ATR-like kinase Mec1 facilitates both chromatin accessibility at DNA replication forks and replication fork progression during replication stress

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Faithful DNA replication is essential for normal cell division and differentiation. In eukaryotic cells, DNA replication takes place on chromatin. This poses the critical question as to how DNA replication can progress through chromatin, which is inhibitory to all DNA-dependent processes. Here, we developed a novel genome-wide method to measure chromatin accessibility to micrococcal nuclease (MNase) that is normalized for nucleosome density, the NCAM (normalized chromatin accessibility to MNase) assay. This method enabled us to discover that chromatin accessibility increases specifically at and ahead of DNA replication forks in normal S phase and during replication stress. We further found that Mec1, a key regulatory ATR-like kinase in the S-phase checkpoint, is required for both normal chromatin accessibility around replication forks and replication fork rate during replication stress, revealing novel functions for the kinase in replication stress response. These results suggest a possibility that Mec1 may facilitate DNA replication fork progression during replication stress by increasing chromatin accessibility around replication forks.

[Keywords: ATR; DNA replication; Mec1; S-phase checkpoint; chromatin; chromatin accessibility; replication fork progression]

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et al. 1998). To alleviate this strong inhibitory effect, eukaryotic cells have evolved multiple mechanisms, such as removal of H2A–H2B dimers by the FACT complex as well as covalent modification of specific histone residues by histone-modifying enzymes traveling with the elongating Pol II (Selth et al. 2010). These results suggest that similar to transcription, chromatin changes may take place during replication to accommodate replisome progression. However, to date, these changes have remained elusive, perhaps because many of the studies to date were done using asynchronously growing cell populations.

The intra-S-phase checkpoint (checkpoint hereafter) plays key roles in both ORI firing and replication progression. Checkpoint protein functions have been best characterized in the presence of replication stress, during which replication forks slow down and/or stall [Friedel et al. 2009]. Replication stress leads to targeting of multiple proteins to stalled replication forks, which initiates a cascade of events to activate the checkpoint response. In S. cerevisiae, a central player in this reaction is the protein kinase Mec1 [ATR in metazoans], which directs a number of events to ensure cell survival [Friedel et al. 2009]. These include delaying the time of late ORI firing [Santocanale and Diffley 1998; Shirahige et al. 1998; Alvino et al. 2007] and prevention of replication fork collapse [Lopes et al. 2001; Tercero and Diffley 2001]. Understanding the molecular mechanisms by which the checkpoint regulates multiple aspects of DNA replication and genome stability is a major challenge in the field.

In this study, we sought to determine the properties of replicating chromatin under replication stress conditions in cells synchronously undergoing DNA replication. Surprisingly, our results indicate that the positions of nucleosomes change very little, if at all, during DNA replication. We extended our analysis of chromatin structure by developing a novel method to measure accessibility of chromatin to micrococcal nuclease [MNase hereafter] that is normalized for histone density, which we named NCAM [normalized chromatin accessibility to MNase] assay. This approach has led to the discovery that nucleosomes at and around DNA replication forks increase accessibility to MNase as compared with nonreplicating chromatin both in normal S phase and during replication stress conditions. We further found that the checkpoint kinase Mec1 is required for normal chromatin accessibility around forks under replication stress, revealing a previously unknown function of this kinase. In addition, using the mec1-100 allele, we provide strong evidence that Mec1 promotes replication fork progression during replication stress. These results suggest an interesting possibility that Mec1 may facilitate replication fork progression by increasing chromatin accessibility.

Results

Chromosome-wide mapping of genomic regions undergoing DNA replication

DNA replication is a very fast and dynamic process that progresses through the entire genome. Therefore, in order to map the regions of active DNA replication for the analysis of chromatin structure, we required the cell populations to proceed synchronously through DNA replication. We therefore performed the first sets of experiments by arresting cells in G1 phase with α-factor, then releasing them into rich medium containing 200 mM hydroxyurea [HU], a drug that decreases the overall rate of fork progression through the reduction of the intracellular dNTP pools [see the Materials and Methods; Koc et al. 2004]. Cells were harvested during G1 arrest (nonreplicating control) and at 60 min post-release into S phase. Both arrest and release steps were monitored by flow cytometry analysis [data not shown]. High-density custom tiling microarrays covering chromosomes III, VI, and XII [Yadon et al. 2010] were used for the subsequent mapping of DNA replication sites and chromatin features.

