Nucleosome occupancy as a novel chromatin parameter for replication origin functions

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Eukaryotic DNA replication initiates from multiple discrete sites in the genome, termed origins of replication (origins). Prior to S phase, multiple origins are poised to initiate replication by recruitment of the pre-replicative complex (pre-RC). For proper replication to occur, origin activation must be tightly regulated. At the population level, each origin has a distinct firing time and frequency of activation within S phase. Many studies have shown that chromatin can strongly influence initiation of DNA replication. However, the chromatin parameters that affect properties of origins have not been thoroughly established. We found that nucleosome occupancy in G1 varies greatly around origins across the *S. cerevisiae* genome, and nucleosome occupancy around origins significantly correlates with the activation time and efficiency of origins, as well as pre-RC formation. We further demonstrate that nucleosome occupancy around origins in G1 is established during transition from G2/M to G1 in a pre-RC-dependent manner. Importantly, the diminished cell-cycle changes in nucleosome occupancy around origins in the *orc1–161* mutant are associated with an abnormal global origin usage profile, suggesting that proper establishment of nucleosome occupancy around origins is a critical step for regulation of global origin activities. Our work thus establishes nucleosome occupancy as a novel and key chromatin parameter for proper origin regulation.

[Supplemental material is available for this article.]

DNA replication is an essential process in all organisms, and faithful completion of replication is required for proper cell division, differentiation, and the maintenance of genome integrity (Bell and Dutta 2002). Eukaryotic DNA replication initiates from multiple discrete sites called origins of replication (hereafter, origins). In *Saccharomyces cerevisiae*, origins have an AT-rich DNA sequence known as an autonomously replicating sequence (ARS) (Marahrens and Stillman 1992). However, the ARS consensus sequence (ACS) alone is not sufficient for defining origins, because many ACSs do not initiate replication (Brewer and Fangman 1991; Dubey et al. 1991). A six subunit origin recognition complex (ORC) binds to a limited number of ACSs throughout the cell cycle (Bell and Stillman 1992; Diffley and Cocker 1992). Then in G1, double hexamers of the minichromosome maintenance (MCM) complex are subsequently recruited to origins to form the pre-replicative complex (pre-RC) (Remus et al. 2009). Once the pre-RC is formed, origins are licensed to fire. However, to prevent re-replication and maintain genomic integrity, origin activation must be tightly regulated; thus, limiting replication factors are distributed to only a subset of origins in a given S phase (Fragkos et al. 2015).

Each budding yeast origin has distinct properties. Some origins tend to initiate replication (fire) early in S phase, whereas others tend to fire late in S phase. Moreover, in a given population of cells, each origin has a distinct probability of activation, which is defined as origin efficiency. Although population studies have demonstrated that each origin has distinct timing and efficiency properties (Raghuraman et al. 2001; Yabuki et al. 2002; Alvino et al. 2007; McCune et al. 2008; McGuffee et al. 2013; Müller et al. 2014), DNA combing experiments have shown that DNA replication occurs stochastically in individual cells and, therefore, is not as deterministic as population analyses suggest (Patel et al. 2006; Czajkowsky et al. 2008). These findings can be reconciled by averaging the heterogeneous replication kinetics of a large number of cells, which recapitulates the observed data from population studies (Czajkowsky et al. 2008). This observation has led to the postulation that in yeast, origin firing is a largely stochastic event in which different origins have different probabilities for firing. Importantly, the probability for a given origin to fire is decided in a cell cycle–regulated manner. For instance, the property of origin ARS501 to fire late in S phase is established at some point between G2/M and G1 (Raghuraman et al. 1997).

What dictates origin properties? The local chromatin environment has been suggested to be one of the key contributors in affecting origin activity. For example, introducing a nucleosome within an ARS resulted in marked reduction of origin activation (Simpson 1990). On the other hand, increasing the distance between the nucleosomes surrounding origins also impairs origin functions (Lipford and Bell 2001). Telomeres, which have a repressive chromatin environment, are associated with late firing origins. Because of these reports, many studies have tried to establish the parameters of chromatin that influence origin firing. We and others have shown that histone modifications, especially acetylation, can affect origin timing and efficiency (Vogelauer et al. 2002; Iizuka et al. 2006; Knott et al. 2009; Unnikrishnan et al. 2010). Moreover, genome-wide nucleosome mapping showed that nucleosome positions dictate the association of Mcm2-7 with origins (Belsky et al. 2015). However, local chromatin structures that distinguish origins based on their properties, such as the efficiencies and timing of firing, are still poorly defined.

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In order to identify chromatin parameters that correlate with origin properties, we performed high-resolution histone chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) and found nucleosome occupancy around origins as a novel chromatin parameter for proper origin functions.

Results

Nucleosome occupancy varies across origins in GI and is ORC-dependent

To identify the chromatin parameters that affect origin properties, we systematically analyzed chromatin structure around all 798 predicted origins in the OriDB database (Nieduszynski et al. 2007; Belsky et al. 2015) in G1 phase. Origins are typically located within nucleosome depleted regions (NDRs), which are surrounded by well-positioned nucleosomes (Eaton et al. 2010). However, nucleosome occupancy around origins has yet to be properly analyzed genome-wide. To measure nucleosome occupancy, we performed chromatin immunoprecipitation (ChIP) using antibodies against histones H3 followed by deep sequencing (ChIP-seq). Nucleosome occupancy is often measured by the height of nucleosome signals after micrococcal nuclease (MNase) digestion of chromatin followed by deep sequencing (MNase-seq). However, the levels of nucleosome signals in MNase-seq are strongly affected by the MNase sensitivity of the nucleosome (Weiner et al. 2010; Rodriguez and Tsukiyama 2013; Rodriguez et al. 2014). This bias is particularly prominent for nucleosomes around nucleosome depleted regions (NDRs), which tend to be sensitive to MNase digestion. As a result, the height of nucleosome signals in MNase-seq can be highly variable around NDRs, depending on the strengths of digestion (Supplemental Fig. S1). Although ultrasonication used for chromatin fragmentation in H3 ChIP-seq has its own bias, ChIP-seq data can be normalized against input samples. This alleviates the bias, making H3 ChIP-seq highly reproducible (Supplemental Fig. S1). Therefore, histone ChIP after sonication reflects a more accurate measure of nucleosome occupancy.

