Replication timing is a crucial aspect of genome regulation that is strongly correlated with chromatin structure, gene expression, DNA repair, and genome evolution. Replication timing is determined by the timing of replication origin firing, which involves activation of MCM helicase complexes loaded at replication origins. Nonetheless, how the timing of such origin firing is regulated remains mysterious. Here, we show that the number of MCMs loaded at origins regulates replication timing. We show for the first time in vivo that multiple MCMs are loaded at origins. Because early origins have more MCMs loaded, they are, on average, more likely to fire early in S phase. Our results provide a mechanistic explanation for the observed heterogeneity in origin firing and help to explain how defined replication timing profiles emerge from stochastic origin firing. These results establish a framework in which further mechanistic studies on replication timing, such as the strong effect of heterochromatin, can be pursued.

[Supplemental material is available for this article.]
that replication timing is regulated by the number of MCMs loaded at each origin.

Results

To test the first prediction of the multiple-MCM model—that early origins have more MCMs loaded than late origins—we examined the genome-wide distribution of MCM by ChIP-seq in G1-arrested cells. As shown on Chromosome 10, the MCM ChIP-seq signal is concentrated at known origins, with more signal at the early origins ARS1012, ARS1014, ARS1018, and ARS1019, than at the late origins ARS1008, ARS1009, ARS1010, and ARS1016 (Fig. 1A). Surprisingly, there is a strong MCM ChIP-seq signal at the late-replicating telomere-proximal origins. The late replication of these origins depends on telomeric heterochromatin (Lian et al. 2011), suggesting that activation of MCM loaded at these origins may be repressed by local chromatin structure (Ferguson and Fangman 1992). Across the genome, there is a significant correlation ($r = 0.42$, $P < 10^{-5}$) between our MCM ChIP-seq signal and the previously inferred origin timing parameter, $n$ (Fig. 1B; Yang et al. 2010). In our mathematical model of replication timing, $n$ is the parameter that describes the firing-time distribution of origins. The model is based directly on the timing of origin firing, not on timing of origin replication (which is influenced both by origin firing and passive replication from the firing of neighboring origins), making $n$ a more direct estimate of origin timing than metrics based on the timing of origin replication. One plausible interpretation of $n$ is that it reflects the number of MCMs loaded at origins (Yang et al. 2010), leading directly to our prediction that MCM ChIP-seq signal should correlate with $n$.

Many origins fall along the MCM/timing diagonal, as predicted. However, many fall above the diagonal. These are origins that have more MCM loaded than would be expected, given their late firing times. These origins include telomeric origins (Fig. 1A), which are known to fire late in a heterochromatin-dependent manner (Ferguson and Fangman 1992; Lian et al. 2011). In addition, the Rpd3 HDAC is known to delay origin firing, presumably through modification of chromatin structure (Knott et al. 2009; Yoshida et al. 2014). Origins controlled by these factors also generally fall above the diagonal (Fig. 1B). Conversely, the Ctf19 kinetochore protein advances pericentromeric origin firing by facilitating the modification of centromeric heterochromatin (Natsume et al. 2013). The Fkh1 transcription factor also affects origin firing, delaying some origins and advancing others (Knott et al. 2012). We find that Fkh1-delayed origins tend to fall above the diagonal; Fkh1-advanced origins show no systematic effect. If these chromatin-influenced origins are excluded from the data set, the correlation between MCM ChIP-seq signal and origin timing increases significantly ($r = 0.54$, $P < 10^{-5}$) (Fig. 1B). Comparing our origin timing data to other published MCM ChIP-seq data sets produces similar correlations ($r = 0.44$ to $r = 0.61$, $P < 10^{-5}$) (De Piccoli et al. 2012; Belsky et al. 2013).
As does comparing our MCM ChIP-seq data to other published estimates of origin timing and/or efficiency \( r = 0.47 \) to \( r = 0.50, P < 10^{-5} \) (Supplemental Fig. S1; Hawkins et al. 2013; McGuffee et al. 2013). From these results, we conclude that the relative number of MCM complexes loaded at origins during G1 regulate their firing timing during S phase. However, these results only elucidate the relative number of MCMs at origins; they do not distinguish between models in which early origins have multiple MCMs (Yang et al. 2010) and those in which early origins have a single MCM complex loaded and late origins have substoichiometric MCM loading (de Moura et al. 2010; Retkute et al. 2011; Hawkins et al. 2013).

