The Swr1 chromatin-remodeling complex prevents genome instability induced by replication fork progression defects

Anjana Srivatsan1, Bin-Zhong Li1, Barnabas Szakal2, Dana Branzei2,3, Christopher D. Putnam1,4 & Richard D. Kolodner1,5,6,7

Genome instability is associated with tumorigenesis. Here, we identify a role for the histone Htz1, which is deposited by the Swr1 chromatin-remodeling complex (SWR-C), in preventing genome instability in the absence of the replication fork/replication checkpoint proteins Mrcl, Csm3, or Tof1. When combined with deletion of SWR1 or HTZ1, deletion of MRC1, CSM3, or TOF1 or a replication-defective mrc1 mutation causes synergistic increases in gross chromosomal rearrangement (GCR) rates, accumulation of a broad spectrum of GCRs, and hypersensitivity to replication stress. The double mutants have severe replication defects and accumulate aberrant replication intermediates. None of the individual mutations cause large increases in GCR rates; however, defects in MRC1, CSM3 or TOF1 cause activation of the DNA damage checkpoint and replication defects. We propose a model in which Htz1 deposition and retention in chromatin prevents transiently stalled replication forks that occur in mrc1, tof1, or csm3 mutants from being converted to DNA double-strand breaks that trigger genome instability.
Genome instability is a hallmark of cancer, and genome rearrangements are often causal mutations in human diseases\(^1\)-\(^4\). Studies using *Saccharomyces cerevisiae* have provided insights into the genes and genetic interactions that prevent the accumulation of gross chromosomal rearrangements (GCRs), and analysis of cancer genome sequences has revealed that the corresponding pathways are affected in human cancers\(^5\)-\(^6\). Recently, we discovered a striking set of genetic interactions between mutations in the genes encoding the Swi2/Snf2-Related chromatin remodeling complex (SWR-C) or its substrate Htz1 and mutations in the genes encoding the replication fork progression proteins Tof1 (human Timeless) and Csm3 (human Tipin) or the Mediator of the Replication Checkpoint protein Mrc1 (human Claspin)\(^6\).

*S. cerevisiae* SWR-C (related to human SRCAP and Tip60/p400 complexes) consists of the Swi1 ATPase and 13 other subunits\(^7\)-\(^9\). This complex mediates incorporation of the only histone variant conserved across eukaryotes, the histone H2A variant Htz1 (human H2A.Z), into chromatin by exchanging chromatin-bound H2A-H2B dimers with Htz1-H2B dimers\(^7\)-\(^8\),\(^11\). Htz1 is enriched at gene promoters, pericentromeric chromatin, interchromatin-euchromatin boundaries, and telomeres\(^12\)-\(^15\), and has been implicated in transcriptional regulation and preventing the spread of heterochromatin\(^16\)-\(^18\).

Several studies have suggested roles for SWR-C/Htz1 in maintaining genome stability. The *htz1Δ* and/or *swr1Δ* mutations cause sensitivity to DNA-damaging agents, decreased sister-chromatid cohesion, defects in recruitment of DNA double-strand breaks (DSBs) to the nuclear periphery, modest defects in resection of DSBs, a small decrease in non-homologous end joining, a small (~twofold) increase in the rate of point mutations when combined with the *pol3-L612M* mutation in DNA polymerase δ that increases base misincorporation rates, and synergistic growth interactions with mutations affecting chromosome segregation\(^18\)-\(^26\). However, these defects are small compared to the effects caused by mutations affecting the core components of the DNA repair pathways that act in these processes.

The *htz1Δ* mutation causes a synthetic growth defect when combined with mutations in replication-related genes, including *MRC1*\(^11\),\(^27\). Loss of SWR-C/Htz1 also causes some phenotypes similar to those caused by loss of Mrc1, such as sensitivity to DNA-damaging agents, impaired sister chromatid cohesion, and a delay in replication progression\(^28\)-\(^33\). During normal replication, Mrc1 couples the leading-strand DNA polymerase ε to the DNA helicase Mcm2\(^7\)-\(^34\)-\(^36\) and interacts with the Tof1-Csm3 protein complex that also promotes replication fork progression; however, *mrc1Δ* defects cause a greater reduction in the rate of replication fork progression than *csm3* or *tof1* defects\(^33\),\(^37\)-\(^39\).

Mrc1 also has other DNA replication functions: (1) maintaining stable fork pausing upon depletion of nucleotides and at natural pause sites; (2) mediating signaling of DNA replication stress by facilitating activation of the downstream Rad53 checkpoint kinase, a step that requires phosphorylation of Mrc1 by the Mec1 and Tel1 checkpoint kinases\(^29\)-\(^31\). In contrast, Tof1-Csm3 is required for fork pausing at protein-DNA blocks and plays a less important role in checkpoint signaling\(^33\),\(^37\)-\(^39\),\(^42\). Defects in *MRC1*, *CSM3*, and *TOF1* also cause defects in cohesion establishment, but these defects appear to define parallel pathways, one involving *MRC1* and the other involving *TOF1* and *CSM3*\(^43\).

Here, we observe that SWR-C/Htz1 and Mrc1/Tof1/Csm3 cooperate to prevent genome instability. Increased replication stress occurs in the absence of Mrc1/Tof1/Csm3, requiring SWR-C/Htz1 to promote efficient replication progression, prevent the accumulation of aberrant replication fork structures, and prevent the accumulation of GCRs. Cells lacking Mrc1/Tof1/Csm3 and Swr1 are hypersensitive to agents that increase replication stress, and under these conditions, HR is essential for cell survival. Together with data from structural analysis of GCRs, these results are consistent with a model wherein transiently stalled replication forks generated in the absence of Mrc1/Tof1/Csm3 are processed in the absence of SWR-C/Htz1 to unusual replication structures that lead to the accumulation of GCRs.

