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γH2A-Binding Protein Brc1 Affects Centromere Function in Fission Yeast

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The coordinated replication and transcription of pericentromeric repeats enable RNA interference (RNAi)-mediated transmission of pericentromeric heterochromatin in fission yeast, which is essential for the proper function of centromeres. Rad3/ATR kinase phosphorylates histone H2A on serine-128/-129 to create γH2A in pericentromeric heterochromatin during S phase, which recruits Brc1 through its breast cancer gene 1 protein (BRCA1) C-terminal (BRCT) domains. Brc1 prevents the collapse of stalled replication forks; however, it is unknown whether this activity influences centromere function. Here, we show that Brc1 localizes in pericentromeric heterochromatin during S phase, where it enhances Clr4/Suv39-mediated H3 lysine-9 dimethylation (H3K9me2) and gene silencing. Loss of Brc1 increases sensitivity to the microtubule-destabilizing drug thiabendazole (TBZ) and increases chromosome missegregation in the presence of TBZ. Brc1 retains significant function even when it cannot bind γH2A. However, elimination of the serine-121 site on histone H2A, a target of Bub1 spindle assembly checkpoint kinase, sensitizes γH2A-deficient and *brc1*Δ cells to replication stress and microtubule destabilization. Collective results suggest that Brc1-mediated stabilization of stalled replication forks is necessary for fully efficient transmission of pericentromeric heterochromatin, which is required for accurate chromosome segregation during mitosis.

Maintenance of genome integrity during successive cycles of cell division is essential to prevent the accumulation of debilitating genetic alterations and chromosome rearrangements. The responsibility for protecting genome stability falls to high-fidelity DNA replication and repair systems acting in conjunction with an accurate chromosome distribution mechanism. DNA damage and mitotic spindle assembly checkpoints regulate these processes and couple their completion to cell cycle progression (1–3).

Accurate chromosome segregation depends on the assembly of kinetochores at a single centromere on each chromosome, which serve as the attachment site for microtubules that form the mitotic spindle. In the fission yeast *Schizosaccharomyces pombe*, the central core domain of each centromere is flanked by large inverted repeats that are themselves composed of shorter repeats. These repeats are the targets of RNA interference (RNAi)-mediated assembly of transcriptionally silent heterochromatin (4–6). A defining feature of this heterochromatin is the dimethylation of histone H3 at lysine 9 (H3k9me2) by the Clr4/Suv39 subunit of Rik1 complex. The heterochromatin protein Swi6/HP1 binds H3k9me2-modified heterochromatin. Failure to maintain pericentromeric heterochromatin impairs centromere function and sister chromatid cohesion near centromeres, leading to increased chromosome loss and sensitivity to the antifungal drug thiabendazole (TBZ), which destabilizes microtubules. Defects in pericentromeric heterochromatin also create a critical requirement for the spindle assembly checkpoint (7). This checkpoint is partly mediated through phosphorylation of serine-121 in the C terminus of histone H2A by Bub1 kinase, which recruits shugoshin (8).

Stabilizing and repairing replication forks are essential for maintaining genome integrity. These activities are regulated by ATR/Rad3/Mec1 checkpoint kinase (9, 10). A notable substrate is the SQ motif at the C terminus of histone H2A in yeast species and H2AX in mammals (11). Phospho-H2A(X), also known as γH2A(X), is formed in large chromosomal domains flanking DNA lesions, where it creates a recruitment platform for DNA repair and checkpoint mediator proteins. As first shown with mammalian MDC1, these proteins bind γH2A(X) through a phosphopeptide docking pocket formed by tandem breast cancer gene 1 protein (BRCA1) C-terminal (BRCT) domains (12). Fission yeast has two γH2A-binding proteins: Crb2, which mediates activation of Chk1 by Rad3 (13–15), and the 6-BRCT domain protein Brc1, which facilitates recovery from stalled and collapsed replication forks (16, 17). Brc1 is structurally related to budding yeast Rtt107/Esc4 and mammalian PTIP, both of which also bind γH2A(X) through BRCT domains (18, 19).