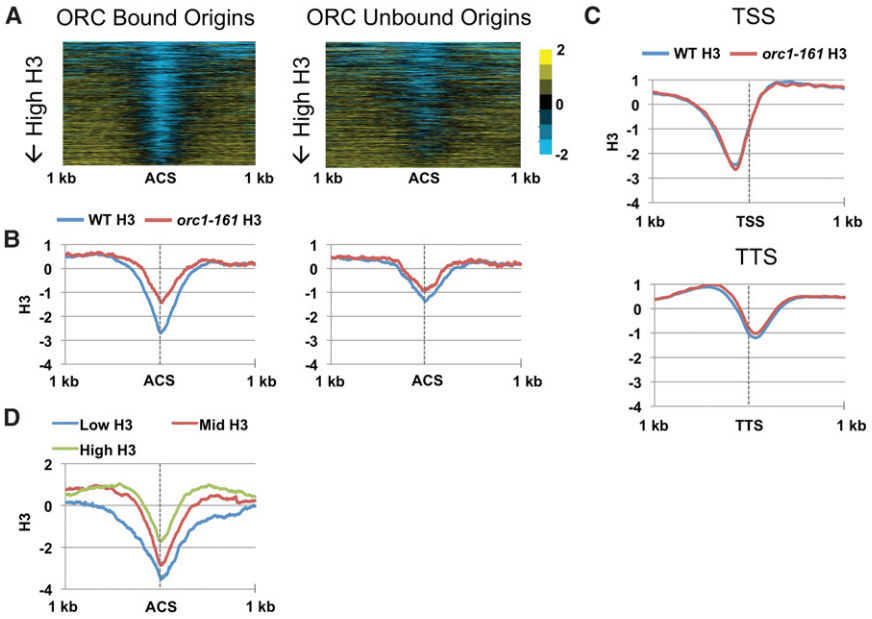

Figure 1. Nucleosome occupancy varies across origins in G1 and is ORC-dependent. (*A*) Nucleosome occupancy (log_2) in G1 aligned around the ACS for 798 predicted yeast origins from the OriDB database (Nieduszynski et al. 2007). Origins are classified based on ORC binding (397 ORC bound and 401 ORC unbound). Origins were aligned from high (*top*) to low (*bottom*) nucleosome occupancies. (*B*) Average nucleosome occupancy after *Z*-score normalization across all ORC-bound (*left*) and ORC-unbound origins (*right*) spanning 1 kb upstream of and downstream from the ACS. The dotted line represents the midpoint of the ACS. WT nucleosome occupancy is shown in blue, and *orc1-161* (at a permissive temperature) H3 is in red. (*C*) Average nucleosome occupancy 1 kb upstream of and downstream from 4551 transcription start sites (TSSs) and 5120 transcription termination sites (TTSs) (Nagalakshmi et al. 2008). (*D*) Average nucleosome occupancy of ORC-bound origins (393) classified based on average H3 levels 500 bp upstream of and downstream from the ACS: Low nucleosome occupancy includes the bottom 33% (blue), mid H3 includes the middle 33% (red), and high H3 includes the top 33% (green).

As expected, nucleosomes are depleted around most ARS consensus sequences (ACSs) as shown previously (Eaton et al. 2010; Belsky et al. 2015). However, surprisingly, nucleosome occupancy in G1 varied very significantly around the 397 ORC-bound origins (Fig. 1A,B, left) (the list of origins, based on Belsky et al. 2015, was provided by the MacAlpine laboratory). As a control, we measured nucleosome occupancy around 401 ORC-unbound ACS sequences (Belsky et al. 2015), which revealed prominently higher levels of occupancy (Fig. 1A,B, right). Because ORC has been shown to position nucleosomes at origins (Lipford and Bell 2001; Eaton et al. 2010), we tested whether ORC affects nucleosome occupancy around origins. The *orc1-161* allele is a temperature sensitive mutation that inhibits ORC from binding DNA at the restrictive temperature (Aparicio et al. 1997). ORC does bind origins at permissive temperatures in *orc1-161*, albeit at slightly reduced levels. All experiments using *orc1-161* allele were performed at a permissive temperature (25°C). H3 ChIP-seq performed in *orc1-161* in G1 revealed higher nucleosome occupancy at ORC-bound origins compared to wild-type cells (Fig. 1B, left). In contrast, nucleosome occupancy was noticeably less affected in the *orc1-161* mutant at ORC-unbound ACSs (Fig. 1B, right), as well as transcription start sites (TSSs) and transcription termination sites (TTSs) (Fig. 1C). Because H3 levels were similar across TSSs in WT and *orc1-161* (Fig. 1C), H3 ChIP signals were *Z*-score normalized at TSSs in the following analyses to account for slight differences in the degree of sonication (for details, see Methods). These results collectively show that nucleosome occupancy greatly varies among origins, and occupancy is reduced in an ORC-dependent manner in G1. We classified ORC-bound origins into three classes (low H3, mid H3, and high H3) based on nucleosome occupancy within 500 base pairs on each side of the ACS (for details, see Methods), and these classes are used in the subsequent analyses (Fig. 1D).