**Results**

Htz1 deposition by SWR-C prevents GCRs in *mrc1Δ* mutants. In our previous study of systematically generated mutant strains, we observed that combining mutations affecting SWR-C or its substrate, Htz1, and the replication fork progression proteins Mrc1, Tof1 or Csm3 resulted in synergistic increases in dGCR rate, as assessed by semi-quantitative patch scores\(^8\). To understand these interactions, we performed GCR rate measurements with newly reconstructed *mrc1Δ*, *swr1Δ*, and *mrc1Δ swr1Δ* strains containing the dGCR, sGCR, or uGCR assay (Fig. 1a, b). The *mrc1Δ swr1Δ* double mutant had synergistic increases in GCR rates in all three assays, with a 5.5- to 91.8-fold increase in GCR rate relative to the higher of the two single-mutant GCR rates (Table 1; Supplementary Data 1). Moreover, deletions of genes encoding Htz1 or the non-essential subunits of SWR-C also caused synergistic increases in dGCR rates when combined with *mrc1Δ* (Fig. 1c; Table 1). The *mrc1Δ swr1Δ* and *mrc1Δ htz1Δ* double mutants and the *mrc1Δ swr1Δ htz1Δ* triple mutant had similar increases in dGCR rates (Supplementary Table 1; Supplementary Data 1), which is consistent with the hypothesis that Htz1 and SWR-C act in the same pathway, i.e., Htz1 deposition, to prevent the accumulation of GCRs in the absence of Mrc1. These experiments utilized strains constructed using gene-disruption methods; however, a subset of strains was also constructed by genetic crosses, and the resulting strains had essentially identical GCR rates (Table 1).

Defects in the genes encoding SWR-C and Htz1 alter gene expression. To evaluate whether altered gene expression possibly results in altered GCR rates, we searched the published transcriptional changes caused by deletion of *HTZ1*\(^16\) for altered expression of the 182 genes that suppress the formation of GCRs\(^8\), and all known DNA replication genes, as replication defects cause increased GCR rates\(^34\). In the *htz1Δ* mutant, none of these genes showed increased expression, and two GCR-suppressing genes, *PHR1* and *YJL218W*, showed a minor ~40% reduction in expression. Defects in *PHR1* and *YJL218W* result in a minor increase in GCR patch score in the sGCR assay, which is of borderline significance, and no increase in the dGCR and uGCR assays\(^5\). It is formally possible that *htz1Δ* alters the expression of a GCR-suppressing gene whose expression is induced by replication stress in *mrc1Δ*, *tof1Δ*, and *csm3Δ* mutants; however, replication stress alters the expression of very few replication, repair or checkpoint genes, and none of these appears to be regulated by Htz1 under normal conditions\(^16\),\(^45\). Therefore it is unlikely transcriptional effects on GCR-suppressing genes can explain the increased GCR rates caused by defects in SWR-C and Htz1.

**Stable chromatin retention of Htz1 suppresses *mrc1Δ* GCRs.** Sumoylated Htz1 promotes relocation of persistent DSBs to the nuclear periphery, and acetylated Htz1 promotes sister chromatid cohesion and the maintenance of telomere heterochromatin boundaries\(^21\)-\(^23\),\(^46\). We tested these roles of Htz1 by eliminating the lysine residues that are the substrates for these post-translational modifications (Supplementary Table 2; Supplementary Data 1). Elimination of Htz1 sumoylation (*htz1-K (126,133)R*) did not increase the dGCR rate individually or when combined with *mrc1Δ*, *tof1Δ*, or *csm3Δ*. Elimination of Htz1...
acetylation (htz1-1-K(4,9,11,15)R) did not increase the dGCR rate individually or when combined with mrc1Δ; these acetylation sites were previously called K3, K8, K10, and K1421,46. Additionally, neither the htz1-1-K(4,9,11,15,126,133)R acetylation- and sumoylation-defective mutation nor the htz1-K(4,9,11,15)Q acetylation-mimic mutation caused an increased GCR rate individually or in combination with an mrc1Δ mutation. In contrast, C-terminal truncations of Htz1, which decrease retention of Htz1^Δc terminus caused increased GCR rates, and combining both genomic alterations engineered into the strains, read sequences spanning the rearrangement junctions. We identified both genomic alterations engineered into the strains, such as the mrc1Δ and swr1Δ deletions (Supplementary Figs. 1, 2), and GCR-associated genomic alterations (Supplementary

**Fig. 1** A mrc1Δ mutation causes increased GCRs when combined with defects in SWR-C/Htz1. a Genome instability was measured using three different GCR assays in which the counter-selectable genes CAN1 and URA3 were inserted as a single cassette in haploid strains at different positions in the non-essential region of the left arm of chromosome V (5FOA), respectively, selects for GCRs with a breakpoint between the CAN1-URA3 cassette and the most telomeric essential gene on the left arm of chromosome V (PCM1); the DNA sequences in this breakpoint region influence the types of GCRs that are formed. The “short repeated sequence” GCR (sGCR) assay, the breakpoint region contains only single-copy sequences. The “short repeated sequence” GCR (sGCR) assay contains single-copy sequences and the canC::PICAL-NAT locus, which introduced two short homologies that mediate GCRs by HR: SUP35, which is a 114-bp gene encoding leucine tRNA, and -100 bp of YCLWdelta5 sequence, which has homology to the long-terminal repeats of Ty1 and Ty2 retrotransposons. The breakpoint region in the “segmental duplication” GCR (dGCR) assay contains the ~4 kb DSFI-HXT13 segmental duplication with divergent homology to regions of chromosomes IV, X, and XIV in addition to SUP35 and the YCLWdelta5 segment. b The uGCR, sGCR, and dGCR assays preferentially select for different kinds of GCRs in wild-type strains49, 50, 77, 78. c Patch test for the formation of GCRs in the dGCR assay in SWR-C/Htz1 single mutants and corresponding double mutants containing the mrc1Δ or mrc1-aq mutation. Increased numbers of papillae correspond to increased GCR rates