γH2A has been studied mostly in the context of double-strand breaks (DSBs), but genome-wide mapping revealed that γH2A is also formed at specific chromosomal locations during an unperturbed DNA synthesis (S) phase (20, 21). These regions include pericentromeric heterochromatin in fission yeast, suggesting that replication forks stall in these chromosomal domains. Indeed, a recent study detected X-shaped DNA structures in pericentromeric heterochromatin (22). These DNA structures are absent in *clr4*Δ cells, suggesting that the alternating pattern of replication origins and noncoding RNA in the pericentromeric heterochromatin of fission yeast results in collisions between the replication and transcription machineries. RNAi-mediated release of RNA polymerase II was proposed to favor fork restart by replisomes associated with Rik1 complex, thereby ensuring efficient transmission of heterochromatin-specific histone modifications in

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TABLE 1 Genotypes of strains used in this study

Strain	Genotype	Source
JW107	*h*$^-$ *ura4-D18 leu1-32* pRep41-N-GFP-*brc1*$^+$	Lab stock
JW397	*h*$^+$ *leu1-32 brc1*::*hphMx*	Lab stock
PR109	*h*$^-$ *leu1-32 ura4-D18*	Lab stock
PR110	*h*$^+$ *leu1-32 ura4-D18*	Lab stock
SR118	*h*$^-$ *leu1-32 ura4-D18 his3-D1 cdc25-22*	Lab stock
SR163	*h*$^+$ *leu1-32 ade6-210 ura4-DS/E otr1R*(SphI)::*ura4*$^+$	Lab stock
SR164	*h*$^+$ *leu1-32 ade-ura4-DS/E imr1R(dg-glu)* NcoI::*ura4*$^+$	Lab stock
SR273	*h*$^+$ *leu1-32 ade6-210 ura4-DS/E otr1R*(SphI)::*ura4*$^+$ *clr4*::*natMX*	This study
SR357	*h*$^+$ *leu1-32 ura4-D18 ade6-M216 swi6*::*ura4*$^+$	Lab stock
SR442	*h*$^+$ *leu1-32 ura4-D18 clr4*::*natMX6* pRep41-N-GFP-*brc1*$^+$	Lab stock
SY01	*h*$^+$ *leu1-32 ade6-210 ura4-DS/E otr1R*(SphI)::*ura4*$^+$ *brc1-T672A*::*natMX*	This study
SY02	*h*$^-$ *leu1-32 ade-ura4-DS/E imr1R*(*dg-glu*) NcoI::*ura4*$^+$ *brc1-T672A*::*natMX*	This study
SY03	*h*$^-$ *leu1-32 ade-ura4-DS/E imr1R*(*dg-glu*) NcoI::*ura4*$^+$ *clr4*::*natMX*	This study
SY04	*h*$^+$ *leu1-32 ade6-210 ura4-DS/E otr1R*(SphI)::*ura4*$^+$ *brc1*::*hphMx*	This study
SY05	*h*$^-$ *leu1-32 ade-ura4-DS/E imr1R*(*dg-glu*) NcoI::*ura4*$^+$ *brc1*::*hphMx*	This study
SY06	*h*$^-$ *leu1-32 ura4-D18 his3-D1 cdc25-22 brc1*::*hphMx*	This study
SY07	*h*$^+$ *leu1-32 ura4-D18 ade6-M216 swi6*::*ura4*$^+$ *brc1*::*kanMx*	This study
SY08	*h*$^-$ *ura4-D18 leu1-32 hta1- S121A-S129A*::*natMX hta2-S121A-S128A*::*kanMx*	This study
SY09	*h*$^-$ *ura4-D18 leu1-32 hta1-S121A*::*natMX hta2-S121A*::*kanMx*	This study
SY10	*h*$^-$ *ura4-D18 leu1-32 hta1-S129A*::*natMX hta2-S128A*::*kanMx*	This study
SY11	*h*$^-$ *ura4-D18 leu1-32 brc1*::*hphMx hta1-S121A*::*natMX hta2-S121*::*kanMx*	This study
SY12	*h*$^-$ *ura4-D18 leu1-32 brc1*::*hphMx hta1-S129A*::*natMX hta2-S128*::*kanMx*	This study
SY13	*h*$^+$ *leu1-32 ura4-D18* pRep41-N-GFP-*brc1 hta1-S129A*::*natMX hta2-S128*::*kanMx*	This study
SY14	*h*$^+$ *leu1-32 ura4-D18* pRep41-N-GFP-*brc1 hta1-S121A*::*natMX hta2-S121*::*kanMx*	This study
SY15	*h*$^+$ *leu1-32 ura4-D18* pRep41-N-GFP-*brc1 hta1-S121A-S129A*::*natMX hta2-S121A-S128A*::*kanMx*	This study

each S phase (22, 23). This model predicts that replication stress responses may contribute to maintenance of pericentromeric heterochromatin and accurate chromosome segregation. Here, we probe the involvement of Brc1 in centromere function.

