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Re-establishment of nucleosome occupancy during double-strand break repair in budding yeast

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Abstract

Homologous recombination (HR) is an evolutionary conserved pathway in eukaryotes that repairs a double-strand break (DSB) by copying homologous sequences from a sister chromatid, a homologous chromosome or an ectopic location. Recombination is challenged by the packaging of DNA into nucleosomes, which may impair the process at many steps, from resection of the DSB ends to the re-establishment of nucleosomes after repair. However, nucleosome dynamics during DSB repair have not been well described, primarily because of a lack of well-ordered nucleosomes around a DSB. We designed a system in budding yeast *Saccharomyces cerevisiae* to monitor nucleosome dynamics during repair of an HO endonuclease-induced DSB. Nucleosome occupancy around the break is lost following DSB formation, by 5' to 3' resection of the DSB end. Soon after repair is complete, nucleosome occupancy is partially restored in a repair-dependent but cell cycle-independent manner. Full re-establishment of nucleosome protection back to the level prior to DSB induction is achieved when the cell cycle resumes following repair. These findings may have implications to the mechanisms by which cells sense the completion of repair.

Keywords

Double-strand break; nucleosome; chromatin; cell cycle; chromatin dynamics

2. Introduction

DNA double-strand breaks (DSBs) are highly deleterious lesions that threaten genomic integrity. Repair of DSBs can be carried out either by nonhomologous end-joining (NHEJ) or by one of several well-conserved homologous recombination (HR) pathways [1]. HR in eukaryotes requires the involvement of many chromatin remodelers, including RSC, Ino80, and Swi2/Snf2 family members. Both the initiation and extension of 5' to 3' resection requires chromatin remodeling by RSC, Ino80 and Fun30 [2–9]. Once resection has created

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long 3'-ended ssDNA tails on which Rad51 recombinase can form a nucleoprotein filament, the subsequent steps of strand invasion and initiation of new DNA synthesis also require chromatin remodeling, by Rad54 [10–12]. In addition, the chromatin remodelers Rdh54, Ino80 and Fun30 have been shown to be involved in regulation of the DNA damage checkpoint that is established in response to the creation of a single DSB [2–4, 13, 14].

In addition to ATP-dependent chromatin remodelers, two histone H3-H4 chaperones, Asf1 and CAF-1, have been shown to have an overlapping role in regulating recovery from the DNA damage checkpoint (DDC) following repair [15]. Cells in which the DSB can be repaired through ectopic gene conversion (GC) have no repair deficiency if both *ASF1* and *CAC1* (the largest subunit of CAF-1) are deleted, but they fail to recover from cell cycle arrest imposed by the DNA damage checkpoint [15]. Recently we have shown that down-regulation of the DDC is controlled by Asf1's interaction with the Rad53 (CHK2) checkpoint kinase [16]. The role of CAF-1 in recovery is still unclear. One intriguing hypothesis is that re-establishment of nucleosome occupancy following repair of a DSB is required to promote recovery from the DSB [15].

Budding yeast mating-type (*MAT*) switching has been extensively used as a model for studying many aspects of HR (reviewed in [17]). The *MAT* locus on chromosome 3 (Chr 3) determines the mating type of the cell. Upon induction of the HO (homothallic) endonuclease, cells suffer a site-specific DSB within the *MAT* locus that is repaired through the process of gene conversion (GC) using one of two heterochromatic *MAT*-homologous cassettes also present on Chr 3 (*HMLα* and *HMRA*). Expressing the HO endonuclease gene from a galactose-inducible promoter allows its rapid induction and repression, causing a well-synchronized and robust formation of a DSB at *MAT* [18, 19].

A combination of Southern blot, PCR and chromatin immunoprecipitation experiments have led to a detailed understanding of the early steps in *MAT* switching [20, 21]. Because the heterochromatic *HML* donor locus has highly positioned nucleosomes [22, 23] it has been possible to observe alterations in nucleosome structure during strand invasion [10]. However, studying nucleosome dynamics around the *MAT* locus itself presents a technical challenge. Whereas nucleosomes in the *HMLα* and *HMRA* are well positioned, the nucleosomes around the HO cut site at the *MAT* locus are not well positioned [22, 23]. To circumvent this problem we inserted the *5S* nucleosome-positioning element from *Xenopus laevis* adjacent to the *MAT* sequences. In these experiments, we first deleted both *HML* and *HMR* in a strain carrying a mutation at the *MAT* locus that prevents HO cleavage (*MATa-inc*). We then inserted a cloned copy of *MATα* on the left arm of Chr 3, with the *5S* nucleosome positioning sequences 350 bp from the cleavage site. Thus the normal *MATa-inc* locus acts as a donor to repair *MATα* and replace it by *a-inc* sequences. We demonstrate that integration of *5S* sequences results in a well-positioned nucleosome that confers a high degree of protection against micrococcal nuclease (MNase) relative to a reference nucleosome. We show that nucleosome occupancy is re-established following repair. This re-establishment does not require cell-cycle progression following repair; however, we find that the relative nucleosome occupancy is significantly lower after repair than before a DSB had been induced. Cells only reestablish full pre-DSB nucleosome occupancy after completing repair and resuming the cell cycle. These observations suggest that repair-

dependent re-establishment of nucleosome occupancy differs from replication-dependent establishment of nucleosome occupancy, but may share some features with the slow re-establishment of heterochromatic gene silencing.