**SWR-C/Htz1 suppress GCRs caused by fork progression defects.** Mrc1 acts in replication fork progression and the replication checkpoint29–32,33. We therefore tested the separation-offunction allele mrc1-1-843, an alternative version of mrc1-c14 that functions the same replication defects (see Methods), and mrc1-aq, which causes a checkpoint signaling defect (Table 1; Supplementary Data 1)30,31. When combined with SWR-C/Htz1 defects, mrc1-aq did not cause an increase in the dGCR or sGCR rate and caused a small increase in the uGCR rate, which was significantly less than the effect seen with mrc1Δ (Table 1; Supplementary Data 1; Fig. 1c). In contrast, the mrc1-1-843 mutation caused large synergistic increases in GCR rates in all three assays when combined with either swr1Δ or htz1Δ. Similarly, tof1Δ and csm3Δ also caused synergistic increases in GCR rates when combined with either swr1Δ or htz1Δ, but not to the extent observed with mrc1Δ or mrc1-1-843 (Table 1; Supplementary Data 1). Combining mrc1Δ or mrc1-1-843 with either tof1Δ or csm3Δ caused increased GCR rates, and combining mrc1Δ or mrc1-1-843 with the tof1Δ swr1Δ or csm3 Δ swr1Δ double mutations caused even greater increases in GCR rates (Supplementary Table 1; Supplementary Data 1). These results suggest that mutations affecting Mrc1 (mrc1Δ and mrc1-1-843) and the Tof1-Csm3 complex (tof1Δ and csm3Δ) impair redundant replication functions, leading to increased GCR rates, which are further exacerbated in the absence of SWR-C/Htz1.

**mrc1 swr1 double mutants have an altered GCR spectrum.** To gain insights into the type of damage caused by combining defects in SWR-C/Htz1 and Mrc1, we determined the structures of the resulting GCRs. We characterized GCRs in the uGCR and sGCR assays, which have more informative product distributions than the homology-mediated rearrangements selected in the dGCR assay (Fig. 1b)39. We performed two types of analyses on these data: (1) we determined the rates of accumulation of individual classes of GCRs (observed rates) to characterize the GCR spectrum for each mutation; and, (2) for each class of GCR, we compared the observed rates to the predicted rates, which were calculated from the mutant GCR rate and the wild-type GCR spectrum (Tables 2, 3).

GCR structures were determined by analysis of paired-end whole-genome sequencing (WGS) of each parental strain and ≥ 18 or ≥ 10 independently derived GCR-containing isolates for each genotype in the uGCR (wild-type, mrc1Δ, swr1Δ, and mrc1Δ swr1Δ) and sGCR (wild-type, mrc1Δ, mrc1-1-843, swr1Δ, mrc1Δ swr1Δ, and mrc1-1-843 swr1Δ) assays, respectively. GCRs were identified using read depth, discordantly mapping read pairs, and read sequences spanning the rearrangement junctions. We identified both genomic alterations engineered into the strains, such as the mrc1Δ and swr1Δ deletions (Supplementary Figs. 1, 2), and GCR-associated genomic alterations (Supplementary
Table 1 GCR rates of strains with defects in SWR-C/Hit1 and Mrc1/Tof1/Csm3

| Relevant genotype | dGCR assay | sGCR assay | uGCR assay |
|-------------------|------------|------------|------------|
|                   | Rate (x 10^-9) | Fold change | Rate (x 10^-9) | Fold change | Rate (x 10^-9) | Fold change |
| Wild type         | 7635 8.1 [6.4-15] | 1.0 | 7964 6.1 [4.3-18] | 1.0 | 8625 1.8 [0.7-4.1] | 1.0 |
| swr1Δ             | 7785 16 [11-34] | 2.0 | 9077 8.4 [5.0-15] | 1.4 | 8808 0.8 [0.6-1.1] | 0.5 |
| hit1Δ             | 8696 14 [10-22] | 1.7 | 9079 7.8 [2.2-4.4] | 0.5 | 8810 0.7 [0.4-1.7] | 0.4 |
| mrc1Δ             | 8301 26 [18-44] | 3.2 | 9081 56 [21-100] | 9.1 | 8804 3.0 [1.4-3.8] | 1.7 |
| mrc1Δ swr1Δ       | 8302 409 [152-964] | 50.3 | 9083 304 [254-402] | 49.5 | 9085 280 [128-514] | 155 |
| mrc1Δ hit1Δ       | 8975 350 [269-456] | 43.1 | n.d. | - | 9087 215 [102-427] | 119 |
| mrc1Δ qg swr1Δ    | 8306 19 [12-43] | 2.3 | 9093 7.0 [4.4-11] | 1.1 | 9095 54 [36-69] | 30 |
| mrc1Δ qg hit1Δ    | 9097 18 [11-55] | 2.2 | 9099 8.3 [5.2-10] | 1.4 | 9101 62 [4.3-110] | 34 |
| mrc1Δyth1Δ        | Δ 8963 22 [15-30] | 2.7 | 9102 24 [18-47] | 3.9 | 8814 2.1 [1.1-4.3] | 1.2 |
| mrc1Δyth1Δ swr1Δ  | 8973 219 [134-321] | 26.9 | 9104 315 [222-444] | 51.3 | 9106 450 [252-623] | 250 |
| mrc1Δyth1Δ hit1Δ  | 9108 219 [175-316] | 6.3 | 9110 148 [103-342] | 24.1 | 9112 76 [51-156] | 42 |
| tof1Δ             | 8963 22 [15-37] | 2.7 | 9114 24 [20-46] | 3.9 | 8816 2.4 [1.5-4.2] | 1.3 |
| tof1Δ swr1Δ       | 8971 51 [36-116] | 6.3 | 9115 64 [42-139] | 10 | 9117 12 [7.6-22] | 6.8 |
| tof1Δ hit1Δ       | 9111 105 [48-201] | 12.9 | n.d. | - | 9121 7.1 [2.8-3.5] | 3.9 |
| csm3Δ             | 8965 34 [20-76] | 4.1 | 9128 25 [10-52] | 4.1 | 8806 5.0 [2.7-9.3] | 2.8 |
| csm3Δ swr1Δ       | 8972 72 [50-95] | 8.9 | 9130 54 [41-67] | 8.8 | 9132 7.6 [5.0-12] | 4.2 |
| csm3Δ hit1Δ       | 9134 90 [59-202] | 11.0 | n.d. | - | 9136 3.2 [1.5-5.7] | 1.8 |