MATERIALS AND METHODS

Strains, media, genetic analysis, and general procedures. Strains are listed in Table 1. Growth media for *S. pombe* were prepared and genetic procedures performed as described previously (24). Growth assays were performed by spotting 5- or 10-fold serial dilutions of exponentially growing cells onto yeast extract with glucose and supplements (YES) plates in the absence or presence of the indicated agents. Plates were incubated at 30°C and photographed after 2 to 7 days of growth. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (21) using anti-dimethyl-histone H3-K9 antibody (UpState Biotechnology) or anti-green fluorescent protein (anti-GFP) antibody (Roche). The percentage of immunoprecipitated DNA (%IP) in the ChIP samples was calculated relative to the amount of DNA in the input samples. ChIP fold enrichment was calculated relative to *act1*. Sequences of qPCR primers are available upon request. Gene-silencing assays at the centromere were performed as described previously (25–28). RNA preparation and quantitative real-time PCR (qRT-PCR) experiments were carried out as previously described (27, 29, 30). All error bars in figures represent the standard errors from a minimum of 3 experiments.

Microscopy. Cells were photographed using a Nikon Eclipse E800 microscope equipped with a Photometrics Quantix CCD camera. GFP-Brc1 was expressed from pRep42-GFP-*brc1*$^+$ or pREP41-GFP-*brc1*$^+$ plasmids (16). Cells were grown in Edinburgh minimal medium (EMM2) in the absence of thiamine for 18 to 20 h at 30°C. For analysis of mitotic abnormalities using the *cdc25-22* arrest and release protocol, cells were grown to log phase at 25°C, shifted to 36°C for 4 h, and then released from the arrest by transfer to 25°C in the absence or presence of 5 μg/ml TBZ. Cells were harvested 60 min later. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) (500 μg/ml), and the spindle was stained with an antitubulin antibody (TAT1).

RESULTS

Heterochromatin-dependent enrichment of Brc1 at centromeres. Brc1 localization at pericentromeric repeat sequences depends largely on γH2A, which appears in these sequences during S phase (21). γH2A enrichment in pericentromeric heterochromatin is largely abolished in *clr4*Δ cells (21). These findings predict that the enrichment of Brc1 in pericentromeric regions should also depend on Clr4. We used ChIP experiments to test this prediction. For these experiments, we expressed Brc1-GFP and performed ChIP assays with anti-GFP antibodies. Using unsynchronized cells from wild-type (*clr4*$^+$) log-phase cultures, we detected ~3-fold enrichments of Brc1 using *cen-dh* and *cen-dg* primers relative to the actin (*act1*) gene locus (Fig. 1A; see Fig. 2 for a centromere map). These Brc1 enrichments in the *otr* repeats were abolished in *clr4*Δ cells, indicating that they likely occur through replication fork stalling in the pericentromeric heterochromatin (Fig. 1A).

We did not detect Brc1 enrichment using *cen-imr* probes (Fig. 1A). This result was consistent with the ChIP analysis of γH2A using asynchronous cells (21). To investigate whether synchronizing cells in S phase reveals Brc1 enrichment with the *imr* or *otr* probes, we repeated the ChIP assays using cells exposed to 12 mM hydroxyurea (HU) for 4 h, which arrests cell cycle progression in early S phase by inhibiting ribonucleotide reductase, required for deoxynucleoside triphosphate (dNTP) biosynthesis. This approach was used previously to characterize enrichment of RNAi and DNA repair factors during S phase (22, 31). S-phase arrest increased Brc1 enrichment at *cen-dh* and *cen-dg* 5- to 6-fold relative to *act1* (Fig. 1B). Brc1 increased 3-fold at *cen-imr* in HU-treated cells (Fig. 1B). As seen for the asynchronous cells, Brc1 enrichment at pericentromeric loci was abolished in HU-treated *clr4*Δ cells that are unable to form heterochromatin (Fig. 1B). The requirement for Clr4 indicates that Brc1 enrichment at pericentromeric loci is not solely a consequence of HU-induced fork arrest but instead depends on replication fork stalling in pericentromeric heterochromatin.

Brc1 promotes gene silencing in the centromeric repeats. Replication and transcription of pericentromeric repeats are coupled to enable RNAi-mediated transmission of heterochromatin and consequent gene silencing (22, 23). The localization of Brc1 at pericentromeric repeats and its role in stabilizing replication forks suggested that it might have a role in gene silencing. To investigate this possibility, we assessed whether centromeric gene silencing is diminished in *brc1*Δ cells. With a *ura4*$^+$ reporter gene inserted in