3. Materials and Methods

3.1 Strains and plasmids

pWH12 was constructed as follows: The *X. laevis* 5S nucleosome position element was PCR amplified from CP1116 (gift of Craig Peterson's lab) using primers 5S-*XcmI* F (5'-ccaaatagggcaatggGAATTCCCGAGGAATTCGGTATTC-3'), which contains 24 bp (uppercase) homology to CP1116 and an *XcmI* restriction site (lowercase underlined) and 5S-*AflIII* R (5'-cttaagCAACGAATAACTTCCAGGGATTATAAGC-3'), which contains 29 bp (uppercase) homology to CP1116 and an *AflIII* restriction site (lowercase underlined). This PCR product was then ligated to pGEM-T (Promega cat. A3600) to create pWH2. The entire *MATa* locus was PCR amplified from a switched isolate of JKM161 using primers Z2-*AflIII* (5'-cttaagTTGATTGTTTGCTTGAGTCTGAG-3'), which contains 23 bp (uppercase) homology to the 1st 23 bp of the Z2-region and an *AflIII* restriction site (lowercase underlined) and *W-KpnI* (5'-ggtaccTTGTATTAGACGAGGGACGGAG-3'), which contains 22 bp (uppercase) homology to the 1st 22 bp of the W-region and a *KpnI* restriction site (lowercase underlined). This PCR product was then ligated to pGEM-T to create pWH1. Next, the 5S nucleosome positioning element was liberated from pWH2 by digesting with *AflIII* and *NotI* and then ligated into similarly digested pWH1, which linked the 5S element to the right of the Z2-region of *MATa* and created pWH11. Finally, the *MATa*-5S fragment was liberated from pWH11 by digesting with *ApaI* and *SacI* and ligated to similarly digested pRS305 (NCBI accession U03437) to create pWH12.

All strains are derivatives of strain WH207 (Table 1). Strain WH207 was constructed by linearizing plasmid pWH12 (*MATa::5S LEU2*) with restriction enzyme *ClaI* at the *LEU2* locus, and transforming into strain JKM146 at the *leu2 3,112* locus. *LEU2* was subsequently deleted using a kanamycin resistance cassette resulting in *leu2::KAN::MATa::5S* (Figure S1A). the donor in strain WH207 is the endogenous *MATa*-inc. Strain MT093 was constructed by transforming a *HPH::MATa* to replace the *KAN::MATa* leaving the 5S intact. In addition, this strain is *bar1::LEU2* (Figure S1B).

3.2 MNase digestion assay

Cultures were grown ON in YEP-raffinose to a concentration of between 5×10^6 and 1×10^7 cells/ml in a given amount of time. Approximately 8×10^8 cells were collected per time point and fixed using formaldehyde at a final concentration of 1%. Cells were incubated with formaldehyde for 10 minutes and the reaction was quenched with glycine at a final conc. of 125 mM. Cells were spun down at 3000 RPM for 5 minutes, washed three times with 1 M sorbitol and frozen on dry ice. To make spheroplasts, pellets were resuspended in 1ml spheroplasting buffer (1 M sorbitol, 1000 units/ml lyticase and 1 mM β -mercaptoethanol) at RT and Incubated at 37°C for 8 min. Spheroplasts were spun down at max. speed for 1 minute, supernatant was discarded, and spheroplasts were resuspended in 1 ml digestion buffer (1 M sorbitol, 1 mM β -mercaptoethanol, 50 mM NaCl, 10 mM pH 7.4 Tris, 1 mM

CaCl₂, 500 μ M spermidine and 0.075% IGEPAL CA 630) and split to 4 epp. tubes (250 μ M each) on ice. Each epp. tube was treated with a different conc. of micrococcal nuclease (0X, 1X, 2X and 4X). MNase units were determined according to pellet size. MNase concentrations ranged from 3.75 units to 60 units in DB buffer (400 mM pH 7.5 HEPES, 10 mM MgCl₂ and 10 mM CaCl₂), vortexed gently and incubated at 37°C for 5 min. Reaction was stopped using 40 μ l stop buffer (250 mM EDTA 5% SDS) and incubated at RT for 10 minutes after which 25 μ l Pronase (10 μ g/ μ l) were added to each reaction, and samples incubated at 65°C ON. 400 μ l phenol-chloroform was added to each reaction, vortexed for 20 sec. and spun 10 min. max speed. Aqueous phase was collected in a new tube, and DNA was precipitated using isopropanol. Pellets were resuspended in 300 μ l 1X TE with 5 μ l RNase (25 μ g/ μ l) and incubated for 30 min. at 37°C. DNA was precipitated using isopropanol and pellets were dried and resuspended in 50 μ l 1X TE. Samples were run on 1.8% agarose gel to determine the extent of MNase digestion.