Table 2 Product distributions in the uGCR assay

| GCR type | No. of isolates/total | Rate for GCR type* | Predicted rate based on wild-type product distributions* | Observed/predicted rate ratio |
|----------|-----------------------|--------------------|--------------------------------------------------------|-----------------------------|
| De novo telomere addition |                       |                    |                                                       |                             |
| Wild type | 15 / 20               | 1.35 [0.52-3.08] x 10^-9 | 1.35 [0.52-3.08] x 10^-9 | 1.00 |
| swr1Δ    | 7 / 18                | 0.32 [0.23-0.43] x 10^-9 | 0.62 [0.44-0.83] x 10^-9 | 0.52 |
| mrc1Δ    | 1 / 18                | 0.17 [0.08-0.21] x 10^-9 | 2.25 [1.05-2.85] x 10^-9 | 0.07 |
| mrc1Δ swr1Δ | 1 / 21               | 13.3 [6.19-24.3] x 10^-9 | 210 [97.5-383] x 10^-9 | 0.06 |
| Intersitial deletion |                       |                    |                                                       |                             |
| Wild type | 1 / 20                | 9.00 [3.50-20.5] x 10^-11 | 9.00 [3.50-20.5] x 10^-11 | 1.00 |
| swr1Δ    | 2 / 18                | 9.11 [6.56-12.2] x 10^-11 | 4.10 [2.95-5.50] x 10^-11 | 2.22 |
| mrc1Δ    | 0 / 18                | <16.7 [7.78-21.1] x 10^-11 | 15.0 [7.00-19.0] x 10^-11 | 1.11 |
| mrc1Δ swr1Δ | 8 / 21               | 10,700 [44000] x 10^-11c | 1400 [650-2550] x 10^-11d | 7.62 |
| Microhomology-mediated translocation |                       |                    |                                                       |                             |
| Wild type | 0 / 20                | <9.00 [3.50-20.5] x 10^-11 | <9.00 [3.50-20.5] x 10^-11 | 1.00 |
| swr1Δ    | 3 / 18                | 13.7 [9.83-18.3] x 10^-11 | <4.10 [2.95-5.50] x 10^-11d | 3.33 |
| mrc1Δ    | 4 / 18                | 66.7 [311-84.4] x 10^-11c | <15.0 [7.00-19.0] x 10^-11d | 4.44 |
| mrc1Δ swr1Δ | 9 / 21               | 12,000 [5900] x 10^-11c | <1400 [650-2500] x 10^-11d | 8.57 |
| Hairpin-mediated inverted duplication |                       |                    |                                                       |                             |
| Wild type | 3 / 20                | 2.70 [1.05-6.15] x 10^-10 | 2.70 [1.05-6.15] x 10^-10 | 1.00 |
| swr1Δ    | 6 / 18                | 2.73 [1.97-3.67] x 10^-10 | 1.23 [0.89-1.65] x 10^-10 | 2.22 |
| mrc1Δ    | 13 / 18               | 21.7 [10.1-27.4] x 10^-10c | 4.50 [2.10-5.70] x 10^-10d | 8.41 |
| mrc1Δ swr1Δ | 3 / 21               | 400 [186-729] x 10^-10c | 420 [195-765] x 10^-10 | 0.95 |

*Observed rate calculated by multiplying the GCR rate for each strain by the fraction of GCRs observed for a specific GCR type. 95% confidence intervals (CI) are displayed in square brackets.

*Predicted rate calculated by multiplying the GCR rate for each strain by the fraction of GCRs observed in the wild-type strain. 95% confidence intervals are displayed in square brackets.

*Rate of a specific type of GCR in the mutant strain has a 95% CI that does not overlap with the 95% CI of the wild-type rate.

*Predicted rate based on the wild-type product distribution has a 95% CI that does not overlap with the 95% CI of the observed rate.

Figs. 3–12 and Supplementary Tables 3–5). A single GCR chromosome was identified in all GCR-containing isolates, and an otherwise normal haploid complement of chromosomes was observed in greater than 80% of the isolates analyzed (Supplementary Fig. 13).

In the uGCR assay, the GCRs in the wild-type strain were predominantly generated by de novo telomere addition (15 of 20 isolates). The observed rate of forming this class of GCRs was reduced relative to the predicted rate in the swr1Δ and mrc1Δ strains, which had uGCR rates similar to wild type, and in the
The changes in the distributions of GCRs in mutant strains are best understood in terms of a modest bias against the accumulation of de novo telomere addition GCRs, which then
leads to the formation of other types of GCRs. The changes in the sGCR assay were subtler than in the uGCR assay due to the ability of the sGCR assay to select for short homology-mediated translocations in most strains. Remarkably, the length of homology at the microhomology-mediated translocation and interstitial deletion breakpoints in these strains was longer (range 2–20 bp, median 10.0 bp; Fig. 2b) than would be expected for random junctions (Fig. 2c51), suggesting that these GCRs are generated by microhomology-mediated end joining or a form of HR that acts on short sequences51. These results suggest that SWR-C/Htz1 plays an important role in preventing the formation of DNA damage and/or preventing DNA damage from being channeled into GCRs, but only plays a minor role in influencing the selection of DNA repair pathways that act to generate GCRs5,44.