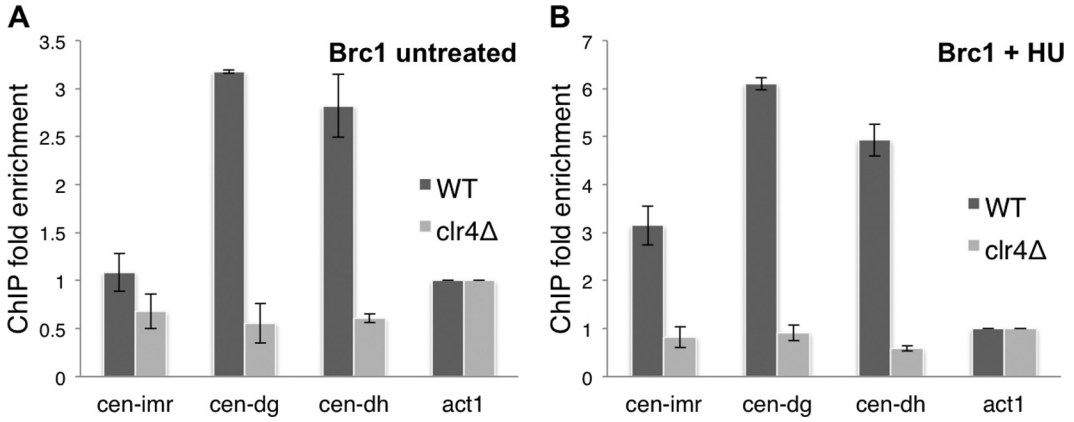

FIG 1 HU treatment increases Clr4-dependent Brc1 enrichment at centromeres. ChIP analysis of Brc1-GFP in log-phase cells (A) and cells treated with 12 mM HU for 4 h (B). Enrichment was calculated relative to the *act1* (actin) locus (see Materials and Methods).

the outer (*otr*::*ura4*$^+$) or innermost (*imr*::*ura4*$^+$) repeat regions (6, 32), we monitored *ura4*$^+$ expression by plating serial dilutions of cells on nonselective (N/S), selective (−URA), or counterselective (5-fluoro-orotic acid [5-FOA]) plates. 5-FOA is a toxigenic substrate for the Ura4 protein. Deletion of *brc1*$^+$ decreased gene silencing at both the *otr*::*ura4*$^+$ and *imr*::*ura4*$^+$ insertion sites, as indicated by *brc1*Δ colony formation on −URA plates and their retarded growth on FOA plates (Fig. 2A). Consistent with these findings, quantitative real-time PCR revealed that *ura4*$^+$ transcripts from *otr*::*ura4*$^+$ were increased ~4-fold in *brc1*Δ cells relative to the wild type (Fig. 2B). As measured by both types of assays, the gene silencing defects in *brc1*Δ cells were partial compared to those in *clr4*Δ cells, which are completely deficient in pericentromeric heterochromatin (Fig. 2A and B).

We also analyzed gene silencing in a *brc1-T672A* mutant that has a missense mutation in the BRCT5,6 region that impairs binding to γH2A (16). In this mutant we detected a silencing defect with *imr*::*ura4*$^+$, although the 5-FOA effect was weaker than that in the *brc1*Δ cells (Fig. 2A). The *brc1-T672A* effect on *otr*::*ura4*$^+$ silencing was even weaker (Fig. 2A). As discussed below, these data indicate that Brc1 retains significant activity even when binding to γH2A is impaired, as previously observed (16).

Brc1 is required for efficient histone H3 lysine-9 methylation in pericentromeric heterochromatin. Methylation of histone H3 on Lys9 (H3K9me2) by Clr4 methyltransferase is a key step in heterochromatin formation. H3K9me subsequently recruits Swi6/HP-1 to facilitate heterochromatin spreading (33, 34). H3K9 dimethylation is also a prominent mark of transcriptional repression. As our data indicated that Brc1 is involved in gene silencing at pericentromeric regions, we used ChIP to measure H3K9me2. Consistent with the silencing defect, reduced H3K9me2 levels at *cen-dg* were observed in *brc1*Δ cells (Fig. 3). As seen for the gene-silencing assays, the defect in *brc1*Δ cells was partial compared to that in *clr4*Δ cells. We repeated the assay with cells arrested in early S phase by HU treatment and found that the H3K9me2 signal was further diminished in *brc1*Δ cells (Fig. 3). These results are con-