Nucleosome occupancy in GI correlates with origin properties

We next sought to determine whether nucleosome occupancy around origins during G1 correlates with various origin properties. Origin firing time can be defined by origin firing patterns in hydroxyurea (HU) (Belsky et al. 2015), because HU depletes dNTP pools, causing replication fork stall and activation of the S phase checkpoint, which strongly suppresses activation of late-firing

origins. Comparison of origin firing time to nucleosome occupancy revealed a statistically significant correlation between low nucleosome occupancy and early firing times ($P = 3.26 \times 10^{-66}$, χ^2 test) (Fig. 2A). Global origin firing efficiencies were previously established from deep sequencing of Okazaki fragments (McGuffee et al. 2013). We found that lower nucleosome occupancy was associated with higher origin efficiencies, whereas higher nucleosome occupancy was associated with lower efficiencies ($P = 1.31 \times 10^{-97}$, χ^2 test) (Fig. 2B). These results suggest that before cells commit to replicate DNA, chromatin structure is more accessible around early firing and efficient origins. Previously, a systematic comparison of ORC binding properties in vitro on naked DNA versus those in vivo led to the classification of origins into DNA-dependent, chromatin-dependent, and weak ORC binding classes (Hoggard et al. 2013): ORC strongly binds origins both in vitro and in vivo in the DNA-dependent class, whereas it binds origins much more strongly in vivo than in vitro in the chromatin-dependent class. Comparison of nucleosome occupancy around origins with these published ORC binding properties revealed a statistically nonsignificant correlation ($P = 0.119$: Fisher's exact test). However, this is likely due to the small number of origins ($n = 66$) that were classified in this manner, as there was a clear trend that DNA-dependent origins were associated with lower nucleosome occupancy, and chromatin-dependent origins were associated with higher nucleosome occupancy (Fig. 2C). It was also recently reported that Forkhead transcription factors, Fkh1 and Fkh2, bind near origins, and their presence can dictate origin timing (Knott et al. 2012). We found no correlation between origins with altered timing in *Δfkh1fkh2* with nucleosome occupancy ($P = 0.194$, χ^2 test), suggesting that Fkh1 and Fkh2 affect origin properties independently of nucleosome occupancy (Fig. 2D). Taken together, these results establish that nucleosome occupancy in G1 significantly correlates with various properties of origins.

Nucleosome occupancy in GI correlates with pre-RC formation

We next investigated whether nucleosome occupancy around origins correlated with pre-RC formation. Comparison of Mcm2-7 ChIP-seq signals (Belsky et al. 2015) at all ORC-bound origins in G1 with nucleosome occupancy revealed that Mcm2-7 loading is highest at origins with low nucleosome occupancy, with the MCM peak centered around the ACS (Fig. 3A). Interestingly, the average MCM ChIP-seq signals at origins with mid and high nucleosome occupancy were virtually indistinguishable, and unlike with low H3 origins, MCM peaks were present both upstream of and downstream from the ACS (Fig. 3A). Initiation of DNA replication depends on ORC bound at origins (Bell and Dutta 2002). Although ORC binding is detectable at most origins throughout the cell cycle by ChIP (Eaton et al. 2010), ORC footprinting by MNase identified two classes of origins: origins that exhibit ORC footprints at both G2/M and G1, and those that do so only in G1, with the former class enriched for origins that fire earlier and more efficiently (Belsky et al. 2015). We found that origins with lower nucleosome occupancy had a higher frequency of ORC footprinting in G2/M, which was statistically significant ($P = 0.000546$, χ^2 test) (Fig. 3B). These results support the notion that lower nucleosome occupancy around origins is associated with pre-RC formation.

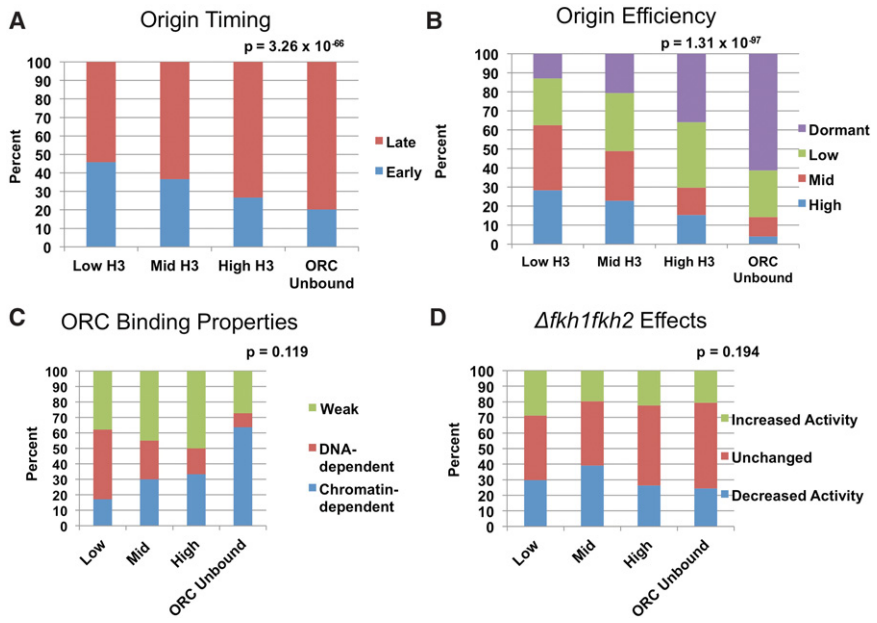

Figure 2. Nucleosome occupancy in G1 correlates with origin properties. (*A*) All origins classified by nucleosome occupancy ($n = 393$) were correlated with origin timing (late or early firing), which was defined by origin usage in the presence of HU (Belsky et al. 2015). (*B*) Correlation of nucleosome occupancy with origin efficiency. Previously established values for origin efficiency metric (McGuffee et al. 2013) were used to classify origins as high (blue), mid (red), low (green), or dormant (purple) efficiency (Methods). (*C*) Nucleosome occupancy correlated with ORC binding properties for 66 origins, which were previously classified as DNA-dependent (red), chromatin-dependent (blue), or weak (green) (Hoggard et al. 2013). (*D*) Nucleosome occupancy correlated with origins where activation depended on Forkhead proteins (Knott et al. 2012). Origins that decrease (blue), increase (green), or are unchanged (red) in activity in the *fkh1fkh2* mutant are shown.