SWR-C/Htz1 defects do not cause checkpoint activation. The synergistic increases in GCR rates in strains with combined defects in Mrc1/Tof1/Csm3 and SWR-C/Htz1 could be due to increased levels of DNA damage or altered processing of damage caused by one of the mutations. We therefore used two assays to evaluate checkpoint activation and DNA damage formation in vivo (Fig. 3 and Supplementary Fig. 14): (1) cytological analysis of Ddc2-GFP foci (Fig. 3a, b), which reflects activation of the Mec1 checkpoint kinase52; and (2) FACS analysis of expression of...
A number of events was 35. Ddc2 foci was slightly decreased in the mrc1 mutant. The mrc1 mutation was caused by HR repair to then the telomere to the broken end; the junctions involved short telomere-like sequences on chromosome V. Interstitial deletions spanned the CANI-URA3 cassette and contained microhomologization at the deletion junctions. Hairpin-mediated GCRs had deletion of the terminal region of chromosome V and hairpin formation, followed by inverted duplication of a region of chromosome V ending in a region of homology, either a Ty delta sequence or PAU gene, and a subsequent secondary translocation with a homologous target elsewhere in the genome. In all hairpin-mediated inverted duplication GCRs, subsequent rearrangements involved a non-reciprocal translocation with a target chromosome, involving duplication of the target chromosome from the targeted homology to the telomere. Homology-mediated inverted duplications are similar to hairpin-mediated inverted duplications except that the fold-back loop is formed by HR between the YCLWdelta5 fragment in can1::PvakZ::NAT and other Ty delta sequences on the left arm of chromosome V, leading to inverted duplication of the flanking sequence. Distribution of the microhomologization lengths at the junctions in the 35 microhomologization-mediated translocations and interstitial deletions observed in the uGCR and sGCR products from the wild-type strain, the mrc1Δ, swr1Δ, and mrc1-1-843 single mutants, and the mrc1Δ swr1Δ and mrc1-1-843 swr1Δ double mutants. Distribution of the breakpoint junction lengths for randomly generated translocations scaled so that the total number of events was 35.

To test if HR acts to repair the damage caused by mrc1Δ in strains without SWR-C/Htz1, we measured the doubling times of wild-type, mrc1Δ, swr1Δ, and mrc1Δ swr1Δ strains with and without a rad52Δ mutation, which causes a substantial HR defect. The rad52Δ mutation caused a modest increase in doubling time in the wild-type, mrc1Δ, and swr1Δ strains and a severely prolonged doubling time in the mrc1Δ swr1Δ strain (Fig. 3f). Therefore in the absence of SWR-C/Htz1, HR plays a crucial role in repairing the DNA damage that occurs in mrc1Δ strains.

To gain insight into the role of the Mrc1/Tof1/Csm3-SWR-C/Htz1 interaction during replication stress as opposed to unperurbed cell growth, we measured the sensitivity of various single and double mutants to methyl methane sulfonate (MMS) and hydroxyurea (HU), which block DNA replication by causing DNA alkyligation damage and depleting dNTP pools, respectively (Fig. 4a, b, and Supplementary Fig. 15). The swr1Δ and htt1Δ single mutants were weakly sensitive to MMS (htt1Δ > swr1Δ) and HU (htt1Δ > swr1Δ), consistent with previous studies. The mrc1Δ and mrc1-1-843 mutations individually caused weak or no sensitivity, respectively, to MMS and HU, but caused strikingly increased sensitivity to both drugs when combined with either swr1Δ or htt1Δ. In contrast, mrc1-1aq did not cause increased HU or MMS sensitivity either as a single mutation or in combination with either swr1Δ or htt1Δ. The tof1Δ and csm3Δ mutations caused similar but weaker patterns of sensitivity to MMS and HU compared to mrc1Δ (Supplementary Fig. 15). In addition, the rad52Δ mrc1Δ and rad52Δ swr1Δ double mutants had synergistically increased sensitivity to HU compared to the rad52Δ single mutant (rad52Δ mrc1Δ > rad52Δ swr1Δ) (Fig. 4c). Consistent with the effect of rad52Δ on the doubling time of the mrc1Δ swr1Δ strain (Fig. 3f), the rad52Δ mrc1Δ swr1Δ triple mutant was extremely sensitive to HU and unable to grow even at the lowest HU concentration tested (10 mM) (Fig. 4c). Thus, the combination of defects in Mrc1/Tof1/Csm3 and SWR-C/Htz1 causes hypersensitivity to replication stress induced by exogenous agents and a requirement for HR for survival under these conditions.

HR repairs DNA damage in strains without Mrc1 and SWR-C/Htz1. Although the mrc1Δ htt1Δ and mrc1Δ swr1Δ double mutants had modest or no increases in checkpoint activation compared to the mrc1Δ single mutant, the mrc1Δ htt1Δ and mrc1Δ swr1Δ double mutants had slower doubling times than any of the respective single mutants (Fig. 3e), which is consistent with defects in resolving the DNA damage caused by mrc1Δ, tof1Δ, or csm3Δ mutations. Sister chromatid HR is a major mechanism for repairing DNA damage in S. cerevisiae without generating GCRs.

Replication stress sensitivity without Mrc1 and SWR-C/Htz1. To gain insight into the role of the Mrc1/Tof1/Csm3-SWR-C/Htz1 interaction during replication stress as opposed to unperurbed cell growth, we measured the sensitivity of various single and double mutants to methyl methane sulfonate (MMS) and hydroxyurea (HU), which block DNA replication by causing DNA alkyligation damage and depleting dNTP pools, respectively (Fig. 4a, b, and Supplementary Fig. 15). The swr1Δ and htt1Δ single mutants were weakly sensitive to MMS (htt1Δ > swr1Δ) and HU (htt1Δ > swr1Δ), consistent with previous studies. The mrc1Δ and mrc1-1-843 mutations individually caused weak or no sensitivity, respectively, to MMS and HU, but caused strikingly increased sensitivity to both drugs when combined with either swr1Δ or htt1Δ. In contrast, mrc1-1aq did not cause increased HU or MMS sensitivity either as a single mutation or in combination with either swr1Δ or htt1Δ. The tof1Δ and csm3Δ mutations caused similar but weaker patterns of sensitivity to MMS and HU compared to mrc1Δ (Supplementary Fig. 15). In addition, the rad52Δ mrc1Δ and rad52Δ swr1Δ double mutants had synergistically increased sensitivity to HU compared to the rad52Δ single mutant (rad52Δ mrc1Δ > rad52Δ swr1Δ) (Fig. 4c). Consistent with the effect of rad52Δ on the doubling time of the mrc1Δ swr1Δ strain (Fig. 3f), the rad52Δ mrc1Δ swr1Δ triple mutant was extremely sensitive to HU and unable to grow even at the lowest HU concentration tested (10 mM) (Fig. 4c). Thus, the combination of defects in Mrc1/Tof1/Csm3 and SWR-C/Htz1 causes hypersensitivity to replication stress induced by exogenous agents and a requirement for HR for survival under these conditions.