Two complementary approaches were taken in order to map the sites of active DNA replication. First, we mapped the sites of active DNA synthesis by generating DNA copy number profiles [DNA profiles hereafter] [Yabuki et al. 2002]. By 60 min in HU, firing activity was detected at 23 ORIs across chromosomes III, VI, and XII [Supplemental Table S1]. We refer to these ORIs as early firing, as previously reported [Supplemental Table S1]. As shown in Figure 1, for the early-firing ORI ARS607 [see Supplemental Fig. S1 for additional early ORIs], the maximal signal in DNA profiles coincided with previously published ORI positions [Supplemental Table S1]. ORIs that had not fired by 60 min in HU were considered as either late firing or inactive, as previously reported [Supplemental Table S1], and were used as nonreplicating control regions [Supplemental Fig. S2]. A total of 18 late-firing ORIs were found across our data set [Supplemental Table S1]. Second, we mapped fork positions by chromatin immunoprecipitation [ChIP] of a Flag-tagged DNA Pol I, a replication fork component [Burgers 1998]. As expected, Pol I-enriched regions were only detected in S-phase samples and precisely mapped around the same early-firing ORIs where we detected DNA synthesis [Fig. 1; Supplemental Figs. S1, S2]. Our chromatin preparation protocol consistently yielded samples sonicated to an average fragment size of 250 bp [Supplemental Fig. S3A], which, together with the use of high-density tiling arrays, provided sufficient resolution to clearly distinguish between left and right forks moving away from ORIs [Fig. 1; Supplemental Fig. S1].

Changes in chromatin structure during DNA replication

We next investigated the properties of chromatin in the regions undergoing DNA replication by first mapping nucleosome positions. Cells for nucleosome mapping experiments were harvested simultaneously with those for DNA profile and replication fork mapping [see the Materials and Methods]. Mononucleosomal DNA and genomic DNA from the same pool of cells were competitively hybridized onto our arrays. Surprisingly, at the levels of synchrony and resolution achieved in our experiments, we did not detect notable changes in nucleosome
positions at and around replication forks (Fig. 1; cf. Supplementary Figs. S1 [Nuc panels] and S2 [nonreplicating regions]). Interestingly, the most prominent change in chromatin structure in S phase was that nucleosome signals at and around replication forks were higher than in the G1 sample (Fig. 1; cf. Supplementary Figs. S1 [Nuc panels] and S2 [nonreplicating regions]).

There are two parameters that could affect the nucleosome signal intensity: nucleosome occupancy (fraction of cells within a population that have a nucleosome formed at a given locus at a given time) and accessibility of nucleosome to MNase (Supplemental Fig. S4). For example, it is possible that nucleosome occupancy is generally higher immediately after nucleosomes are newly deposited behind replication forks and gradually decreases as the cell cycle progresses. If this were the case, even if the sensitivity of each nucleosome to MNase stayed constant between G1 and S phases, higher nucleosome signals would be expected from S-phase chromatin. To test whether higher nucleosome occupancy caused the increase in nucleosome signals at forks, we measured histone density independently from MNase by histone H3 ChIP following extensive chromatin sonication (Lee et al. 2004; Fan et al. 2008, 2010). Interestingly, histone H3 density at and around forks in S phase was not higher, but similar to or lower, than in G1 phase (Fig. 1; Supplemental Fig. S1). No significant changes in H3 density were detected between G1 and S phase at nonreplicating regions (Supplemental Fig. S2). Similar results were obtained by ChIP using anti-histone H2B antibodies (data not shown). These results demonstrated that increases in nucleosome density are not the cause for the increases in nucleosome signals around replication forks. Rather, the higher nucleosome signals were obtained from regions that have similar or lower nucleosome occupancy.