3.3 qPCR analysis of nucleosome occupancy

The 5S nucleosome occupancy was monitored using quantitative PCR (qPCR) with an array of primers (Table 2). A 1:8 dilution curve (1:8, 1:64, 1:512, 1:4096) was created from the 0 h untreated sample. For each primer pair (including the reference *PHO5*+1 locus), the dilution curve and a 1:40 dilution of each treated time point sample was run to determine the amount of DNA signal relative to the 0 h untreated and to verify that the sample is within the linear amplification range. The signal from each time point was then normalized to either treated *PHO5*+1 or untreated *PHO5*+1 signal (indicated on the graphs). Protection was plotted of a graph where the X-axis is distance from the DSB and the Y-axis is percent protection. Occupancy was calculated as the peak protection.

3.4 Viability assays

Strains were grown in YEPD overnight, washed twice in YEP-lactose or YEP-raffinose and grown in YEP-lactose or YEP-raffinose for 6 h. Cells were then counted using a hemocytometer and 100 cells were plated on YEPD and YEP-galactose plates.

3.5 Repair assays

For qPCR-based repair assay, a primer in the 5S sequence and a primer in the *Z1* region were used (Table 2). A 1:8 dilution curve (1:8, 1:64, 1:512, 1:4096) was created from the 0 h untreated sample. For each primer pair (including the reference *PHO5*+1 locus), the dilution curve and a 1:40 dilution of each untreated time point sample was run to determine the amount of DNA signal relative to the 0 h. In WH207 a product only appears after repair has commenced. The formation of this product was quantified by qPCR and normalized to the amount of product to a similarly treated strain that had already switched and could not be cleaved by HO. In MT093, a product appears before HO induction, disappears after HO cleavage, and reappears after primer extension begins [20]. In this strain the re-formation of product was normalized to the amount level of product before HO induction.

3.6 Synchronization in G1/S phase

We integrated an auxin-inducible degron (AID) [24] into Cdc7 and Dbf4 into strain MT093. Cdc7-AID Dbf4-AID cells were grown for an MNase time course as described above. After cells reached log-phase, cells were synchronized in G1 using α -factor. Arrest was monitored visually. After 95% arrest 500 μ M 3-indoleacetic acid (IAA) was added to the media [24]. One hour following auxin addition the culture was filtered and washed twice with DDW, and resuspended in new media containing 500 μ M IAA. One hour after release from α -factor HO was induced with 2% galactose and samples were collected at the indicated times. Six hours after galactose induction, the cultures were filtered and washed twice in DDW ("release"), and resuspended into YEP-raffinose with galactose.

3.7 Flow cytometry analysis of DNA content

Cells were fixed by ethanol. 3 ml of culture were collected by centrifuging. Supernatant was removed and pellet was resuspend in 1.5 ml of water. 3.5 ml of 95% ethanol were slowly added. Cells were fixed ON +4C or 1 h on the bench. Cells were spun 4min 4000 rpm, and sup. was discarded. Pellet was resuspended in 500 μ l DDW and transferred to epp. tube. Cells were spun 4min 4000 rpm, and sup. was discarded. Cells were resuspended in RNase buffer (50 mM Tris pH = 8.0, 15 mM NaCl, 40 μ l of 25mg/ml RNase). Cells were incubated 2 h to ON in 37° C. 50 μ l of 10mg/ml Proteinase K were added, and incubated 1h 50° C. Cells were spun 4min 4000 rpm, and sup. was discarded. Cells were resuspended in 50mM Tris 7.5 and 0.2 μ l/ml Quant-iT™ PicoGreen®.

3.8 BrdU incorporation assay

MT174 cells (Table 1) were arrested in G1 using α -factor. 3 h after G1 arrest 500 μ M IAA was added for 1 h. Cells were then released into G1/S (500 μ M IAA), G2/M (15 μ M nocodazole) or allowed to resume cell cycle progression. 400 μ g/ml bromodeoxyuridine (BrdU) were added to the media prior to release, and 45 ml of cells were collected and pelleted every time point. Genomic DNA was purified using phenol extraction. Cells were resuspended in 450 μ l extraction buffer (5% SDS, 250 mM EDTA pH 8) and 450 μ l phenol (Sigma-Aldrich). 500 μ l acid washed beads were added and cells were vortexed for 2 minutes. Cells were then spun 10 minutes 13,000 RPM and aqueous phase was taken to a new epp tube to which 600 μ l isopropanol and 40 μ l LiCl 4 M were added. Tubes were mixed by inversion 20 times and spun 10 minutes maximum speed. Supernatant was discarded and pellet was resuspended in 300 μ l 1 X TE. 25 μ l RNase (10 μ g/ml) was added and incubated at 37° C for 30 minutes. 300 μ l isopropanol and 25 μ l NaCl (5N) was added, mixed by inversion and spun for 10 minutes. Pellets were washed with 75% EtOH, dried and resuspended in 100 μ l 1 X TE.

DNA concentration was determined using NanoDrop (Thermo-Scientific). DNA was denatured by boiling for 10 min. at 95° C and loaded on a nitrocellulose membrane by vacuum (slot blot). BrdU was detected using α -BrdU antibody (Roche).