Cell cycle defects without Mrc1 and SWR-C upon HU treatment. Plate-based HU and MMS hypersensitivity reflects the effect of replication stress over the course of 25–40 rounds of cell division. We next investigated if this sensitivity causes defects...
during the progression of a single cell cycle. Cells were first arrested in G1 phase, then allowed to progress to mid-S phase in medium containing 200 mM HU, and then released into medium lacking HU. We assessed cell cycle recovery by monitoring DNA content by FACS analysis at different time points (Supplementary Fig. 16). Upon release from HU, the cell cycle progression in the presence of HU but recovered upon HU release (Supplementary Fig. 16). The tof1Δ single mutant had a similar profile to wild-type cells; however, the tof1Δ swr1Δ and tof1Δ htz1Δ double mutants showed little progression into S-phase in the presence of HU and were highly defective in recovering from HU treatment. Compared to the tof1Δ strain, the mrc1Δ strain had a greater defect in S-phase progression in the presence of HU, and because of the severity of this defect, the differences between the mrc1Δ single mutant and the mrc1Δ swr1Δ and mrc1Δ htz1Δ double mutants were less striking than between the corresponding tof1Δ single and double mutants (red curves in Supplementary Fig. 16). Upon release from HU, the mrc1Δ swr1Δ and mrc1Δ htz1Δ double mutants showed more severe replication defects compared to the mrc1Δ single mutant. Overall, the effect of swr1Δ was similar to but slightly more severe than that of htz1Δ, both as single mutations and when combined with tof1Δ or mrc1Δ. Together these data suggest that loss of Mrc1/Tof1/Csm3 in combination with loss of SWR-C/Htz1 leads to defects in replication during recovery from replication stress.

**Fork defects without Mrc1/Tof1 and SWR-C upon HU treatment.** To better characterize the DNA replication defects caused by absence of Mrc1/Tof1/Csm3 and SWR-C/Htz1, we used two-
consistent with the observed cell cycle pro-
yby the reduced intensity of the bubble arc. These results are
(Fig. 5b, compare the 4 h FACS time points).

Fig. 16A) and explain the slight delay in S phase progression
and Y-arc molecules (Fig. 5b; for example, see the 2-h time point),
and recombination-like structures. Notably, small Y-molecules and
cone structures were observed in mrc1Δ cells, resembling those
reported for rad53 checkpoint mutants replicating in the presence
of HU60,61, where the structures were attributed to resection of
stalled forks and formation of reversed forks61–64 (Supplementary
Fig. 17).

The tof1Δ swr1Δ and tof1Δ htz1Δ mutants had greatly
exacerbated replication defects with substantially reduced origin
firing, persistence of replication intermediates, and accumulation of
cone structures (Fig. 5b and Supplementary Fig. 18). In
contrast, unusual replication intermediates did not accumulate at
detectable levels in the mrc1Δ swr1Δ and mrc1Δ htz1Δ double
mutants; however, this likely reflects the extreme replication
defects of these double mutants, as evidenced by both the low
intensity of the bubble arc and limited progression into S-phase as
measured by FACS (Supplementary Fig. 17 - compare to Fig. 5b;
Supplementary Fig. 16).

Because the aberrant DNA replication intermediates were more
easily observable in the tof1Δ swr1Δ and tof1Δ htz1Δ double
mutants, we tested whether these would be resolved if the strains
were allowed to recover from a transient HU block. To test this,
cells were first synchronized in G1 phase, then released into 200
mM HU for 4 h, and finally released into medium without HU for
2 h. The wild-type, swr1Δ, htz1Δ, and tof1Δ strains were able to
recover from HU treatment over the course of 2 h, although some
persistent replication intermediates were still observed in swr1Δ,
htz1Δ, and tof1Δ cells during the first hour of recovery

![Image](http://example.com/image1.png)
In contrast, the  tof1Δ swr1Δ and tof1Δ htz1Δ double mutants were more defective in cell cycle progression than the single mutants, and the levels of cone structures forming in the ARS305 region decreased over the 2-h time course as they were converted to other structures or DSBs (Supplementary Fig. 18). Together, these results indicate that SWR-C/Htz1 helps promote the processing of stalled DNA replication fork intermediates that are induced by HU and defects in Mrc1 and Tof1, facilitating replication progression.

Discussion

Previous studies have suggested a role for SWR-C/Htz1 in genome maintenance; however, the magnitude of the defects in genome maintenance-related functions caused by mutations affecting SWR-C and Htz1 are small. Here, we found that loss of SWR-C/Htz1 plays a profound role in genome maintenance in the presence of replication defects caused by mrc1Δ, mrc1-1-843, tof1Δ, or csm3Δ. These combined defects caused: (1) significant increases in GCR rates and modest changes in the spectrum of GCRs formed, (2) hypersensitivity to chemical agents that induce replication stress, (3) a requirement for Rad52 for promoting cell division and survival under conditions of replicative stress, and (4) severe HU-induced replication defects leading to increased levels of aberrant replication fork structures. The mrc1Δ, tof1Δ, and csm3Δ mutations caused constitutive activation of the DNA damage checkpoint response, but this was not exacerbated by additional mutations in SWR1 or HTZ1. Together, these data support a model in which the absence of functional Mrc1 or Tof1-Csm3 causes replication defects.
progression and replication stress signaling. The NATURE COMMUNICATIONS | 2018 | 9:3680 | DOI: 10.1038/s41467-018-06131-2 | www.nature.com/naturecommunications

resulting in DNA damage, necessitating chromatin-bound Htz1 for replication fork dynamics to prevent genome instability (Fig. 6).