Nucleosome accessibility increases at and around DNA replication forks

The results above suggested that nucleosomes around replication forks might be more accessible to MNase (Supplemental Fig. S4). Consistent with this, it was recently reported that elevated MNase accessibility leads to higher nucleosome signals at or around transcription start sites of Pol II genes (Weiner et al. 2010). To address this hypothesis, we directly measured the sensitivity of chromatin to digestion by MNase. G1- and S-phase chromatin samples were digested with MNase and analyzed by Southern blot hybridization with probes corresponding to replicating and nonreplicating regions. If our hypothesis is correct, mononucleosomes should be produced more readily from chromatin around replication forks than that from nonreplicating control regions upon MNase digestion. Indeed, larger fractions of mononucleosomal signals were obtained from S-phase samples compared with G1 samples when hybridized with a probe from a replicating region adjacent to ARS606 (Fig. 2A). To confirm that the shift in nucleosome signal was replication dependent, the same membrane was rehybridized with a probe corresponding to a nonreplicating control region in chromosome VI. In this case, we did not detect any significant increase in the relative amount of mononucleosomal signals between G1- and S-phase chromatin (Fig. 2B). Quantification and normalization of these results...
showed that chromatin around replication forks is more readily digested by MNase (Fig. 2C). Similar results were obtained by using a probe adjacent to the early-firing ORIs ARS306 and ARS607 (Fig. 2C). Taken together, these results establish that chromatin is more accessible to MNase around replication forks.

Establishment of a normalized measure of chromatin accessibility to MNase

The finding that chromatin around replication forks exhibits elevated accessibility to MNase prompted us to determine chromatin accessibility during DNA replication in a global fashion using tiled microarrays. Supporting this approach, a recent study demonstrated that more accessible nucleosomes yield higher signals when mapped on microarrays (Weiner et al. 2010). However, as discussed in Supplemental Figure S4, nucleosome signal strengths are affected by both histone density and MNase accessibility of chromatin at any given locus. Therefore, the intensity of nucleosome signals alone cannot be used to assess MNase accessibility. Indeed, currently, there is no available method to determine MNase accessibility of chromatin independently from nucleosome density in a genome-wide fashion. We therefore sought to measure MNase accessibility of chromatin by normalizing nucleosome signals by histone H3 ChIP signal. This method, the NCAM assay, calculates the degree of chromatin digestion by MNase per amount of histone [see the Materials and Methods for details]. We validated the utility of this method by measuring NCAM at gene promoters. As shown in Supplemental Figure S5, NCAM at promoters correlated well with the transcriptional levels of genes at a global level.

We then performed the NCAM assay during DNA replication. As shown in Figure 3A and consistent with results in Figure 1, cells in S phase displayed an increase in NCAM at early efficient ORIs ARS305 and ARS606 but not at late ORIs, where neither DNA synthesis nor Pol I enrichment was detected (Fig. 3B). Importantly, the replication-dependent increase in NCAM does not peak at the sites of maximal DNA signal and extends ahead of Pol I and DNA synthesis regions—in some cases, for several thousands of base pairs. We then averaged data for the most efficient early ORIs in our data set, which, based on their DNA profiles, had fired in 90% of cells, on average (Fig. 3C, early efficient ORIs). To facilitate visualization and quantification of the averaged data, we defined a fork region around ORI midpoints, where the level of Pol I enrichment is above the genome average. The regions upstream of and downstream from the fork region were considered ahead of forks. In agreement with
single ORI analysis (Fig. 3A), NCAM increased by 60 min in HU at and ahead of the fork region, with a distribution similar to that of Pol I ChIP signals and clearly different from DNA copy number profiles (Fig. 3D). At forks, the increase in NCAM is mainly driven by an increase in nucleosome signal and, to a much lesser extent, by a decrease in histone H3 occupancy (Fig. 3D, nucleosome and H3 panels). On the other hand, the increase in NCAM ahead of forks, which extends a minimum of 7 kb (see Supplemental Fig. S6 for broader chromosomal regions),
is exclusively driven by an increase in nucleosome signal, as no significant H3 loss is detected (Fig. 3D, nuc and H3 panels, up and down regions). Similar results were obtained for less efficient early ORIs, except that the decreasing levels of ORI activity [Fig. 3C, early mid and low columns] were associated with decreasing NCAM levels both at the forks and ahead of them [Supplemental Fig. S7, NCAM panels]. In contrast to early ORIs, no NCAM increase was detected at late ORIs, where no replication activity was detected by 60 min in HU, as shown by complete absence of Pol I and DNA signals [Fig. 3E]. In summary, the results above clearly establish that a previously unknown increase in chromatin accessibility occurs at and ahead of replication forks in the presence of replication stress.