Nucleosome occupancy around ORC-bound origins is cell-cycle regulated

It was previously shown that the late firing property of ARS501 is established between G2/M and G1 (Raghuraman et al. 1997). To determine whether nucleosome occupancy is cell-cycle regulated, we compared nucleosome occupancy around origins in G2/M and G1. This analysis showed that nucleosome occupancy around ORC-bound origins is higher in G2/M, suggesting that nucleosomes around these origins are lost between G2/M and G1 (Fig. 4A). Subtraction of nucleosome occupancy in G1 from that in G2/M (note that the *y*-axis is in $\log_2$ scale) showed that nucleosome occupancy decreases almost twofold on average (Fig. 4B). Interestingly, the strongest loss in nucleosome occupancy takes place immediately adjacent to the ACS, which overlaps with MCM ChIP signals in G1 (Fig. 3A). Ranking origins by the degree of nucleosome loss from G2/M to G1 revealed that almost all ORC-bound origins exhibit significant changes in histone levels within the NDR (Fig. 4C). In contrast, nucleosome occupancy around ORC-unbound ACSs and TTSs changed much less

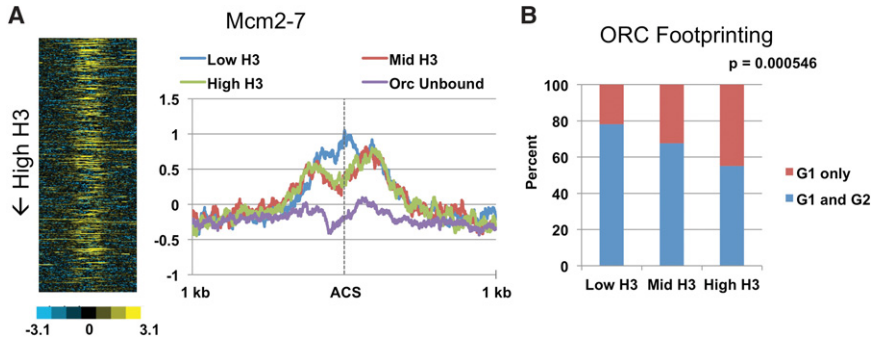

Figure 3. Nucleosome occupancy in G1 correlates with pre-RC binding. (*A*) Mcm2-7 ChIP signals (Belsky et al. 2015) were correlated with nucleosome occupancy: (*left*) heat map of Mcm2-7 signals across all ORC-bound origins, ranked based on nucleosome occupancy; (*right*) average Mcm2-7 binding aligned at the ACS classified based on nucleosome occupancy, with low H3 in blue, mid H3 in red, high H3 in green, and ORC unbound in purple. (*B*) Nucleosome occupancy classes were correlated to cell-cycle ORC footprinting data (Belsky et al. 2015). Blue denotes origins with ORC footprints in both G1 and G2, whereas red denotes those with ORC footprints in G1 only.

between G2/M and G1 (Fig. 4A–C). To determine how much nucleosome loss after G2/M contributes to nucleosome occupancy in G1, we measured the average nucleosome occupancy loss among different classes of origins. This revealed that origins with lower nucleosome occupancy in G1 tend to exhibit greater loss of nucleosomes between G2/M to G1, and vice versa (Fig. 4D). These results demonstrate that the nucleosome loss between G2/M and G1 plays a major role in determining the occupancy in G1.

It was recently reported that nucleosome positions around some origins shift between G2/M and G1, and origins were classified by these nucleosome shifts; those that remain static ("static" class), those that shift upstream of the ACS ("upstream" class), or those that shift downstream from the ACS ("downstream" class) (Belsky et al. 2015). Comparison of the occupancy changes with nucleosome shifts between G2/M and G1 revealed statistically significant correlations ($P = 0.015$, χ^2 test) (Supplemental Fig. S2A), suggesting that origins that are surrounded by changes in nucleosome occupancy tend to exhibit changes in nucleosome positions. Consistent with this conclusion, comparison of cell-cycle changes in occupancy among the three classes of positioning changes revealed that occupancy changes more in "upstream" and "downstream" classes than in the "static" class (Supplemental Fig. S2B). Furthermore, nucleosome occupancy changes slightly more downstream from origins in the "downstream" class. However, cell-cycle changes in occupancy take place similarly on both sides of origins among "upstream" class origins, and occupancy changes are observed among the "static" class of origins, albeit to a lesser degree. Collectively, these results suggest that