Mrc1/Tof1/Csm3 are involved in normal replication fork progression and replication stress signaling. The mrc1Δ and tof1Δ mutants have increased levels of aberrant replication fork structures, and mrc1Δ, tof1Δ, and csm3Δ mutants also have increased DNA damage checkpoint activation. Although Mrc1, Tof1, and Csm3 have similar roles at the replication fork, mutations affecting MRC1, TOF1, and CSM3 cause distinct phenotypes: (1) Tof1-Csm3 promotes the Mrc1-replisome association, but the converse is not true; (2) loss of Mrc1 causes a more severe reduction in replication fork speed than the absence of Tof1-Csm3; (3) Tof1-Csm3 is required for fork pausing at protein-DNA blocks, whereas Mrc1 is not; and (4) Mrc1 and Tof1-Csm3 function in distinct pathways that promote the establishment of sister chromatid cohesion, albeit not to the same extent as cohesin itself. Consistent with this, we found that: (1) mrc1Δ and the replication-defective mrc1-1-843 mutation cause large synergistic increases in GCR rates in combination with tof1Δ, csm3Δ, swr1Δ, or htz1Δ mutations, and in triple mutants containing tof1Δ or csm3Δ and swr1Δ or htz1Δ; and (2) mrc1Δ causes higher GCR rates than tof1Δ or csm3Δ when combined with swr1Δ or htz1Δ. Together with the observation that the checkpoint-defective mrc1-aq mutation caused much lower or none of the defects described above, depending on the assay, these

Fig. 6 Hypotheses for the role of Htz1 in preventing genome instability. a Schematic of a normal replication fork showing key components including the Cdc45-Mcm2-7-GINS helicase complex, the leading (Polε) and lagging strand (Polα and Polβ) DNA polymerases, Ctf4, which couples the helicase and Polε, and Mrc1, which couples the helicase and Polε and interacts with Tof1-Csm3. Other replisome components are omitted for clarity. Also shown are nucleosomes containing the canonical histone H2A (grey circles) or the histone variant H2A.Z/Htz1 (red circles), which is incorporated by the SWR-C. b (Fork Stability Model) In the absence of Mrc1, replication fork progression is slowed and replisome uncoupling can occur during replication stress. The DNA damage response (DDR) is constitutively induced. The presence of Htz1 in normal chromatin prevents further replisome uncoupling or fork regression and collapse, preventing genome instability. The absence of Tof1 or Csm3 leads to similar albeit weaker defects. c In the absence of Mrc1 and Htz1, replication fork progression is severely impeded. Although DDR signaling is not elevated relative to the loss of Mrc1 alone, abnormal replication intermediates are formed, which are processed into DSBs. HR repairs the DNA damage and is required for survival during replication stress. Repair of the DNA damage using non-allelic targets leads to elevated genome instability. d (Collapsed Fork Stability Model) In the absence of Mrc1, increased levels of collapsed forks are formed. Remaining single-stranded DNA regions induce checkpoint activation, but incorporation of Htz1 near the sites of damage supresses Rad51 filament formation and other HR-mediated repair processes, allowing the collapsed fork to be repaired by approach of an oppositely oriented fork. e In the absence of Mrc1 and Htz1, the collapsed fork is subjected to processing by structure-specific helicases and endonucleases, resulting in DSBs and other substrates for intermolecular HR, which generate aberrant replication fork structures and GCRs.
How does SWR-C/HTz1 promote the repair of replication damage? The similar dGCR rates of the $\Delta$htz1 mutant and the $\Delta$htz1 $\Delta$swr1 $\Delta$mrc1 double mutant indicate that SWR-C suppresses GCRs by incorporating Htz1 into chromatin. Analysis of separation-of-function alleles of HTZ1 indicates that the known roles of Htz1 in promoting the relocation of persistent DSBs to the nuclear periphery, promoting sister chromatid cohesion, and maintaining telomeric heterochromatin boundaries do not suppress the formation of GCRs in the absence of Mrc1. Moreover, the roles of SWR-C/HTz1 in preventing the spread of heterochromatin at silenced regions are unlikely to explain the effects observed here: (1) only 2 of ~240 genes involved in DNA replication or genome stability show modestly (~40%) reduced expression in the $\Delta$htz1 mutant, and in these two genes only a minor, if any, role in preventing GCRs; (2) replication stress alters the expression of very few replication, repair, or checkpoint genes in none of these appears to be regulated by Htz1 under normal conditions; and (3) the aberrant replication structures in strains with mutations in $\Delta$mrc1 $\Delta$TOFI and $\Delta$swr1 $\Delta$HTZ1 were observed at ARS305, which does not correspond to an Htz1-activated domain. Finally, C-terminal truncation mutations of HTZ1 encoding Htz1 mutants that are not stably retained in chromatin result in synergistic increases in GCR rates when combined with $\Delta$mrc1, like $\Delta$htz1. Thus, stable incorporation of Htz1 by SWR-C is required for preventing genome instability in the presence of $\Delta$mrc1-induced replication damage.