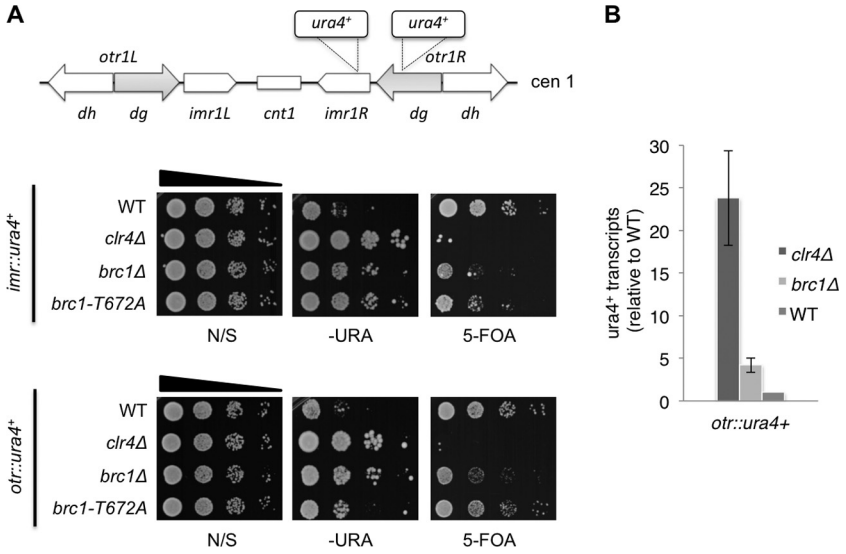

FIG 2 Brc1 enhances gene silencing in pericentromeric heterochromatin. (A) Diagram of centromere I (*cen1*) with *ura4*$^+$ insertion sites (top panel). Gene-silencing assays in nonselective (N/S), selective (−URA), and counterselective (5-FOA) media (bottom panels). (B) qRT-PCR analysis of *ura4* (*otr*::*ura4*$^+$) transcript levels relative to a control transcript, *act1*$^+$, normalized to the wild type.

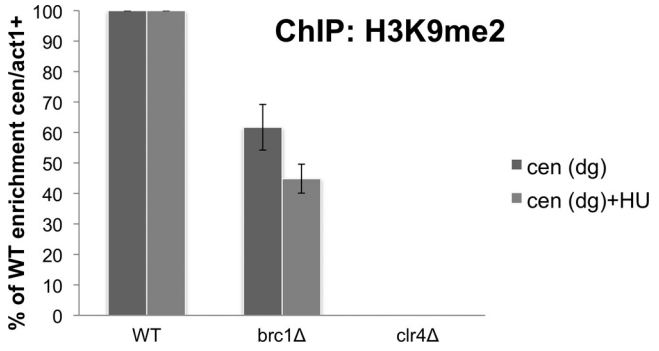

FIG 3 Brc1 assists heterochromatin-specific modification of the histone H3 tail at the centromere. ChIP analysis using anti-dimethyl-histone H3K9 (H3K9me2) antibodies at *cen*(*dg*) relative to *act1*$^+$, normalized to the wild type. Cells were either untreated or exposed to 12 mM HU for 4 h.

sistent with the gene-silencing assays and suggest that Brc1 is required for efficient maintenance of heterochromatin-specific modifications of histone H3 in pericentromeric regions.

Chromosome missegregation in *brc1*Δ cells. Maintenance of pericentromeric heterochromatin is essential for robust cohesion of chromosome arms in pericentromeric regions and for proper centromere function (35, 36). Defects in centromeric heterochromatin cause sensitivity to TBZ. Interestingly, we found that *brc1*Δ cells are sensitive to TBZ (Fig. 4A). This TBZ-sensitive phenotype of *brc1*Δ cells was moderate, but it was strongly synergistic with elimination of Swi6/HP1 (Fig. 4A).

Genetic assays established that Brc1 is required for mitotic chromosome stability, which is indicative of a role in chromosome segregation (37). To further investigate the effect of TBZ in *brc1*Δ cells, we monitored chromosome segregation in cultures synchronized by the *cdc25-22* block and release protocol, which arrests cells in late G_2 phase and synchronously releases them into mitosis. Cells were released from the G_2 arrest in either the presence or the absence of a low concentration (5 μg/ml) of TBZ. DNA and tubulin staining revealed a large increase of lagging chromosomes in TBZ-treated *brc1*Δ cells compared to wild-type cells (16.4% in *brc1*Δ cells versus 3.0% in wild-type cells) (Fig. 4B and C). These data suggest that defects in centromere function sensitize *brc1*Δ cells to TBZ.

γH2A defect enhances TBZ sensitivity of spindle assembly checkpoint mutant. In contrast to *brc1*Δ cells, TBZ sensitivity was not observed in *htaAQ* (*hta1-S129A hta2-S128A*) cells lacking the C-tail SQ phosphorylation site in both H2A genes (Fig. 5A). This relationship was also observed in replication stress survival assays (Fig. 5A) (16), implying that Brc1 retains substantial activity in the absence of γH2A. This relationship is shared with other γH2A-binding proteins (13, 18, 38) and is likely explained by their γH2A-independent scaffolding functions (39) and the compensatory activities of other genome protection mechanisms (21). To investigate the latter possibility, we tested whether elimination of γH2A enhances the TBZ-sensitive phenotype in cells lacking the histone H2A serine-121 site phosphorylated by Bub1. Replacement of serine-121 by alanine (*hta1-S121A hta2-S121A*) to generate the *htaSA* genotype causes substantial sensitivity to TBZ (8) (Fig. 5A). Combining the *htaSA* and *htaAQ* mutations to generate an *htaSA.AQ* strain (*hta1-S121,129A hta2-S121,128A*) further enhanced sensitivity to TBZ (Fig. 5A). Interestingly, the *htaAQ* and *brc1*Δ mutations had similar effects in the *htaSA* background, and *htaSA* increased sensitivity to replication stress agents in the *htaAQ* and *brc1*Δ backgrounds (Fig. 5A). Importantly, the *htaSA* genotype did not reduce Brc1 localization at *cen-imr* or *cen-dh* (Fig. 5B). These data indicate independent compensatory activities of the γH2A-Brc1 replication stress response and the Bub1-mediated spindle assembly checkpoint.