4. Results

4.1 System to study nucleosome occupancy around a DSB during HR

The *MAT* locus in budding yeast is devoid of highly positioned nucleosomes, especially in comparison to the heterochromatic donors *HML α* and *HMR α* [22, 23]. To examine chromatin changes, we modified the *MAT α -inc* strain JKM146 lacking *HML* and *HMR*. On the opposite side of the centromere from *MAT α -inc*, at the *leu2* locus, we inserted a copy of *MAT α* locus which had been modified to carry a 207-bp 5S nucleosome-positioning element from *Xenopus laevis* [25], adjacent to the Z1Z2 region of *MAT α* (Figure 1A, 1B and S1A, and Materials and Methods). The *MAT* proximal boundary of the 5S sequence lies approximately 350 bp from the HO cleavage site. Here, a DSB will be created at the *leu2*-integrated *MAT α ::5S* locus and the donor for repair will be *MAT α -inc* at the endogenous *MAT* locus, which cannot be cleaved. The resulting strain, WH207, expressing both *MAT α* at the *leu2* locus and *MAT α -inc*, is non-mating and impervious to synchronization in G1 by α -factor. We then replaced *MAT α ::5S* with *MAT α ::5S*; this *a*-mating strain, MT093, is therefore responsive to α -factor (Figure S1B).

4.2 Homologous recombination is proficient in the 5S strains

The insertion of the 5S nucleosome-positioning element in strains WH207 and MT093 did not apparently interfere with efficient repair, as both WH207 and MT093 exhibited near-complete cleavage of *MAT α ::5S* and approximately 90% viability when grown on galactose plates on which HO is induced (Figure 1C). We tested if the kinetics of repair were affected by the 5S sequence, employing a primer extension assay [20], using an antisense primer in the 5S sequence and a sense primer in the *Ya* sequence at the *MAT* locus. In strain WH207, there will be a qPCR product only after strand invasion and new DNA synthesis has commenced (Figure 1D). In strain MT093, the primers initially detect *MAT α* ; the near-complete drop in the PCR product after HO induction is a measure of the efficiency of HO cleavage and its increase reflects repair. In both WH207 and MT093, 6 h after induction of HO repair was approximately 90% complete (Figure 1D). These kinetics are similar to the kinetics reported for mating type switching [10].

4.3 Nucleosome occupancy near the *MAT α* cassette

To determine the steady state nucleosome occupancy around the integrated *MAT α ::5S* cassette in strain WH207 we used a MNase digestion assay followed by qPCR (see Materials and Methods). To determine changes in occupancy we compared the protection conferred by different primer pairs across the *Z1Z2* and 5S loci, relative to the protection conferred by a primer pair that detects the *PHO5*+1 nucleosome, previously shown to be stable during different cell cycle and transcriptional states [26–28]. This normalization strategy improved the reproducibility of the data compared using total DNA input as the reference (Figure S2A).

Two peaks of occupancy can be detected in the 327 bp *Z1Z2* region in yeast, but there is much less protection than when the same sequences are in heterochromatin at *HML* [10]. The occupancy at this region (which is found both at *MAT α* and *MAT α -inc*) exhibits approximately 40% of the *PHO5*+1 protection (Figure 1E). Because the *Z1Z2* region is

found at both *MAT* loci, we examined the protection in the *ZIZ2* region in the parental strain JKM146, in which the *MATa-5S* cassette was not inserted. The peak of MNase protection detected in the parental *MATa-inc* strain at *ZIZ2*, when normalized to input DNA, was at the same distance from the mutated HO cut site as at the *ZIZ2* locus in strain WH207 (Figure S2B). We conclude that the level of occupancy detected at *ZIZ2* in strain WH207 is contributed by both the *ZIZ2* region at the substrate locus (*MATa-5S*) and the *ZIZ2* region at the donor (*MATa-inc*).

The *5S* nucleosome-positioning element is present only at the recipient locus; therefore it should be well suited to monitor nucleosome occupancy and positioning around a DSB, avoiding the confounding effect of additional copies of the locus. *In vitro*, the nucleosome at the *5S* sequence can occupy several positions with a preference for a dominant position, giving a protection pattern approximately twice as broad as the predicted 146 bp [29]. We find a similar broad pattern of protection *in vivo* using our assay when we normalize the signal to either the treated or the untreated DNA (Figure 1E and S2A). To confirm that the broad pattern of protection seen in the *5S* locus is specific to that locus we monitored nucleosome protection of the *PHO5*+1 nucleosome. We find that the well-positioned *PHO5*+1 nucleosome only protects a narrow region under our MNase treatment (Figure 1F).

4.4 5S nucleosome dynamics during HR

We then tested the dynamics of nucleosome occupancy during repair in MT093 in which *MATa* switches to *MATa-inc*. The initial maximum *5S* nucleosome occupancy in this strain was approximately 70% of the *PHO5*+1 occupancy (Figure 2A). 2 h after HO induction occupancy decreased to 15%. This loss of occupancy may be a result of active eviction by ATP dependent chromatin remodelers such as the RSC complex [7, 8] but is more likely to be the result of resection (see below).