**Chromatin accessibility increases around replication forks during normal S phase**

The results above prompted us to ask whether chromatin accessibility also increases around replication forks during normal S phase [in the absence of replication stress]. To address this question, cells were released from α-factor arrest into rich medium at a temperature of 24°C to slow down forks. To compensate for the higher replication fork asynchrony later in S phase, which is caused by heterogeneous ORI firing and faster fork progression, cells were harvested at an earlier time point (30 min) for NCAM assay. Despite the fact that a much smaller fraction of cells within a population fired early ORIs and that replication forks progress less synchronously compared with the experiment in the presence of HU [cf. Figs. 3D and 4A [DNA and Pol1-3Flag panels]], we observed a sharp increase in NCAM around replication forks [Fig. 4A]. The increase in NCAM was not detected in non-replicating control regions [Fig. 4B]. These results clearly demonstrate that an increase in chromatin accessibility around replication forks takes place during normal S phase.

**Increased chromatin accessibility at DNA replication forks during replication stress is Mec1-dependent**

Because the S-phase checkpoint controls many aspects of DNA replication under replication stress [Friedel et al. 2009], we considered the possibility that it might affect chromatin accessibility around forks in the presence of replication stress. To test this hypothesis, we deleted *MEC1*, a key kinase in this checkpoint pathway. *SML1* was simultaneously deleted to allow viability of the *mec1*-null mutants [Zhao et al. 2001]. The *sml1* strain was used as a control. Similar to experiments with wild-type cells, *mec1 sml1* and *sml1* cells were arrested in G1 and released into S phase in the presence of HU. Cells were harvested at an earlier time point in S phase [30 min post-release from α-factor] to compensate for the faster replication progression of the *sml1* background [Poli et al. 2012]. Both *sml1* and *mec1 sml1* mutants showed increases in NCAM at and around forks [Fig. 5A, NCAM panels]. However, quantification by signal integration of the increase in NCAM at early efficient ORIs showed that *mec1 sml1* double mutants have a 45% reduction in accessibility compared with *sml1* cells [Fig. 5C]. This is likely an underestimation, as firing activity at early efficient ORIs was, on average, 13% higher in *mec1 sml1* cells compared with *sml1* cells at the time point at which they were harvested [Fig. 5D].

We next tested whether increased NCAM around replication forks during normal S phase is also Mec1-dependent. As shown in Supplemental Figure S8, NCAM around replication forks in *mec1 sml1* and *sml1* mutants is very similar, showing that the roles of Mec1 in chromatin accessibility control are a part of the replication stress response. Together, these results suggest that Mec1 has a previously unknown role in promoting an increase in chromatin accessibility at and around stalled DNA replication forks in response to replication stress.
Mec1-dependent increase in chromatin accessibility at forks is associated with faster fork progression