DISCUSSION

The key conceptual advance that emerges from this study is that Brc1 contributes to centromere function in fission yeast. Our experimental evidence suggests that Brc1 enhances the maintenance of pericentromeric heterochromatin and thereby increases the

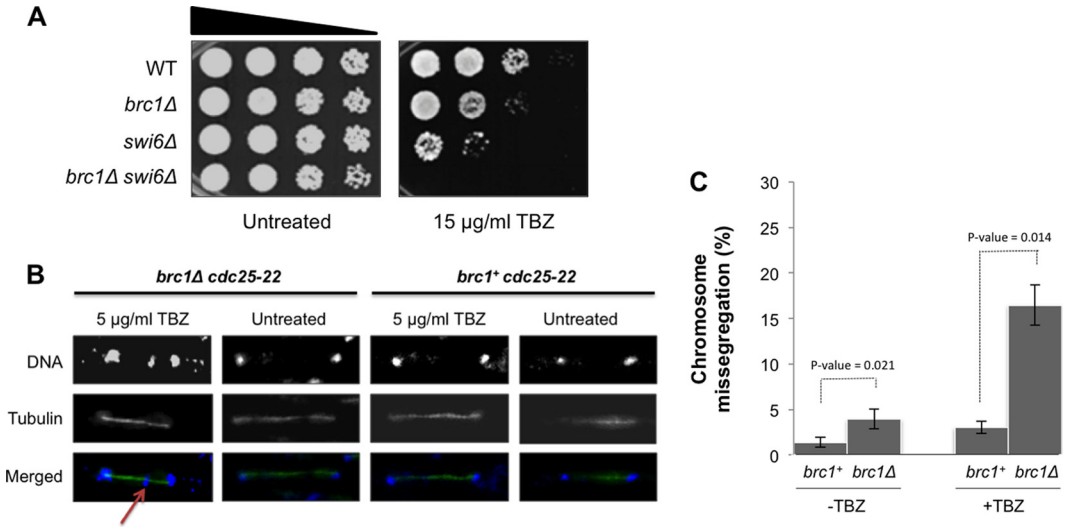

FIG 4 TBZ sensitivity in *brc1*Δ cells. (A) TBZ sensitivity caused by *brc1*Δ is additive to that caused by *swi6*Δ. Tenfold serial dilutions were incubated at 30°C for 2 days. (B) Mitotic abnormalities in *brc1*Δ cells released from a *cdc25-22* arrest in the absence or presence of 5 μg/ml TBZ. Micrographs were taken at 60 min after release from the arrest. DNA was stained with DAPI and antitubulin antibody (TAT1). The arrow points to a lagging chromosome (or chromosome fragment) in *brc1*Δ cells treated with TBZ.