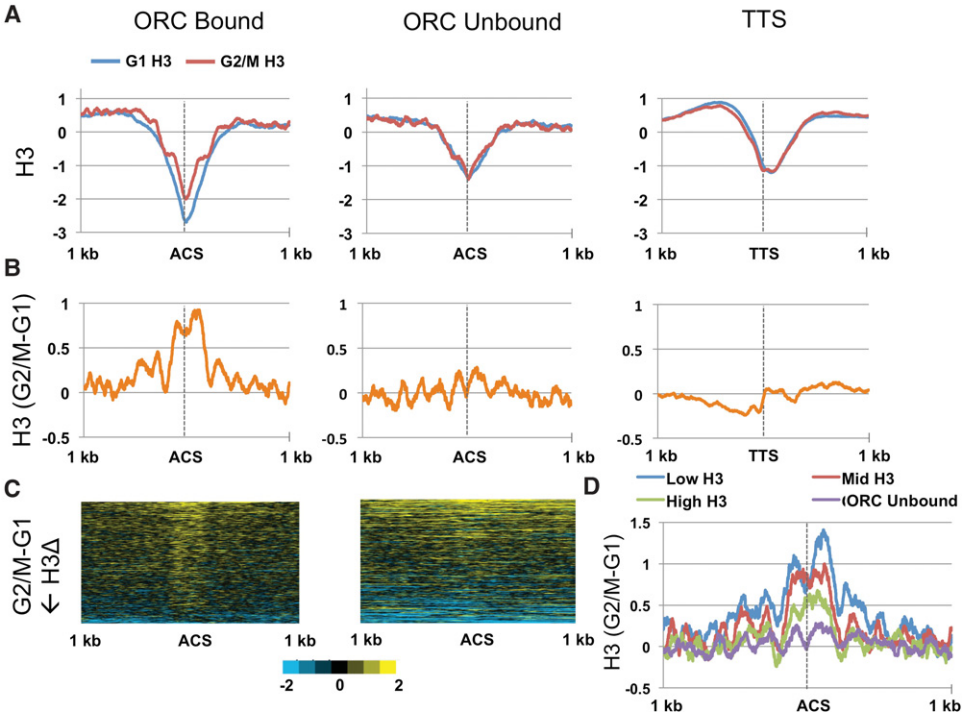

Figure 4. ORC-bound origins lose nucleosomes during the G2/M to G1 transition. (*A*) Average nucleosome occupancy ($\log_2$) within a 2-kb window of ACS for ORC-bound origins (*left*), ORC-unbound origins (*center*), and TTSs (*right*) in G1 (blue) and G2/M (red). The dotted line represents the midpoint of the ACS. (*B*) The difference in nucleosome occupancy ($\log_2$) between G2/M and G1 (G2/M-G1) for ORC-bound origins (*left*), ORC-unbound origins (*center*), and TTSs (*right*). (*C*) Heat maps of the changes in nucleosome occupancy from G2/M to G1 within a 2-kb window of the ACS for ORC-bound (*left*) and ORC-unbound (*right*) origins. Origins are ranked based on the degree of the changes in nucleosome occupancy. (*D*) The decrease in nucleosome occupancy between G2/M and G1 was correlated to origins classified by occupancy in G1. Low nucleosome occupancy class is shown in blue, mid nucleosome occupancy class is shown in red, high nucleosome occupancy class is shown in green, and ORC-unbound ACSs is shown in purple.

cell-cycle changes in nucleosome occupancy and positions are partially correlated.

Changes in nucleosome occupancy between G2/M and G1 are dependent upon pre-RC components

Based on our results that G1 nucleosome occupancy around origins is partially dependent on ORC and is strongly affected by nucleosome loss between G2/M and G1, we hypothesized that nucleosome loss around origins may depend on pre-RC formation. To test this model, we first performed H3 ChIP-seq in *orc1-161* in G2/M at a permissive temperature (25°C). We found that nucleosome occupancy in the *orc1-161* mutant in G2/M is much higher than that of wild-type cells (Fig. 5A, left and center). In addition, there is a much smaller degree of loss in nucleosome occupancy between G2/M and G1 in this mutant as compared to wild-type cells (Fig. 5B, left and center). This was the case across all ORC-bound origins, including those that exhibit the biggest loss of histones between G2/M and G1 in wild-type cells (Fig. 5C, left and center). To test whether the MCM complex affects nucleosome occupancy, we constructed an auxin degron (Nishimura et al. 2009) allele of *MCM2* (*mcm2-AID*). To measure G2/M nucleosome occupancy, we arrested cells in G2/M, added auxin to degrade Mcm2, then performed H3 ChIP-seq. For G1 occupancy, we arrested cells in G2/M, added auxin, then released cells into alpha factor in the presence of auxin (for details, see Methods), followed by H3 ChIP-seq. These experiments showed that the loss of MCM during G2/M does not appreciably affect nucleosome occupancy around origins in G2/M (Fig. 5A, right). However, the loss of histone occupancy between G2/M and G1 is almost entirely abolished in the absence of Mcm2 (Fig. 5A,B, right) to a higher degree than in the *orc1-161* mutant. This was the case across all ORC-bound origins (Fig. 5C). Together, these results demonstrate that cell cycle–dependent changes in nucleosome occupancy at origins are dependent upon the pre-RC.

Changes in nucleosome occupancy at origins are associated with altered origin activities

We next sought to address whether the decrease in nucleosome occupancy between G2M and G1 is important for origin activities. To this end, we took advantage of the fact that the *orc1-161* allele causes a significant loss in cell cycle–dependent nucleosome occupancy changes even at the permissive temperature, a condition in which cells do not exhibit severe growth defects. Genome-wide BrdU profiling experiments revealed that origin firing is markedly altered in the *orc1-161* mutant (see, example, Fig. 6A). At a time point in which cells have undergone similar amounts of global replication, some origins that have relatively low activity in wild-type cells exhibit higher activity in the *orc1-161* mutant, whereas other origins exhibit lower activities in the mutant. Importantly, this pattern is observed across the genome. Although replication profiles are similar between the individual biological replicates for wild type and *orc1-161* ($r^2 = 0.99$ and 0.92), they exhibit markedly different origin activities on a global scale ($r^2 = 0.47$) (Fig. 6B).

To further analyze the effects of the *orc1-161* mutation, we separated origins by timing, then ranked them by efficiency as described (Fig. 6C; McGuffee et al. 2013; Belsky et al. 2015). Columns 1 and 5 show changes in nucleosome occupancy between G2/M and G1 in wild-type cells at early and late firing origins, respectively. Consistent with our preceding conclusions, early and efficient firing origins tend to have greater losses (more yellow) in nucleosome occupancy between G2/M and G1 in WT. Columns 2 and 6 show changes in nucleosome occupancy between G2/M and G1 in *orc1-161* cells at early and late firing origins, respectively. Again, consistent with earlier conclusions, much of the cell-cycle change in occupancy dissipates in *orc1-161*. Columns 3 and 7 demonstrate the effects of the *orc1-161* mutation on cell-cycle changes in nucleosome occupancy, with yellow denoting decreased cell-cycle changes in the mutant, and blue denoting increased cell-cycle changes in the mutant. These columns show that many, but not all, early firing origins exhibit decreased occupancy changes in the mutant, whereas many late firing origins, especially inefficient ones, gain cell-cycle occupancy changes. We then examined how this ranking correlates with the effects of the *orc1-161* mutation on origin activity by taking the difference between WT and *orc1-161* BrdU profiles (columns 4 and 8). Yellow denotes origins that show decreased activity in *orc1-161*, whereas blue indicates origins with increased activity in *orc1-161*. This analysis revealed that the majority of