Importantly, the results above also meant that we could use the mec1-100 mutant to test whether Mec1 affects the fork progression rate. We used DNA content analysis to estimate the progression rate of forks originating from early efficient ORIs in the presence of 200 mM HU. The fork rate was estimated measuring the distance traveled by forks between 30 and 60 min post-release into S phase (see the Materials and Methods; Supplemental Fig. S10 for complete details). In wild-type cells, the fork rate from the average early efficient ORI was 38 ± 4 bp min⁻¹ [Fig. 6A,B]. Deletion of SML1, which increases dNTP levels [Zhao et al. 1998], resulted in a higher fork rate of 76 ± 4 bp min⁻¹ (P < 0.05, two-tailed t-test) [Fig. 6A,B]. This is in agreement with previous findings that Sml1 increases the fork rate and reduces fork stalling [Zhao et al. 1998]. The results thus suggest that Mec1-dependent chromatin accessibility at forks is positively correlated with fork progression rate.
agreement with a recent report (Poli et al. 2012) that dNTP levels regulate fork progression. We next measured the fork rate in the mec1-100 sml1 mutant. Surprisingly, double-mutant cells displayed an average fork rate of 31.6 ± 2 bp min⁻¹, much slower than that of sml1 cells [P < 0.005, two-tailed t-test] (Fig. 6A,B). The decrease in fork rate in mec1-100 sml1 compared with sml1 is a general phenomenon, as every single fork from early efficient ORIs was significantly slower in the double mutant compared with the single mutant [Supplemental Figs. S11, S12]. These results revealed that Mec1 facilitates replication fork progression during replication stress conditions.

We next asked whether previously described defects in mec1 mutants could explain the slow fork rate in the mec1-100 sml1 strain. It has been shown that Mec1 is required for up-regulating dNTP levels during replication stress conditions (Huang and Elledge 1997), which, as shown above, can impact fork rates. Recently, the intracellular dNTP levels in several checkpoint mutants have been determined (Poli et al. 2012), which revealed that the dNTP levels are higher in mec1-100 sml1 than in

Figure 5. MEC1-dependent increase in chromatin accessibility around replication forks. (A) DNA (DNA, top panels) and NCAM (NCAM, bottom panels) profiles at early efficient ORIs [n = 8] by 30 min in S phase in the presence of 200 mM HU in sml1, mec1-100 sml1, and mec1-100 sml1 mutant strains. (B) Same as in A for Pol1-3Flag signal profiles. (C) Quantification of the relative increase in NCAM between G1 and S phases from A in sml1, mec1 sml1, and mec1-100 sml1 cells upstream of, within, and downstream from replication fork regions. The value upstream of the replication fork region in sml1 cells is set as 1.0. (D) Origin firing efficiencies, as judged by DNA profile analysis, of early efficient and late-firing ORIs in sml1, mec1 sml1, and mec1-100 sml1 cells at the time of the experiment (30 min in HU). Values in panels C and D express the mean ± standard error of the mean from two independent biological replicates.
Replication fork rate is compromised in mec1-100 cells in HU. (A) Average DNA profiles at 30 min [gray line] and 60 min [black line] from early efficient ORIs (n = 8) in wild type (WT), sml1, and mec1-100 sml1 cells. Data were smoothed using a moving average window of 800 bp. (B) Average replication fork rates from two independent biological replicates measured from 30 to 60 min in 200 mM HU in the same strains as in A. Error bars indicate the standard error of the mean.

Figure 6.

Discussion

NCAM assay around replication forks

In this study, we described the NCAM assay, a new method to measure chromatin accessibility to MNase in a way normalized for histone density. Although it has been shown that MNase accessibility strongly affects the strength of nucleosome signals upon nucleosome mapping (Weiner et al. 2010), a genome-wide measurement of chromatin accessibility to MNase has not been reported. The utility of the NCAM assay as a biologically meaningful parameter of chromatin structure was demonstrated by applying it to chromatin around replication forks and gene promoters. DNase I, the most commonly used endonuclease to measure chromatin accessibility, preferentially digests DNA in the long linkers between nucleosomes, such as nucleosome-depleted regions [NDR] at gene promoters (Bell et al. 2011), as well as sites where DNA structure is distorted (Zhu and Thiele 1996). In contrast, the NCAM assay will be a useful tool in the study of chromatin structure in a wide variety of contexts in which access of protein factors to chromatin plays significant roles.

