       | 61.9 ± 1.6 | 2.6 ± 0.5 | 2.4% (20/22) | 0.163            |

The mean ± SE from at least three independent experiments with three individual strains are shown. % dTA was calculated by multiplying the fraction of dTA positive colonies identified from the 20 ade<sup>*</sup> G418<sup>R</sup>/Hyg<sup>S</sup> colonies examined (indicated in brackets) by the % LOH. * denotes values as previously described, shown here for comparison (Cullen et al., 2007).

### Table 4. DSB-induced marker loss and dTA in minichromosome

| Ch16-MGTASTel (strain number) | % ade<sup>*</sup> G418<sup>R</sup> (NHEJ/SCR/uncut) | % ade<sup>*</sup> G418<sup>R</sup> (Ch16/loss/other) | % ade<sup>*</sup> G418<sup>R</sup> (LOH) | % ade<sup>*</sup> G418<sup>R</sup> (dT) | P-value (LOH relative to wildtype) |
|---------------------------------|---------------------------------|----------------|----------------|----------------|------------------|
| Wild type                       | 17.4 ± 4.0                      | 79.3 ± 4.17 | 3.3 ± 0.6 | 0.0% (0/20) | 1.000            |
| rqh1Δ                           | 50.8 ± 0.4                      | 44.8 ± 0.8 | 4.3 ± 0.6 | 3.0% (14/20) | <0.005           |
| exo1Δ                           | 51.0 ± 0.8                      | 43.2 ± 0.7 | 5.8 ± 0.1 | 5.8% (20/20) | <0.005           |
| rad55Δ                          | 47.2 ± 3.9                      | 39.7 ± 1.8 | 13.0 ± 2.3 | 13% (20/20) | <0.005           |
| rqh1Δ exo1Δ                    | 23.3 ± 2.9                      | 25.0 ± 4.5 | 51.7 ± 1 | 41.4% (16/20) | <0.005           |
| rqh1Δ rad55Δ                   | 31.3 ± 0.8                      | 15.5 ± 1.1 | 53.2 ± 1.9 | 45.2% (17/20) | <0.005           |

The mean ± SE from at least three independent experiments with three individual strains are shown. % dTA was calculated by multiplying the fraction of dTA positive colonies identified from the 20 ade<sup>*</sup> G418<sup>R</sup> colonies examined (indicated in brackets) by the % LOH.
Rad55 may also contribute to post-synaptic break-induced DSB induction in an

The mean ± SE from at least three independent experiments with three individual strains are shown. % dnTA was calculated by multiplying the fraction of dnTA positive colonies identified from the 20 ade+ G418S colonies examined (indicated in brackets) by the % LOH.

Determinants of chromosome healing

We found Rad51 to be required for efficient dnTA. This was unexpected as efficient Rad51 loading suppresses dnTA. Accordingly, dnTA levels were significantly reduced in a rad51Δ rqh1Δ rad55Δ strain compared to an rqh1Δ rad55Δ background. Similarly, preventing Rad51 loading by simultaneously disrupting both Rad55–Rqh1 and Swi5–Sfr1 heterodimers significantly reduced dnTA in swi5Δ rqh1Δ rad55Δ background. Taken together, our data are consistent with the hypothesis that Rad55–Rqh1 and Swi5–Sfr1 have distinct roles in Rad51 assembly (68). It has been shown that Rad51-foci form less efficiently in Swi5–Sfr1 compared to that telomerase may preferentially bind in the absence of both Rqh1, and Rad55. Non-homologous MATa site (dark grey) within the minichromosome (light grey) reduced resection, shortened and or an altered Rad51 nucleofilament structure is likely to play a critical role in defining the fate of broken chromosome ends. Pathways A and B may be non-exclusive. See text for details.

with and stimulates BLM to disrupt Holliday junctions in vitro (67). The observation that a DSB lacking a homologous distal chromosome arm significantly further increased dnTA levels in rqh1Δ rad55Δ or rqh1Δ exo1Δ backgrounds is consistent with a model in which the post-synaptic HR events of second end capture or strand annealing compete with dnTA (Figure 5).

Figure 5. Model for efficient dnTA within HR intermediates. (A) Presynaptic break-induced dnTA in an rqh1Δ rad55Δ background. Following DSB induction at the MATa site (dark grey) within the minichromosome (light grey) reduced resection, shortened and or an altered Rad51 nucleofilament structure facilitates presynaptic dnTA (black arrows). (B) Post-synaptic break-induced dnTA in an rqh1Δ rad55Δ background. Following DSB induction Rad51-dependent strand invasion of ChIII (black) leads to D-loop formation, which is stabilized in the absence of both Rqh1, and Rad55. Non-homologous MATa 3’ ends remain unprocessed and are extruded from the D-loop, facilitating dnTA. Removal of the second homologous arm significantly further increases dnTA in this context suggesting that second end capture or strand annealing efficiently suppresses dnTA. (C) Over-expression of Rad51 in an rqh1Δ rad55Δ background increases gene conversion. Thus Rad51 loading and subsequent nucleofilament structure plays a critical role in defining the fate of broken chromosome ends. Pathways A and B may be non-exclusive. See text for details.
such dntA. Such resection may facilitate Rad51 binding as indicated above. Reduced dntA is associated with increased NHEJ/SCR following Exo1 deletion in an rqh1 Δ rad55 Δ background. However, further studies will be required to elucidate the precise role of Exo1 in this context.

Mechanisms of telomere addition

The finding that efficient dntA was observed at or near the MATα site was unexpected, as this region lacks the canonical GGTTACA S. pombe telomeric repeat sequence (61). Studies in S. cerevisiae have shown dntA was restricted to very short regions of homology to the telomerase guide RNA that were likely to facilitate annealing of such RNA (71,72). Thus, efficient dntA observed in our study may result from recognition of degenerate telomeric sequences by guide RNA or other telomere recruitment factors. Alternatively, telomere recruitment may be achieved in a sequence-independent manner through interaction between ssDNA binding factors and telomerase (73).

Chromosome healing and genome instability

Our findings indicate that dntT4 has the capacity to stabilize broken chromosomes. However, such a role comes at the price of potentially extensive loss of genetic material centromere-distal to the break site. While dntT4 is predicted to result in loss of viability in a haploid setting, such extensive LOH in a diploid or polyploidy cells may be tolerated. Thus, dntT4 may provide an important back-up mechanism to rescue broken chromosomes, thus facilitating cell survival.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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