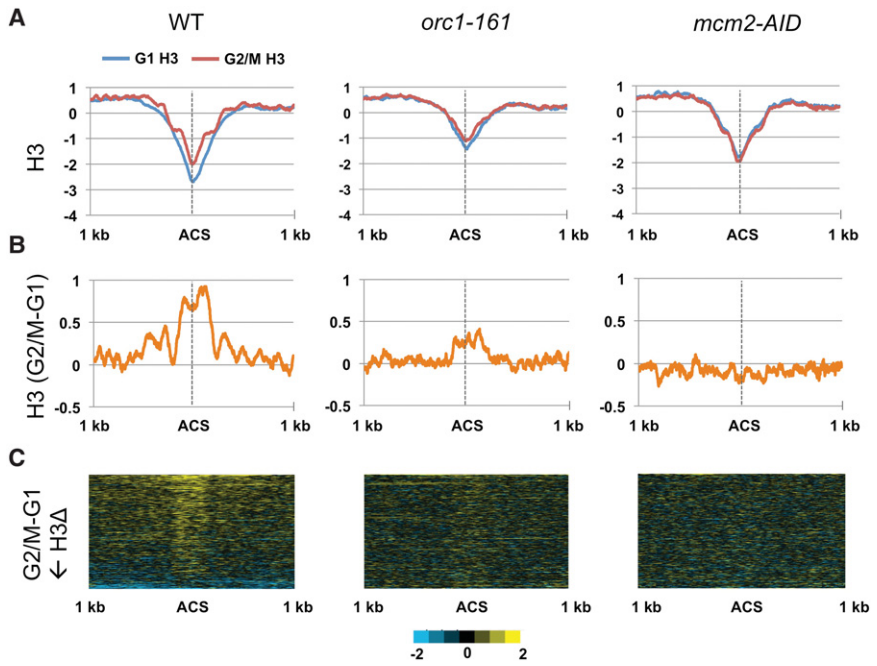

Figure 5. The decrease in nucleosome occupancy around origins from G2/M to G1 is pre-RC dependent. (*A*) The average nucleosome occupancy around ORC-bound origins in wild type (WT, *left*), *orc1-161* (*center*), and *mcm2-AID* (*right*) cells in G1 (blue) and G2/M (red). (*B*) The degree of nucleosome loss between G2/M and G1 ($\log_2$) in WT (*left*), *orc1-161* (*center*), and *mcm2-AID* (*right*) cells. (C) Heat maps of nucleosome occupancy loss at all ORC-bound origins that are ranked by the degree of the loss between G2/M and G1 in WT (*left*), *orc1-161* (*center*), and *mcm2-AID* (*right*) cells. For the purposes of comparison, wild-type profiles are shown again from Figure 4.

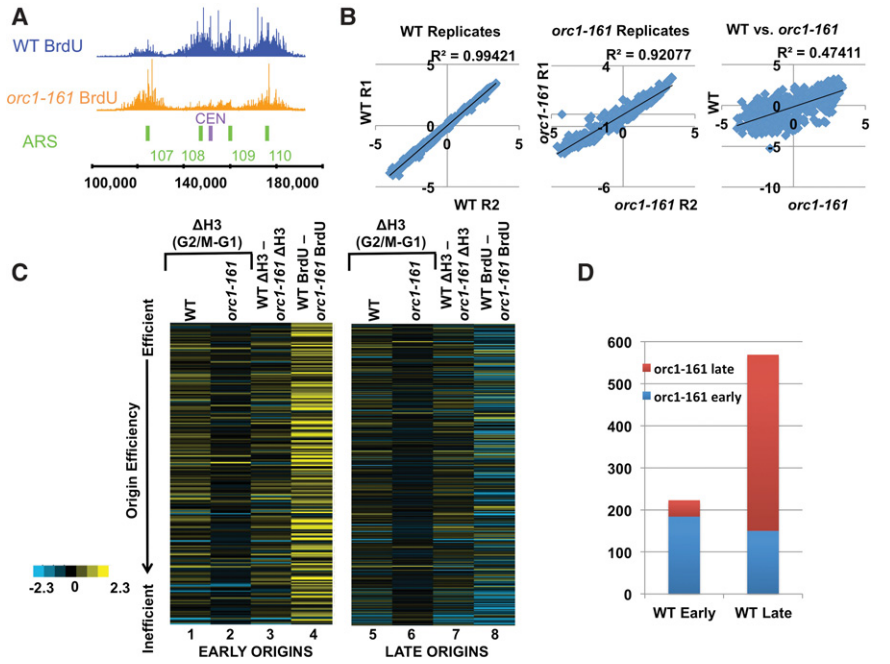

Figure 6. Reduction of nucleosome occupancy loss is accompanied with changes in origin usage profiles in *orc1-161*. (*A*) A snapshot of BrdU profiles for WT in blue and *orc1-161* in orange. The region spans from 100,000 to 200,000 base pairs on Chromosome I. The green bars indicate annotated ARSs, and the purple bar indicates the centromere. (*B*) Graphs correlating averaged BrdU signals from all origins. Biological replicates of BrdU profiles from WT (*left*) and *orc1-161* (*center*) were correlated. WT and *orc1-161* profiles (*right*) were correlated with each other. (C) Heat maps showing the effects of the *orc1-161* mutation on nucleosome occupancy and origin activity. Origins were separated by early and late firing, then ranked based on efficiency (OEM = −1 to 1) (McGuffee et al. 2013; Belsky et al. 2015). Each line represents an origin. Columns *1* and *5* show the changes in nucleosome occupancy between G2/M and G1 in wild-type cells. Columns *2* and *6* show the same in *orc1-161* cells. Columns *3* and *7* show the differences in nucleosome occupancy changes between wild-type and *orc1-161* cells (columns *1* subtracted by *2*, and *5* subtracted by *6*, respectively). Columns *4* and *8* show the differences in origin activity (BrdU signals) between wild-type and *orc1-161* cells. (*D*) The effects of the *orc1-161* mutation on origin firing time. BrdU profiling was performed in *orc1-161* cells in the presence of 200 mM HU; origins that fired under this condition were designated as early firing origins, and those that do not fire were designated as late firing in the mutant. The *left* column shows the origins that fire early in wild-type cells, and the fraction of origins that are early and late firing in *orc1-161* cells. The *right* column shows the origins that fire late in wild-type cells, and the fraction of origins that are early and late firing in *orc1-161* cells.

early firing origins lose activity (column 4), whereas many late firing origins gain activity (column 8) in the *orc1-161* mutant. Comparison of columns 3 with 4, and 7 with 8 revealed that the changes in nucleosome occupancy modestly but significantly correlate with changes in origin activities in *orc1-161* cells ($r = 0.088$, $P = 0.014$, Pearson correlation). These results support the idea that establishing appropriate levels of nucleosome occupancy is important for proper origin usage genome-wide.