By measuring NCAM at different genomic locations as they undergo replication, we found that accessibility of chromatin to MNase significantly increases around replication forks during normal S phase as well as in the presence of replication stress caused by HU. How does chromatin accessibility increase around replication forks? This change is mainly driven by increased nucleosome signals and, to a much lesser extent, by decreased nucleosome occupancy. This means that the increased MNase sensitivity of nucleosomes is mainly responsible for the increased NCAM. It has been proposed that nucleosomes newly deposited after replication are hypersensitive to nucleases (Seale 1975, 1978; Hildebrand and Walters 1976). However, the increase in normalized chromatin accessibility coincides with the presence of the replisome [DNA Pol I ChIP signals], not with newly synthesized DNA on which nucleosomes are newly deposited. Moreover, increased accessibility extends several kilobases ahead of the replisomes. Therefore, it is highly unlikely that the nuclease hypersensitivity of newly deposited
nucleosomes is the major cause for the increased chromatin accessibility that we observed. We envision that changes in the properties of nucleosomes, such as altered histone–DNA contacts or topological constraints on nucleosomal DNA, may be responsible for the increased accessibility of chromatin to MNase around replication forks. Besides changes in nucleosome accessibility, we also detected a reduced histone density at the regions where Pol I ChIP signals peak. The peak of Pol I marks the regions where the replisome is present in the highest fraction of cells within the population. The fact that the same decrease in histone density was detected using antibodies against both histones H3 and H2B likely reflects transient loss of entire nucleosomes at replication forks, as previously suggested (Tabancay and Forsburg 2006). This result strongly argues against the possibility that the increase in chromatin accessibility at the forks is due to the presence of nucleosomes lacking specific histones.

**Mec1 promotes chromatin accessibility and replication fork progression**

The S-phase checkpoint regulates diverse aspects of DNA replication under stress conditions that are directed to ensure cell survival, among which are the stabilization of replication forks and the delay in late ORI firing. The highly conserved Mec1 kinase plays central roles in the S-phase checkpoint [Friedel et al. 2009]. Here, we present evidence that Mec1 function is required for the maintenance of high levels of both chromatin accessibility around replication forks and replication fork progression rates under replication stress conditions. One exciting possibility is that Mec1 facilitates the replication fork rate by increasing chromatin accessibility at and ahead of replication forks, although we cannot exclude other possibilities. For example, because Mec1 phosphorylates a large number of replisome components [Friedel et al. 2009], it may also facilitate replication fork progression by activating some of them. Our results suggest that, besides generally promoting a slower S-phase progression through the inhibition of late ORI firing [Santocanale and Diffley 1998; Shirahige et al. 1998], the S-phase checkpoint specifically helps forks maintain their progression rate. We hypothesize that both functions contribute to alleviate the deleterious effects of the nucleotide depletion imposed by HU: While the delay in late ORI firing helps maintain higher dNTP levels and other resources for replication forks originated from early ORIs, the checkpoint facilitates the progression of the large replication complex, possibly by making chromatin more accessible, such that S phase would not be unnecessarily long during this vulnerable state.

How does Mec1 promote chromatin accessibility? One possibility is that Mec1 increases chromatin accessibility through modification of replisome components. Alternatively, Mec1 may function through activation of chromatin remodeling factors, some of which are targeted to stalled replication forks [Papamichos-Chronakis and Peterson 2008; Shimada et al. 2008; Vincent et al. 2008]. It is also possible that Mec1 activates and/or recruits complexes that covalently modify histones. The fact that the two amino acid substitutions in the mec1-100 allele reside outside of its kinase domain (Paciotti et al. 2001) leads us to speculate that these mutations impair the ability of Mec1 to interact directly with the regulators of chromatin accessibility. In any case, it is worth noting that NCAM does not completely diminish in mec1 and mec1-100 mutant cells, thus suggesting the presence of unidentified mechanisms that function in a MEC1-independent manner to promote chromatin accessibility around replication forks. In addition, the fact that the mec1-100- and mec1-null mutations cause a very similar degree of loss in chromatin accessibility indicates that the functions of Mec1 to promote chromatin accessibility and protect replication forks are genetically separable.