After repair is complete (6 h after HO induction), *5S* nucleosome occupancy was partially restored, but only to about 50% of the initial level of protection (35% relative to *PHO5*+1, Figure 2A), even though repair was approximately 90% complete (Figure 1C and 1D). As previously shown [30], in a *rad52* strain, where recombination is absent (Figure 1D), there was no re-establishment of nucleosome protection (Figure 2A and S2C). The loss of nucleosome protection is gradual over the first 3 h, consistent with removal of sequences by 5' to 3' resection [4, 31] (Figure S2C). Because the unrepaired locus showed no protection of the *5S* region, we hypothesize that the reestablishment of nucleosome protection reflects the formation of the repaired product. Working under this assumption, we normalized the nucleosome occupancy seen at 6 h to the repaired DNA product detected by the qPCR repair assay at the same time.

We observed similar results in exponentially growing strain WH207. The initial protection conferred by the *5S* nucleosome was approximately 80% relative to the *PHO5*+1 nucleosome (Figure 1E and Figure 2B). Two h after HO induction, nucleosome occupancy was significantly reduced in the *5S* region to 15% of the reference locus. As seen for strain MT093, when repair is 90% complete (6 h after HO induction) the level of protection at the *5S* region was partially restored to 50%, or about 62% of its initial value (Figure 2B).

The partial restoration of the occupancy in the *5S* region may be transient or may represent a new and heritable state. To differentiate between these two possibilities we examined *5S* nucleosome occupancy in WH207 cells from a colony that had completed repair and grew for twenty or more generations (termed a survivor). *5S* nucleosome occupancy in the survivors was comparable to the occupancy seen before induction of a DSB, indicating that the lower relative occupancy seen immediately after repair is complete is not permanent, but is restored after one or more cell cycles (Figure 2C). After repair is complete, cells deactivate the DNA damage checkpoint and resume cell division. It was possible that reestablishment of occupancy does not occur after repair, but only occurs in the subset of cells that have repaired and resumed the cell cycle, passing through S phase before the 6 h time point. To test if resumption of the cell cycle following repair is required for the partial reestablishment seen in WH207 6 h after HO induction, we monitored nucleosome dynamics in cells that were already arrested in G2/M by treatment with nocodazole. Three hours after treatment with nocodazole, approximately 90% of the cells appeared arrested as dumbbell-shaped cells, consistent with G2/M arrest. We then induced HO and monitored both repair and nucleosome dynamics. As previously reported, repair in nocodazole is less efficient than in cycling cells [32]. Six h after induction of HO, repair in WH207 was approximately 60% complete, when compared to a survivor (Figure 1D). The relative occupancy at the *5S* nucleosome before HO induction was comparable to that in cycling cells (Figures 2B and 2D). As seen in cycling cells, 2 h after induction of a DSB, *5S* nucleosome protection decreased to 15% and then, 6 h after HO induction, occupancy in G2/M-arrested cells was partially re-established to approximately 60% (Figure 2D).

To determine if the reduced in peak protection seen following repair reflects reduced total occupancy at the *5S* locus we calculated the area under the curve in steady state (0 h) and following repair (6 h) in our different conditions (Figure 2E). As seen for the peak protection, the total occupancy 6 h following HO induction was reduced in MT093, WH207 and WH207 arrested in G2/M using nocodazole, while in the survivors total occupancy was completely reestablished. This result supports our observation that partial re-establishment of occupancy during HR occurs as a consequence of repair, without need for progression through a particular stage of the cell cycle; however progression through one or more cell cycles is required for complete re-establishment of nucleosome positioning.

4.5 A single cell cycle is sufficient to re-establishment of nucleosome occupancy following HR

We then wanted to see if a single passage through the cell cycle is sufficient for reestablishment of pre DSB nucleosome occupancy. Cells arrested at the “start” point where Cdk1 (Cdc28) is inhibited cannot complete DSB repair [33, 34]; however cells arrested beyond “start” but before the initiation of DNA replication, by inhibiting Cdc7, the catalytic subunit of the Dbf4 dependent kinase (DDK) are repair-proficient [33, 34]. We integrated an auxin-inducible degron (AID) [24] into both Cdc7 and Dbf4 in strain MT093. In this a-mating background we synchronized cells in G1, using the mating type pheromone α -factor, and then added auxin to degrade Cdc7 and Dbf4, ensuring that all cells arrested prior to S phase. We confirmed arrest prior to S phase by flow cytometry (Figure S3A). MT093 treated with α -factor arrest in G1 3 h after treatment (Figure S3A). Likewise, Cdc7-AID Dbf4-AID

cells treated with 500 μ M auxin (3-indoleacetic acid) arrested prior to S phase 3 h after treatment (Figure S3A). To further validate the arrest imposed by the Cdc7-AID and Dbf4-AID we transformed our Cdc7-AID Dbf4-AID with a vector containing herpes simplex virus thymidine kinase (HSV-TK) and human equilibrative nucleoside transporter (hENT1) [35]. This vector allows the incorporation of the thymidine analogue bromodeoxyuridine (BrdU) into the genome. BrdU incorporation can be detected using an antibody against BrdU. We synchronized cells in G1 using α -factor for 3 h, added BrdU and auxin for 1 h and then released them by filtration and two washes with DDW into either auxin arrest (G1/S), nocodazole (G2/M) or allowed them to cycle. If Cdc7-AID and Dbf4-AID degradation imposed an efficient G1/S arrest no BrdU incorporation should be detected even 6 h after release from α -factor. We analyzed BrdU incorporation by a slot-blot method (see Materials and Methods). Cells that were released into nocodazole or allowed to cycle incorporated BrdU following release, demonstrating that the arrest imposed by degradation of Cdc7-AID and Dbf4-AID is reversible and that replication proceeds efficiently with 2 – 4 hours following release. Cells that were arrested in G1/S using the AID degrons failed to incorporate BrdU even 6 h after release (Figure S3B), indicating that replication is inhibited under these conditions.