Given that the BrdU profiles in Figure 6C columns 4 and 8 were collected at an early time point after release from G1 arrest (Methods), the increased BrdU signals at late firing origins are most likely a result of advanced firing time in *orc1-161* mutants. To test this possibility, we measured origin firing in the presence of 200 mM HU in the *orc1-161* mutant, as the operational definition of early origins in this manuscript is those that fire in HU. We found that the majority (82.5%) of origins that fire early in wild-type cells (Belsky et al. 2015) do so in *orc1-161* cells (Fig. 6D), whereas a significant portion (26.4%) of the origins that fire late in wild-type cells fire early in *orc1-161* cells. This result suggests that the changes in replication profile in the *orc1-161* mutant are at least partially due to altered timing of origin usage.

Discussion

For the first time, we show that origins are surrounded by varying degrees of nucleosome occupancy in G1, and that the loss of nucleosomes between G2/M and G1 plays a major role in establishing nucleosome occupancy around origins. These results suggest that nucleosome occupancy around origins is reset after S phase, then reestablished in every cell cycle for DNA replication. We also provide evidence supporting the notion that this change in nucleosome occupancy between G2/M and G1 around origins is a significant determinant of origin properties. It should be noted that nucleosome occupancy measured by H3 ChIP-seq reflects the fraction of cells within a population having nucleosomes at any given locus. This means that a large fraction of cells lose nucleosomes around origins between G2/M and G1, and that the fraction that loses nucleosomes varies among origins. This nature of nucleosome occupancy is therefore consistent with findings from single-cell analyses that origin firing is stochastic and that origin properties define the probability of origin activation (Czajkowsky et al. 2008).

Our results suggest that there are multiple factors that affect origin properties, and nucleosome occupancy is one of these factors. For example, Fkh1 and Fkh2 (Knott et al. 2012), and the proximity to telomeres and centromeres are known to affect origin properties, but nucleosome occupancy does not significantly correlate with these parameters. Indeed, although *orc1-161* abolishes major changes in nucleosome occupancy at origins between G2/M and G1 and greatly disrupts proper origin usage profiles, changes in nucleosome occupancy do not perfectly correlate with the changes in origin activities. Hoggard et al. (2013) previously showed that ORC binding, and subsequently origin activation, was dependent upon DNA sequence for only a subset of origins, whereas the local chromatin environment was more important for other origins. Our analysis revealed that "chromatin-dependent" origins tend to have higher nucleosome occupancy in G1 and tend to lose nucleosomes to a lesser degree between G2/M and G1. We suspect that "chromatin-dependent" origins are regulated by other chromatin factors, which make these origins rely less on nucleosome occupancy to control their properties. It is not unexpected that origins are regulated by multiple factors, given that strict regulation of origin activation is necessary for proper DNA replication.

However, the fact that most early firing origins lose activities whereas many late firing origins gain activities in the *orc1-161* mutant supports our conclusion that nucleosome occupancy around origins play critical roles in origin usage profiles genome-wide. It was recently shown that co-overexpression of several limiting replication factors cause late firing to fire earlier and/or inefficient

origins to fire more efficiently (Mantiero et al. 2011; Tanaka et al. 2011), leading to a model in which the access of limiting replication factors to origins strongly influences global origin usage patterns. Our results are consistent with this model and provide evidence that nucleosome occupancy is likely one of the key features that dictate which origins are targeted by limiting replication factors.

What is the molecular basis for the loss of nucleosome occupancy around origins between G2/M to G1? Histone-DNA interactions within nucleosomes are very stable, and active mechanisms are usually used to slide, evict, or replace histones at gene promoters for transcriptional regulation (Li et al. 2007). One possibility is that the pre-RC recruits chromatin regulators to facilitate the removal of nucleosomes around origins. The histone chaperone facilitates chromatin transcription (FACT) was shown to bind histones together with Mcm2-7 and maintain proper levels of nucleosome occupancy at subtelomeric regions (Foltman et al. 2013). The *mcm2-2A* mutation in the amino terminal tail prevents Mcm2-7 from binding histones with FACT, but is dispensable for DNA replication (Foltman et al. 2013). We tested whether Mcm2-7 and FACT could regulate changes in nucleosome occupancy from G2/M to G1 by measuring nucleosome occupancy in the *mcm2-2A* mutant. However, the mutant did not exhibit significant changes in the loss of nucleosome occupancy around origins between G2/M and G1 (Supplemental Fig. S3). Although this result does not exclude the possibility that the MCM complex collaborates with unidentified factors, it does suggest that the histone binding activity of Mcm2 alone is not sufficient for regulating levels of nucleosome occupancy at origins. Another possibility is that chromatin regulators could affect pre-RC binding, which in turn can alter nucleosome occupancy at origins. Indeed, nucleosome positions were found to be important for loading Mcm2-7 onto origins in a specific manner (Belsky et al. 2015). We preliminarily screened for potential chromatin regulators using H3 ChIP-seq, but thus far have not found any factor responsible for the reduction in nucleosome occupancy around origins at G1. Although we cannot exclude the possibility that pre-RC formation alone can reduce nucleosome occupancy, this seems highly unlikely because the affinity of ORC to origins alone (Hoggard et al. 2013) cannot explain the nucleosome occupancy in G1. Furthermore, Mcm2-7 ChIP-seq revealed that Mcm2-7 localizes to origins to a similar extent in both mid and high H3 classes of origins, supporting the idea that the change in nucleosome occupancy is a regulated process and not a simple consequence of pre-RC binding. Given that genetic screens for mutants that alter origin properties are likely difficult to establish, biochemical screens for such factors may be more fruitful. Recently established in vitro DNA replication systems (Heller et al. 2011; Yeeles et al. 2015), if combined with nucleosomal templates, will likely be a very powerful tool for this purpose.