An important clue to the defects caused by loss of SWR-C/HTz1 is the suppression of growth defects of the $\Delta$mrc1 $\Delta$swr1 $\Delta$htz1 double mutant strain by HR and the accumulation of aberrant DNA structures during replication in the $\Delta$tof1 $\Delta$swr1 and $\Delta$tof1 $\Delta$htz1 strains. These results argue that the replication damage occurring in the absence of Mrc1/Tof1/Csm3 and SWR-C/HTz1 is processed into substrates for HR, such as DSBs or single-stranded gaps, whereas HR plays a less important role in strains lacking Mrc1/Tof1/Csm3 when SWR-C/HTz1 is present. Potential roles for SWR-C/HTz1 that are consistent with the analysis of replication intermediates are: (1) normal incorporation of Htz1 into chromatin may help directly stabilize damaged replication forks by preventing fork reversal (“Fork Stability Model”, Fig. 6); or (2) incorporation of Htz1 into chromatin could occur at sites of replication damage after the collapse of the replication fork and prevent processing of the replication damage to DSBs by helicases and endonucleases (“Collapsed Fork Stability Model”, Fig. 6). This stabilization could occur via direct Htz1-replication fork interactions, which could slow the MCM DNA helicase when it becomes uncoupled from the remaining replisome, and/or by formation of a specialized chromatin domain around the damaged site that may occur after replication fork collapse, which may be consistent with the role of Htz1 in antagonizing the formation of Rad51 filaments at HO endonuclease-induced DSBs. In either case, the formation of DSBs in the absence of Mrc1/Tof1/Csm3 and SWR-C/HTz1 is consistent with the requirement for HR in the $\Delta$mrc1 $\Delta$swr1 strain and the formation of aberrant DNA structures at replication origins.

SWR-C/HTz1 and Mrc1 are evolutionarily conserved. Their homologs are significantly mutated (SRCAP in prostate cancer and glioblastoma; CLASPIN in gliomas and breast cancer) or overexpressed (H2A.Z in liver, colorectal, and metastatic breast cancer) in human cancers, and these alterations are predicted to play important roles in carcinogenesis. It will be interesting to examine whether the genetic interactions described here are conserved in mammalian cells and play a role in cancer development.
3′ ends were adenylated using Klenow DNA polymerase (NEB). Indexed Illumina adapters were then ligated to the A-tailed DNA fragments using Quick DNA Ligate (NEB), and the samples were purified using the MinElute kit. Size selection was performed using gel extraction to obtain 600–800 bp fragments, and the adapter-ligated fragments were enriched by PCR using the KAPA library amplification readmix (KAPA Biosystems) with primers AATGATACGGCGACCACCGA-GATCTACAC and CAAGCAGAAGACGGCATACGAGAT. The libraries were then pooled using gel extraction to select for 600–800 bp fragments. The library concentrations were measured in a Qubit fluorometer using the Qubit dsDNA HS assay kit. Sets of 12 libraries (10 nM each) were mixed for multiplexing and sequenced on an Illumina HiSeq 2000 instrument using the Illumina GAII sequencing procedure for paired-end short-read sequencing to obtain 50- bp reads.

Analysis of GCR structures from WGS data. Individual reads from all read pairs were mapped to the S288c reference genome using bowtie. The Pyrus suite (https://sourceforge.net/projects/pyrus-seq/) was used to determine genomic alterations including GCR structures from mapped sequence reads.10

Drug sensitivity assay. To test sensitivity to chronic exposure to HU and MMS, tenfold serial dilutions of cultures of selected strains grown in YPD medium at 30 °C were spotted on plates containing drugs at the indicated concentrations. Two independent isolates were tested for each strain, and the plating was performed in duplicate. The plates were photographed after 2 days of incubation at 30 °C or after 6 days for rad52Δ-containing strains. Representative images are shown.

Measurement of doubling times. Logarithmic-phase cultures grown in YPD medium at 30 °C were sampled at appropriate intervals, and their OD600 was measured using a Nanodrop spectrophotometer with 1.5-ml cuvettes. The doubling time was calculated from the logarithmic phase of the growth curves.

Measurement of Dsd2-GFP foci. Cells were grown in complete synthetic medium to log phase and examined by live imaging using an Olympus BX43 fluorescence microscope with a 60 × 1.42 PlanApo N Olympus oil-immersion objective. GFP fluorescence was detected using a Chroma FITC filter set and captured with a Qimaging QIClick CCD camera. Images were analyzed using Meta Morph Advanced 7.7 imaging software, keeping processing parameters constant within each experiment.

Fluorescence-activated cell sorter (FACS) analysis. Cell-cycle analysis was conducted using a standard protocol.12 In brief, 1 × 10^6 cells were collected by centrifugation and resuspended in 70% ethanol for 16 h. Cells were then washed in 0.25 M Tris-HCl (pH 7.5), resuspended in the same buffer containing 2 mg/ml of RNaseA and incubated at 37 °C for at least 1 h, then treated overnight with proteinase K (1 mg/ml) at 37 °C. Cells were then resuspended in 200 mM Tris-HCl (pH 7.5) buffer containing 200 mM NaCl and 80 mM MgCl2 and stained in the same buffer containing 1 μM Sytox-green (Invitrogen). Samples were then diluted tenfold in 50 mM Tris-HCl (pH 7.8) and analyzed using a Becton Dickinson FACSscan instrument. This FACS analysis verified that all of the strains used in the experiments reported in this study were haploid.

To measure Hug1-GFP expression, 1-ml samples of logarithmic-phase cultures were spotted on plates containing drugs at the indicated concentrations. Two tenfold serial dilutions of cultures of selected strains grown in YPD medium at 30 °C were sampled at appropriate intervals, and their OD600 was measured using a Nanodrop spectrophotometer with 1.5-ml cuvettes. The doubling time was calculated from the logarithmic phase of the growth curves.

To test sensitivity to chronic exposure to HU and MMS, tenfold serial dilutions of cultures of selected strains grown in YPD medium at 30 °C were sampled at appropriate intervals, and their OD600 was measured using a Nanodrop spectrophotometer with 1.5-ml cuvettes. The doubling time was calculated from the logarithmic phase of the growth curves.

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Author contributions
A.S., C.D.P. and R.D.K. conceived the overall experimental design. A.S. performed strain construction, quantitative rate measurements, drug sensitivity studies, and Hug1-GFP measurement. A.S. and C.D.P. analyzed the GCR structures derived from WGS data. B.-Z.L. analyzed Ddc2-GFP foci formation. B.S. and D.B. performed the 2-D gel electrophoresis and FACS experiments. A.S., C.D.P., D.B. and R.D.K. wrote the paper, and all other authors revised and modified the paper.

Additional information
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