It has been suggested that chromatin structure negatively influences DNA replication fork progression, as the replisome has to progress through nucleosome arrays for this process [Groth et al. 2007]. However, compared with other DNA-dependent processes such as transcription and DNA repair, much less is known about how chromatin structure is regulated for specific steps in DNA replication. Here we showed that chromatin accessibility increases around replication forks during normal S phase and replication stress conditions. We also found that this chromatin regulation is partially dependent on the checkpoint kinase Mec1 during replication stress, revealing a novel aspect of replication stress response. Finally, our work revealed that Mec1 facilitates replication fork progression during replication stress. Based on these results, we suggest a model in which Mec1 facilitates replication fork progression through increased chromatin accessibility. Together, these results provide a step toward elucidating the molecular mechanisms by which chromatin is regulated to control DNA replication and replication stress response.

**Materials and methods**

**Yeast strains, cell culture synchronization, and sample cross-linking**

The yeast strains used in this study (Supplemental Table S2) are congenic to W303-1a in which the weak rad5 mutant allele in the original W303 has been corrected [Thomas and Rothstein 1989, Zhao et al. 1998]. At least two independent biological replicates were used for each experiment. Cells were grown in YPD medium at 30°C, arrested in G1 with 5 μg/mL α-factor, and released into YPD containing 200 mM HU, as previously described [Unnikrishnan et al. 2010]. Cells for genomic DNA, nucleosome harvest, ChIP, and flow cytometry analysis were simultaneously collected and cross-linked at the time of harvest in G1 and S phase [30 and 60 min after α-factor release]. Samples were cross-linked with 0.5% (genomic DNA and nucleosome harvest samples) or 1% (ChIP samples) formaldehyde for 20 min at room temperature. Cross-linking reactions were stopped with 0.125 M glycine.

**Genomic DNA copy number profiles**

For each S-phase time point, DNA copy number profiles were obtained by competitive hybridization of S-phase DNA against G1 DNA as previously described [Yabuki et al. 2002].
Nucleosome harvest

Mononucleosomal DNA was prepared as previously described (Whitehouse et al. 2007), typically obtaining 60%-80% mononucleosomal DNA [Supplemental Fig. S3B,C]. Nucleosome maps were obtained by competitive hybridization of labeled nucleosomal DNA against labeled genomic DNA from the same time point.

ChIP

Chromatin was sonicated using a Bioruptor sonicator bath (Diagenode) to an average fragment size of 250 bp [Supplemental Fig. S3A], as extensive sonication has been previously shown to increase ChIP resolution (Fan et al. 2008). Soluble chromatin was immunoprecipitated as previously described [Gelbart et al. 2005; Lindstrom et al. 2006] using antibodies against Flag (Sigma), H3 C terminus [Active Motif], or H2B [Active Motif]. Immunoprecipitation efficiency was routinely checked by Western blot. Libraries for array hybridization of both immunoprecipitated and input DNA were obtained using ligation-mediated PCR (LM-PCR) or whole-genome amplification (WGA, Sigma) strategies.

Tiling array hybridization and data processing

Samples were fragmented and labeled as previously described (Whitehouse et al. 2007). Labeled samples were competitively hybridized to custom tiling microarrays (Roche Nimblegen), as previously described [Yadon et al. 2010]. Both strands of chromosomes III, VI, and XII were tiled with 50-mer probes overlapping by an average of 42 bp, covering 14% of the yeast genome. Only data corresponding to the forward strand were used in this analysis. NimbleScan software was used to obtain Tukey by-weight mean-adjusted log2 Cy5/Cy3 ratio files. Both raw and analyzed hybridization files are available for download at http://labs.fhcrc.org/tsukiyama. A pseudomedian smoothing method was applied to all ratio files to eliminate outliers (Royce et al. 2007) using a 25-bp window for smoothing nucleosome data and a 50-bp window for the rest of the data files. A set of custom-made Perl scripts was used to align data according to multiple genomic features and generate all of the averaged profiles.