Thus far, understanding mechanisms of origin control through chromatin regulation has proven to be difficult because of the contribution of multiple factors. Origin initiation must be strictly controlled but also able to adapt to diverse cellular environments. We propose nucleosome occupancy is an important factor in origin regulation and believe further work will help elucidate how nucleosome occupancy functions, together with other determinants of origin activation, in the regulation of origin activities.

Methods

Yeast strains

The yeast strains used in this study are listed in Supplemental Table S1. All yeast strains are MAT**a** and congenic to W303-1a with a correction for the weak *rad5* allele in the original W303 (Thomas and Rothstein 1989). All the experiments using *orc1-161* allele were performed at a permissive temperature (room temperature). Strains used for BrdU profiles had an integrated BrdU vector as previously described (Viggiani and Aparicio 2006).

Cell synchronization

Yeast strains were grown at 30°C (25°C for *orc1-161*) to early log phase to a density of OD_{660} = 0.2–0.25. Cells were arrested in G1 phase by alpha factor (5 µg/mL) or in G2/M phase by nocodazole (15 µg/mL). After 2 h of G2/M arrest in the *mcm2-AID* strain, 500 µM of IAA was added for 30 min. Half the culture was taken for the G2/M time point, then the rest of the culture was released into alpha-factor and 500 µM IAA for 90 min.

Chromatin preparation and ChIP

Cells were harvested and chromatin was prepared as previously described (Rodriguez et al. 2014). Thirty microliters of Protein G beads (Invitrogen) was conjugated to 5 µL of H3 C-term rabbit polyclonal antibody (Active Motif). Bead conjugation and the ChIP were performed as previously described (Rodriguez et al. 2014).

BrdU profiling

G1 arrested cells were filtered on a 0.45-mm nitrocellulose membrane, washed twice with YPD, and released into half the volume of prewarmed YPD containing BrdU (800 µg/mL) for 33 min at 24°C. For replication profiling of the *orc1-161* mutant in HU, cells were released into YPD containing BrdU and 200 mM HU at room temperature for 60 min. G1 and S phase cells were harvested by adding a 0.1% final concentration of sodium azide. Genomic DNA was prepared, and BrdU-labeled DNA was immunoprecipitated as previously described (Viggiani et al. 2010). To ensure conversion of ssDNA to dsDNA before library prep, 10 ng of DNA was denatured for 5 min at 95°C and incubated with 0.17U Klenow exo- (NEB), 1.2 mM dNTPs, and 1 ng/µL random hexamers (Invitrogen) for 10 min at 22°C and for 30 min at 37°C for a total of two cycles.

Sequencing

ChIP and input sequencing libraries were prepared using the manufacturer's protocol from the NuGEN Ovation Ultralow System v2 (Nugen). Libraries were deep sequenced, aligned, and normalized as previously described (McKnight and Tsukiyama 2015; McKnight et al. 2015).

Analysis and ranking of nucleosome occupancy

Z-score normalization was performed as described previously using nucleosome occupancy from 1 kb upstream of and downstream from 4551 transcription start sites (TSSs) (Nagalakshmi et al. 2008). As a control locus, H3 occupancy was averaged at 5120 transcription termination sites (TTSs) (Nagalakshmi et al. 2008). H3 occupancy was determined for 798 total origins from OriDB (Nieduszynski et al. 2007) aligned at the highest scoring ACS (Eaton et al. 2010). Origins were classified into 397 ORC bound and 401 ORC unbound based on ORC footprinting by MNase-seq (Belsky et al. 2015). To calculate the difference in H3 occupancy between G2/M and G1, the $\log_2$ ratios were subtracted. H3 occupancy was ranked as low, mid, and high based on averaged H3 signal 500 bp upstream of and downstream from the midpoint of the ACS for 393 ORC-bound origins, excluding four origins that

were too close to the ends of chromosomes or poorly annotated regions.

Analysis of origin properties and pre-RC binding

Origin timing data was obtained from hydroxyurea (HU) experiments (Belsky et al. 2015), and origin efficiency data was determined from deep sequencing of Okazaki fragments (McGuffee et al. 2013) for 393 origins. Origin efficiency was classified as follows: The numerical origin efficiency metric (OEM) of 0.6–1.0 was assigned high efficiency, 0.3–0.6 was assigned mid efficiency, 0–0.3 was assigned low efficiency, and 0 and −1 either detected no firing or were in fork merger zones so they were considered as indeterminable. We correlated H3 occupancy with 66 origins that were classified based on differences in ORC binding properties (Hoggard et al. 2013) and used the Fisher's exact test to calculate statistical significance due to the small sample size of origins. A study reported 350 origins that were Fkh1 and Fkh2 dependent (Knott et al. 2012), which was used in our analysis. Mcm2-7 ChIP-seq and ORC footprinting data sets (Belsky et al. 2015) were used for analysis of pre-RC binding. Unless stated otherwise, statistical significance was calculated using the χ^2 test for all correlations.

Analysis of DNA replication

To compare replication, BrdU ratios of wild type or *orc1-161* biological replicates were linearly correlated. BrdU signal was averaged 1 kb upstream of and downstream from the midpoint of the ACS, and the difference in replication was calculated by subtracting $\log_2$ ratios of *orc1-161* from wild type.

Data access

All genomics data, including nucleosome occupancy and replication profile data, from this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE81028.

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Author contributions: J.R. and L.L. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. B.L. performed experiments. T.T. designed experiments, interpreted data, and wrote the manuscript.

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