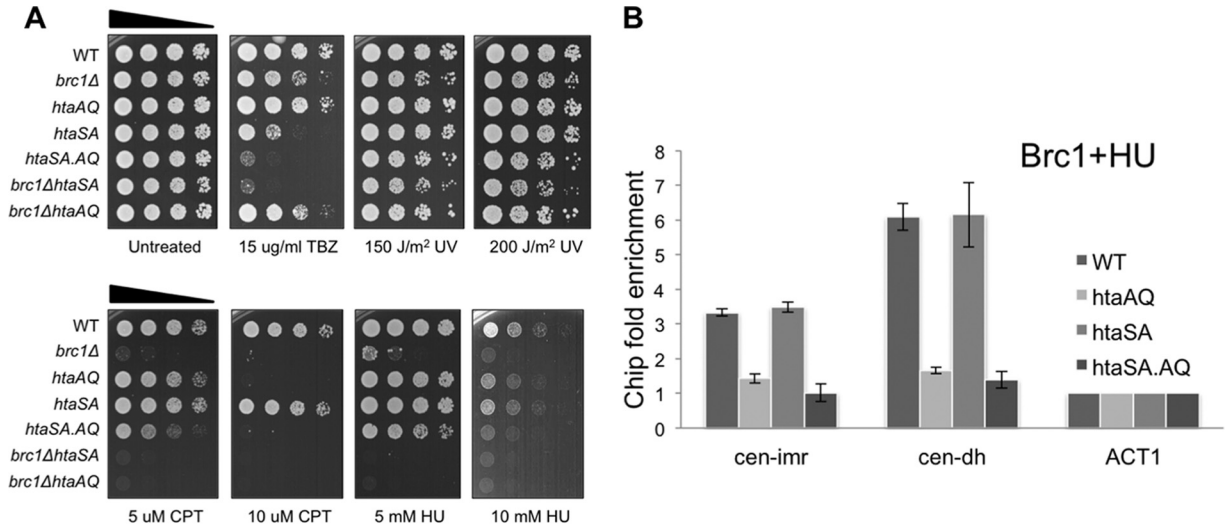

FIG 5 Loss of γH2A or Brc1 increases TBZ sensitivity in cells lacking the serine-121 site in histone H2A that is phosphorylated by Bub1. (A) Histone H2A mutants lacking the γH2A site (*htaAQ* [*hta1-S129A hta2-S128A*]) or the Bub1 phosphorylation site (*htaSA* [*hta1-S121A hta2-S121A*]) alone, or in combination (*htaSA.AQ*), or with *brc1*Δ were tested with TBZ or the indicated genotoxins. Cells were incubated at 30°C for 2 days. (B) Phosphorylation of histone H2A at serine-121 is not required for localization of Brc1 at centromeres. ChIP analysis of Brc1-GFP in cells treated with 12 mM HU for 4 h. Enrichment was calculated relative to the *act1* locus (see Materials and Methods).

probability of proper chromosome segregation, especially when spindle function is impaired. The discovery of this relationship is consistent with evidence that γH2A and Brc1 are enriched in pericentromeric heterochromatin during S phase in a Clr4-dependent manner (21). The data are furthermore consistent with studies indicating that RNAi-mediated release of RNA Pol II is necessary to complete replication of pericentromeric heterochromatin with replisomes that remain associated with the Rik1 holocomplex (22). Taken together, the data indicate that the γH2A-Brc1 module and the RNAi pathway work together to ensure the efficient transmission of pericentromeric heterochromatin during each S phase.

These studies indicate a physiologically significant role for Brc1 in the maintenance of pericentromeric heterochromatin, but pericentromeric gene silencing is not fully ablated in *brc1*Δ cells. In this respect, Brc1 is similar to 12 other genes recently identified in a targeted-deletion screen for loss of pericentromeric silencing (40). Several of these candidates are involved in replication stress responses, including the nonessential Nse5 subunit of Smc5-Smc6 structural maintenance of the chromosome holocomplex (31). As seen with γH2A and Brc1, the Smc5/6 holocomplex is transiently enriched at pericentromeric heterochromatin during S phase in a Clr4-dependent manner (41). Brc1 is a high-copy-number suppressor of *smc6-74* and is required for viability in strains having hypomorphic mutations of essential subunits of Smc5/6 holocomplex (17, 37). These data suggest that multiple proteins involved in the response to replication stress contribute to the maintenance of pericentromeric heterochromatin.

Eliminating γH2A does not sensitize cells to TBZ, implying that Brc1 retains significant activity without binding γH2A. This result is consistent with our previous study showing that Brc1 enrichment at *cen-dh* is reduced in *htaAQ* cells but it remains above the Brc1 signal at the *act1* locus (21). As mentioned above, γH2A(X)-independent activity occurs for many γH2A(X)-binding proteins, and for Crb2 this activity is at least partly explained by its multiple independent protein interactions (39). However, genetic interaction tests can reveal subphenotypic effects of mutations, as we have observed when the *htaSA* and *htaAQ* genotypes are combined. This particular genetic interaction also highlights how distinct genome protection pathways are propagated through C-tail phosphorylations of histone H2A.

Brc1's involvement in heterochromatic gene silencing may extend to its *Saccharomyces cerevisiae* homolog *RTT107*. This gene was initially identified through its regulation (i.e., repression) of ty1 retrotransposon transposition (42). Chromatin structure strongly impacts retrotransposon integration frequencies and site selection (43). Indeed, defects in chromatin assembly factor 1 (CAF-1) and HIR chromatin assembly complexes synergistically increase Ty1 transposition rates (44). Interestingly, centromeres from many plant species contain sequences related to retrotransposon long terminal repeats (45). *RTT107* was later uncovered as *ESC4* in a screen for genes that can establish silent chromatin at the *HMR* silent mating type cassette (46). The C-terminal BRCT5,6 domains of Esc4 bound Sir3, a subunit of the silent information regulator holocomplex that is required for chromatin silencing at transcriptionally repressed mating type donor cassettes and telomeric heterochromatin (47). These observations suggest that Rtt107/Esc4 may have retained chromatin assembly and modification functions even as the mechanism of chromatin-mediated transcriptional repression has diverged in *S. cerevisiae*.