NCAM assay

Nucleosome mapping data were normalized against histone H3 ChIP data by subtraction of the log2 values. The fact that whole nucleosome signals, not just peak heights or valleys, are integrated in this analysis makes the NCAM assay insensitive to differences in translational nucleosome positioning, as such differences do not alter the overall nucleosome signal across large genomic regions. To normalize for sample-to-sample differences in NCAM signals due to differences in MNase digestion, which have been shown to significantly affect nucleosome signals (Weiner et al. 2010), we performed Z-score transformation (Cheadle et al. 2003). In the Z-score transformation, values are expressed as a unit of standard deviation (SD) from a normalized mean of 0, therefore allowing for direct sample-to-sample comparisons. Briefly, for each NCAM data file, the mean and SD were calculated from a region encompassing 1 kb upstream of and 2 kb downstream from 673 ORFs aligned at their transcription start sites. None of the ORFs included in this analysis were replicated downstream from 673 ORFs aligned at their transcription start sites. None of the ORFs included in this analysis were replicated downstream from 673 ORFs aligned at their transcription start sites. None of the ORFs included in this analysis were replicated downstream from 673 ORFs aligned at their transcription start sites.

Orn classification based on firing activity

A list of all ORI positions included in this analysis as well as their firing activity in HU and ACS locations can be found in Supplemental Table S1. ORI midpoints used in this study were derived from the maxima of the spline-smoothed DNA copy number profiles, similar to previously described [Raghouraman et al. 2001]. ORIs were initially classified as early (n = 23) and late (n = 18) firing based on their activity in 200 mM HU-containing medium such that late ORIs were those exclusively firing in S-phase checkpoint mutants in the presence of HU, as previously reported [Santocanale and Diffley 1998, Shirahige et al. 1998]. ORIs were further classified based on firing activity in a wild-type strain by 60 min in S phase in the presence of 200 mM HU [Supplemental Table S1]. For each ORI, firing activity was calculated as the fraction of cells that had fired the ORI, measured as the maxima of the spline-fitted DNA profile. Thus, firing activity ranged from 0 [nearly replicating] to 1 [fully replicated]. ORIs were classified as being early efficient (firing activity >0.7, n = 8), mid (between 0.7 and 0.40, n = 8), or low (<0.4, n = 7). ORIs were aligned based on the orientation of the T-rich strand, as recently published [Xu et al. 2006, Berbenetz et al. 2010, Eaton et al. 2010].

Southern blot analysis of MNase chromatin accessibility

Following a described method [Fazzio and Tsukiyama 2003], cells from G1 and S phase (30 and 60 min) were treated with increasing amounts of MNase (100, 200, and 400 U) for 5 min at 37°C. For each sample, ~1.5 μg of MNase-digested DNA was loaded onto a 1% agarose gel, transferred to a nylon membrane, and hybridized with radioactive probes. Signals from exposed membranes were quantified using Image Quant software (Amersham), and the fraction of mononucleosome signal versus the total signal in the lane was calculated. We first used a probe corresponding to a region upstream of ARS606 that replicates by 30 min in HU, as seen in DNA profiles. This blot was then stripped and rehybridized with a probe corresponding to a region in chromosome VI that does not replicate during the course of the experiment (negative control). The mononucleosome signal fraction from the replicating probe was normalized to that of the nonreplicating probe. Two additional membranes were similarly probed against two other replicating regions adjacent to ARS506 and ARS607 and their nonreplicating control probes in chromosomes III and VI, respectively. The PCR primers used to generate the probes are listed in Supplemental Table S3.

Fork progression rate

The rate of fork progression was measured as the distance traveled between DNA peak edges in successive time points, divided by the time difference in minutes between the two time points, as previously described (see Supplemental Fig. S10 for further details, Sekedat et al. 2010). Data were normalized before the analysis so that nonreplicating regions had an average signal intensity equal to 0. DNA synthesis rates are expressed as mean base pairs per minute ± standard deviation.

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