We then monitored repair efficiency in cells arrested prior to S phase by Cdc7-AID Dbf4-AID, as described above, before inducing HO by adding galactose to the medium. In G1/S arrested cells, 6 h after treatment with galactose, the product reached 60% of the level before HO induction (Figure 3A). The nucleosome occupancy at the *.5S* locus, 6 h after HO induction in G1/S, was significantly lower than the occupancy in cycling cells, as seen in the other repair events in G2/M arrested cells and cycling cells (Figure 3B, $p=0.03$ Wilcoxon rank sum test).

Lastly we asked when cells that are released from G1/S following repair are able to reestablish occupancy at the repaired locus. We allowed cells to repair for 6 h in G1/S, after which we released them and allowed them to resume cell cycle progression. Three hours after release (9 h following HO induction) repair remained at 60%, and reached 80% nine hours following release (15 h following HO induction) (Figures 3A). This increase can be attributed either to continuing repair following release, or to the fact that cells that had completed repair would undergo mitosis following release, and outgrow cells that have failed to repair and are still arrested by the DNA damage checkpoint. The occupancy at the *.5S* locus increased 3 h following release from the Cdc7/Dbf4 block, and was significantly higher than the level seen 6 h following HO induction in the G1/S-arrested cells ($p=0.004$, Wilcoxon rank sum test), even though the level of repair had not changed (Figure 3B). The occupancy 3 h following release from IAA was not different than the occupancy in cycling cells ($p=0.39$, Wilcoxon rank sum test), indicating that a single cell cycle was sufficient to lead to complete reestablishment of nucleosome occupancy. The level of occupancy 9 h following release from G1/S arrest (15 h following HO induction) did not increase further and remained similar to the level of occupancy 3 h after release (9 h following HO induction, $p=0.09$, Wilcoxon rank sum test) (Figure 3B) supporting the conclusion that reestablishment of occupancy is completed within one cell cycle following repair.

5. Discussion

Although chromatin remodeling has been shown to be important for both homologous recombination and DNA damage checkpoint regulation, little is known about nucleosome dynamics during homologous recombination. Previous studies employed ChIP for histones around the *MAT* locus during mating type switching to infer chromatin dynamics during repair [30]. While this study has provided suggestions about the role of chromatin remodelers and histone modifications during repair, it did not identify the exact position of re-established nucleosomes nor the kinetics of achieving full occupancy. Inserting the *5S* nucleosome-positioning element from *X. laevis* to the right of the *ZIZ2* region in *MAT α* provided us with a well-positioned nucleosome that shows high degree of occupancy in the proximity of a DSB. Using this system we provide evidence of the complex dynamics of repair-dependent reestablishment of nucleosome occupancy.

5S nucleosome occupancy is reduced 2 h after induction of a DSB, and is completely lost 2 h to 3 h following HO induction in a *rad52*⁻. Similarly, sequences near the DSB are only fully resected approximately 2 h to 4 h after DSB induction [4, 31]. It is possible that resection leads to the eviction of the *5S* nucleosome 2 h after DSB induction, although previous studies show that the RSC complex is responsible for repositioning nucleosomes around an irreparable DSB [7, 8]. These studies have suggested that 1 h after the DSB nucleosome remodeling is restricted to the two nucleosomes surrounding the DSB, and this remodeling is mostly expressed as sliding of these two nucleosomes; however it should be noted that at the normal *MAT* locus there is only very weak nucleosome positioning to analyze. Sliding or repositioning of the *5S* nucleosome would appear as the migration of the peak occupancy to a new position or a broader positioning landscape and lower occupancy due to the distribution of protection in the population. We do not detect repositioning of the *5S* nucleosome or broader distribution of occupancy, but rather a loss of occupancy with no change of positioning. This observation indicates that at the time points we measure, the *5S* nucleosome is likely evicted, although we cannot entirely rule out sliding of this nucleosome prior to its eviction.

In the system we designed, repair - as monitored by primer extension - is complete 6 h after induction of a DSB. Although nucleosome occupancy is partially re-established at the *5S* locus at this time, it is significantly lower than the occupancy seen before induction of the DSB and only completely re-established in a survivor, suggesting that cell cycle resumption following repair is required for complete reestablishment of occupancy. Indeed, we show that a single passage through the cell cycle led to a significant increase in occupancy in the *5S* locus and resulted in complete reestablishment of protection.