The involvement of H2AX-binding DNA damage response proteins in gene silencing is not limited to yeast species. Mammalian Mdc1, which binds γH2AX through its C-terminal BRCT domains and functions in checkpoint signaling (12, 48), is critical for inactivation of sex chromosomes in male mouse meiosis (49).

Genetic and biochemical evidence supports a model in which collisions between the replisome and the RNA polymerase II transcription complex stall replication forks in pericentromeric heterochromatin of fission yeast (22, 23) (Fig. 6). According to this model, RNAi releases Pol II by processing pre-small interfering

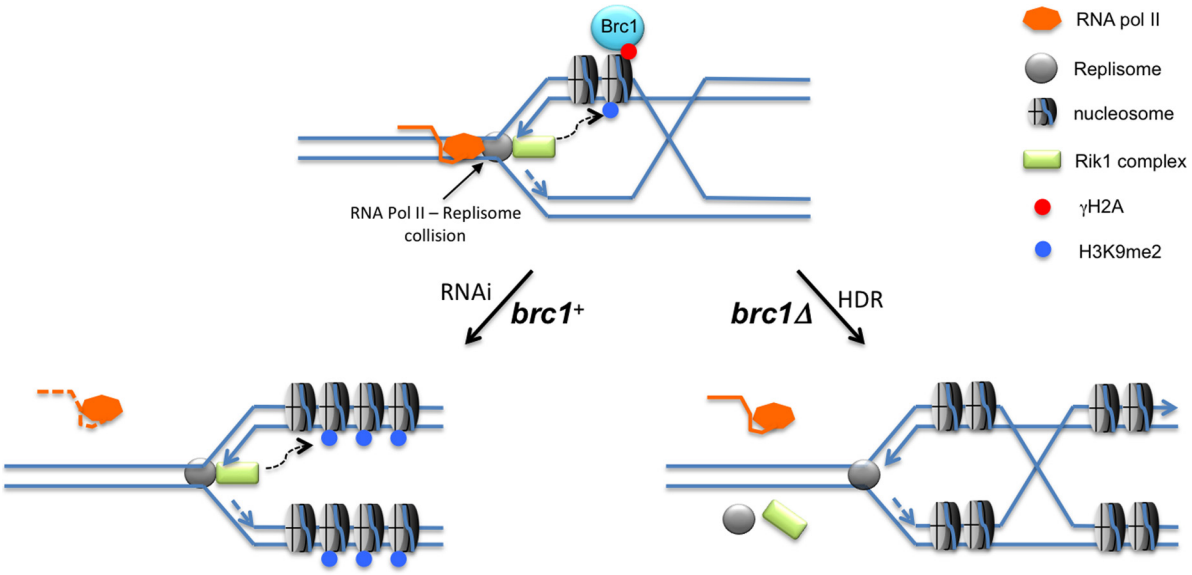

FIG 6 Model for Brc1 stabilizing replication forks in pericentromeric heterochromatin. Collisions between the replisome and RNA Pol II trigger γH2A formation by Rad3 (not shown), resulting in recruitment of Brc1. Stabilization of the replication fork by Brc1 favors RNAi-mediated disassociation of RNA Pol II from the chromosome, allowing fork restart by the Rik1-associated replisome (22). Loss of Brc1 destabilizes the fork and increases the frequency of HDR-mediated fork restart by a reassembled replisome that is not associated with the Rik1 complex. See the text for additional description.

RNA (pre-siRNA) transcripts, allowing the completion of DNA replication. The leading-strand DNA polymerase associates with the Rik1 histone modification complex, which includes Clr4, ensuring efficient transmission of H3K9 methylation. RNAi defects are proposed to prolong replisome stalling, leading to an alternative pathway of replication fork restart requiring homology-directed repair (HDR) (22). This pathway engages a replisome that lacks the associated Rik1 complex, leading to the loss of histone modifications required for heterochromatic gene silencing (Fig. 6). Aberrant repair via this alternative pathway may underlie the formation of isochromosomes, which account for ~50% of spontaneous gross chromosomal rearrangements (GCRs) in fission yeast (50). Rad3 suppresses these GCRs, supporting a model in which they originate from a failure to stabilize stalled replication forks near centromeres. Mutants lacking Brc1 have increased spontaneous foci of Rad52 (Rad22) HDR protein, and there is a severe synergistic growth defect when *brc1*Δ and *rad22*Δ mutations are combined, indicating that stalled replication forks are more likely to be restarted via an HDR pathway in *brc1*Δ cells (16). Incorporating the data presented in this study, the most parsimonious model is that Brc1 stabilizes replication forks stalled in pericentromeric heterochromatin, favoring the restart pathway in which the replisome maintains its association with the Rik1 complex (Fig. 6).

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