The re-establishment of highly positioned nucleosomes is reminiscent of the re-establishment of nucleosomes at *HML* or *HMR* when the Sir2 histone deacetylase complex is restored to full activity [36, 37]. This process was shown to require a single passage through S phase but not replication. It is possible, however that the restoration of nucleosome occupancy is separate from re-establishment of histone marks, as recent studies of histone modifications in mammals and yeast have shown that some modifications occur slowly, over several DNA replication cycles [38, 39].

Nucleosome dynamics have been shown to play a role in transcription and replication in yeast and mammals [40–43]. The abundance of nucleosome remodelers and histone chaperones involved in repair of a DSB and regulation of the DNA damage response suggests that nucleosome dynamics play an important role during repair [2–4, 7, 8, 11, 12, 14, 44, 45]. Previous work has shown the involvement of histone chaperones Asf1 and CAF-1 in recovery from the DNA damage checkpoint [15, 30]. Interestingly, a previous study suggested that the acetylation of histone H3-K56 played a key role in the apparent establishment of nucleosome position and the extinguishing of the DNA damage checkpoint [30]. In our present study, nucleosome positioning is not complete when cells are held prior to the initiation of S phase, or when the cells are held in G2/M, but is significantly increases when cells pass through S phase. This suggests that nucleosomes that are repositioned in S phase confer higher protection than nucleosomes in other cell cycle stages. Some histone modifications such as H3-K56 acetylation have been shown to be S phase specific [46–50]. Therefore, an intriguing possibility is that the improved protection in S phase is a result of S phase-specific histone modifications.

Recently we have found that the role of Asf1 in recovery is, at least in part, to bind Rad53 following repair [16]. The role of CAF-1 in recovery, however, remains unclear. CAF-1 has been shown to interact with histone H3 that is methylated at lysine 79 in late S phase, and this may contribute to nucleosome positioning [47]. Like H3-K56 acetylation, H3K79 methylation peaks in S phase [47]. CAF-1, therefore, might be required to regulate reestablishment of nucleosome positioning following repair of DSB. Indeed, both Asf1 and CAF-1 have been shown to be involved in reestablishment of nucleosomes following repair in mammalian cells [51]. A recent study implicated both HIRA replication-independent and CAF-1 replication-dependent pathways in reestablishment of nucleosomes following repair by NHEJ [52]. The results presented here suggest a mechanistic difference between reestablishment of nucleosome occupancy during DSB repair and replication and described a powerful system to further assay the involvement of chromatin remodelers and histone chaperones in re-establishment of nucleosome occupancy following repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We engineered a genetic system to monitor nucleosome dynamics in budding yeast.
- We monitor nucleosome dynamics during repair of a DNA double-strand break.
- Nucleosome protection is lost around a break and re-established following repair.
- Nucleosome protection following repair is only partially re-established.
- A single passage through the cell cycle increases nucleosome protection.

Figure 1. system to monitor nucleosome dynamics around a DSB

A) Schematic of the *MATα-5S* cassette. B) Schematics of repair in strain WH207. C) Viability assays in WH207 (n=3) and MT093 (n=3). Error bars represent standard error. D) qPCR primer extension assay to monitor product formation in strain WH207, MT093 and MT093 *rad52Δ*. WH207, MT093 and WH207 nocodazole n = 3, error bars represent standard error. MT093 *rad52Δ* n=1. E) qPCR assay to monitor nucleosome protection following MNase digestion relative to protection conferred by the *PHO5*+1 nucleosome (termed occupancy) proximal to the DSB. n = 4, error bars represent standard error. F) Protection

conferred by the *PHO5*+1 nucleosome normalized to input DNA. n=3, error bars represent standard error.

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Figure 2. nucleosome dynamics during homologous recombination

A) Nucleosome dynamics at the 5S locus during repair in strain MT093 WT (n=4, error bars represent standard errors) and 6 h following HO induction in strain MT162 (MT093 *rad52Δ*, purple line, n=1). B) Nucleosome dynamics at the 5S locus during repair in strain WH207 (n=4, error bars represent standard errors). C) Reestablishment of nucleosome occupancy at the 5S locus in a survivor following repair in strain WH207 (n=3, error bars represent standard errors). D) Nucleosome dynamics at the 5S locus during repair in G2/M arrested WH207 cells. Cells were treated with 15 μ M nocodazole 3 h prior to HO induction. Arrest

was monitored visually (>90% dumbbells, n=4, error bars represent standard errors). E) Total nucleosome occupancy at the *5S* locus was inferred by calculating the area under the protection curve in 2A–D for 0 h and 6 h. * represents $p<0.05$.

Figure 3. nucleosome dynamics during homologous recombination in G1/S arrested cells

A) Primer extension in strain MT181 arrested in G1/S, allowed to repair in G1/S for 6 h then released and allowed to cycle. n=4, error bars represent standard error. B) Nucleosome dynamics at the *5S* locus during repair in strain MT181 under following release from the G1/S arrest (n=4, error bars represent standard errors).

Table 1

strain list

Strain name	genotype
<i>JKM146</i>	<i>hml ::ADE1 hmr ::ADE1 MATa-inc ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL::HO</i>
<i>JKM161</i>	<i>HMLα MATa hmr ::ADE1 ade1-100 leu2-3, 112 lys5 trp1::hiG⁺ ura3-52 ade3::GALHO</i>
<i>WH207</i>	<i>hml ::ADE1 hmr ::ADE1 MATa-inc ade 3::GAL-HO leu2-3, 112::KAN::MATα-5S</i>
<i>MT093</i>	<i>hml ::ADE1 hmr ::ADE1 MATa-inc ade3 ::GAL-HO leu2-3, 112::HYG::MATa-5S bar1::LEU2</i>
<i>MT128</i>	<i>hml ::ADE1 hmr ::ADE1 MATa-inc ade3 ::GAL-HO leu2-3, 112 ::HYG::MATa-5S bar1::LEU2 yku80 ::NAT</i>
<i>MT174</i>	<i>hml ::ADE1 hmr ::ADE1 MATa-inc ade3 ::GAL-HO leu2-3, 112 ::HYG::MATa5S bar1 ::TIR1::URA3 CDC7-AID::KAN DBF4-AID::LEU2 pJH2082 (p405 – BrdU – Inc)</i>
<i>MT162</i>	<i>hml ::ADE1 hmr ::ADE1 MATa-inc ade3 ::GAL-HO leu2-3, 112 ::HYG::MATa-5S bar1 ::LEU2 rad52 ::KAN</i>
<i>MT181</i>	<i>hml ::ADE1 hmr ::ADE1 MATa-inc ade3 ::GAL-HO leu2-3, 112 ::HYG::MATa-5S bar1 ::TIR1::URA3 CDC7-AID::KAN DBF4-AID::LEU2</i>

Table 2

oligos to assay protection at the 5S nucleosome

Oligo name	sequence	pair position (center of the product relative to HO cut site in bp)
Z1Np4 Z1Np3-RC	CGCAACAGTATAATTTATAAACCC CTTCAGCATAATTATTCGCAACC	28bp
Z1Np3 Z1Np2-RC	GGTTGACGAATAATTATGCTGAAG CAGTACTCGAAAGATAACAACC	95bp
Z1Np5 Z1Np6-RC	GAAGATGTGTTTGTACATTTGGCC GGGGAGTTTCAAATAGGATAGC	142bp
Z1Np2 Z1Np1-RC	GGTTGTTTATCTTTTCGAGTACTG GAGTGTATAACAAACATTGGGAAC	178bp
Z1Np6 Z2Np2-RC	GCTATCCTATTTGAACTCCCC CAAAAGAGGCAAGTAGATAAGGG	221bp
Z1Np1 Z2Np1-RC	GTTCCCAATGTTTGTATACACTC GCTTGAGTCTGAGTAATATCATATTTATAC	256bp
Z2Np2 5Sp10-RC	CCCTTATCTACTTGCCTCTTTTG CCCTGGAAGTTATTCGTTGC	299bp
Z2Np1 5Sp2-RC	GTATAAAATATGATATTACTCAGACTCAAGC GGGTCAGGGATGTTATGACGTC	329bp
5Sp10 5Sp11-RC	GCAACGAATAACTTCCAGGG GTAGGCTCTTGCTTGATGAAAG	379bp
5sp14 5sp15-RC	GGGATTTATAAGCCGATG ATATTCAGCATGGTATGGTCG	396bp
5Sp2 5Sp1	GACGTCATAACATCCCTGACCC GTGATCGGACGAGAACCGG	407bp
5sp16 5sp17-RC	CCCTTTAAATAGCTTAACCTTC CTATGCTGCTTGACTTCGG	433bp
5Sp11 5Sp12-RC	CTTTCATCAAGCAAGAGCCTAC CCAAGTACTAACCGAGCCC	451bp
5sp15 5sp18-RC	CCATACCATGCTGAATATACC CCAGGCGGTCTCCCATCC	471bp
5Sp1-RC 5Sp3	CCGGTTCTCGTCCGATCAC GAATTCCCAGGAATTGCGTATTC	485bp
5sp17 5sp19-RC	CGAAGTCAAGCAGCATAGG GATCCAAATAGGCAATGGG	511bp
5Sp12 5Sp13-RC	GGGCTCGGTTAGTACTTGG GGCCGCACTAGTGATCC	525bp
5sp18 5sp20-RC	GGATGGGAGACCGCTGG CTCCCATATGGTCGACCTG	546bp
5Sp3-RC 5Sp4	GAATACCGAATTCCTCGGGAATTC CTATAGGGCGAATTGGAGC	565bp
5Sp13 5Sp5	GGATCACTAGTGC GGCC CAAGGCGATTAAAGTTGGG	625bp
5Sp4-RC 5Sp5	GCTCCAATTCGCCCTATAG CAAGGCGATTAAAGTTGGG	638bp
5Sp5-RC 5Sp6	CCCAACTTAATCGCCTTG CATTCAGGCTGCGCAACTG	722bp
R3p3 5Sp10-RC	GTAGTTCATAAATAACGTATGAGATC CCCTGGAAGTTATTCGTTGC	0 bp (HO cut site)

Oligo name	sequence	pair position (center of the product relative to HO cut site in bp)
Pho5orf+50 Pho5orf+100	CGCTTCTTTGGCCAATGC GGGTACCAATCTTGTCGAC	

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