To directly assay the number of MCM complexes loaded at origins and thereby test the second prediction—that early origins have multiple MCM complexes loaded—we used a single-origin purification strategy. We engineered different origins into the TALO8 plasmid affinity purification system (Unnikrishnan et al. 2010) and introduced a single binding site for the zinc-finger DNA binding protein Zif268, which binds to its 10-bp recognition site with sub-nanomolar affinity (Elrod-Erickson and Pabo 1999), as an internal control. We tagged MCM2 and ORC2 with the HA epitope and expressed an HA-tagged Zif268 (Supplemental Fig. S2A,B). We then purified TALO8 plasmids containing different origins from G1-arrested cells and determined by Western blotting how many MCM complexes were loaded in vivo, relative to the Zif268 control (Fig. 2A,B). On average, about three MCM complexes (with two molecules of Mcm2 in each MCM complex) are loaded on each plasmid containing the early origin ARS1 (Fig. 2B).

**Figure 2.** ARS1 has multiple MCM complexes loaded in vivo. (A) HA Western blot of protein from Mcm2-HA, Orc2-HA, Zif268-HA cells (yFS853) before and after affinity purification of an ARS1-containing TALO8 plasmid. G1 cells were arrested in α-factor; G2 cells were arrested in nocodazole. (B) Quantitation of eluate data in A. \( n = 7 \) for G1; \( n = 3 \) for G2; error bars represent SEM. (C) Western blot as in A from G1-arrested cells carrying an ARS1-containing (yFS853) or ARS316-containing (yFS855) TALO8 plasmid. (D) Quantitation of data in C. \( n = 7 \) for ARS1, \( n = 3 \) for ARS316, error bars represent SEM.

Assuming that the loading of MCM complexes is not cooperative, \(~90\%\) of the plasmids in the population should have between one and five MCM complexes loaded, and \(~5\%\) should have no MCMs loaded, consistent with an ARS1-plasmid loss rate of \(~3\%\) (Strich et al. 1986). Another early origin, ARS305, also has an average of more than two MCM complexes loaded (Supplemental Fig. S2C, D). Loading of MCMs is specific to G1, with only trace MCM signal seen in metaphase-arrested cells (Fig. 2A,B). Since ORC occupancy at ARS1 has been reported to be high in vivo (Difffley et al. 1994), we attribute the substoichiometric recovery of ORC to loss of ORC binding during purification. We compared the level of MCMs loaded between the early firing ARS1 and a late-firing origin, ARS316. ARS316 has, on average, less than one MCM complex loaded per plasmid (Fig. 2C,D), consistent with its later firing time, its low efficiency, and its high rate of plasmid loss (Poloumienko et al. 2001). These results demonstrate that multiple MCMs can be loaded at a single origin and provide additional evidence for the model that MCM loading regulates origin timing.

To test the third prediction—that reducing the number of MCMs loaded at an origin will delay its average replication time—we compared replication timing between ARS1 and an ARS1 mutation, ARS1-ΔB2, which reduces MCM loading without affecting ORC binding (Zou and Stillman 2000). We verified the MCM-loading defect of ARS1-ΔB2 using the TALO8 system (Fig. 3A,B) and tested replication timing of wild-type ARS1 and ARS1-ΔB2 using a copy number variation assay (Yabuki et al. 2002). Cells containing chromosomal alleles of ARS1 or ARS1-ΔB2 were released from a G1 arrest and sampled throughout S phase (Supplemental Fig. S3). Genomic DNA was prepared, and copy number at selected loci was determined using the NanoString nCounter approach (Geiss et al. 2008). The ΔB2 mutation causes a 13-min delay in the average replication timing \( \langle t_{rep} \rangle \) of ARS1 (Fig. 3C), transforming ARS1 from an early origin to a late origin. These results suggest that the number of MCMs loaded at an origin directly affects its average firing time. Nonetheless, this conclusion rests on the manipulation of MCM levels at only one origin; the ability to manipulate MCM at other origins would increase the generality of this approach.

**Discussion**

The work presented here provides mechanistic insight into how replication timing is regulated in budding yeast. It supports a model (Rhind et al. 2010; Yang et al. 2010) in which replication timing is determined by the stochastic firing of origins that compete for rate-limiting activators (Fig. 4; Patel et al. 2008; Mantiero et al. 2011; Tanaka et al. 2011). In this model, origins that compete more efficiently for limiting activators have a higher probability of firing and thus, on average, replicate earlier. Our results suggest that the number of MCMs loaded at an origin contribute significantly to this competition and influence the origins efficiency and timing. Although our model posits that individual MCMs at an early origin are no more likely to fire than MCMs at a late origin, an origin with more MCMs is more likely to fire early, because the greater number of MCMs increases the chances of one of the MCM firing. The loading of multiple MCMs provides simple and biochemically plausible mechanisms for regulating origin efficiency and timing.

An advantage of the multiple-MCM model is that it does not require an explicit timing mechanism during S phase. Once programmed by MCM loading, origins will naturally fire at characteristic times in S phase without any mechanism that distinguishes early S from late S (Rhind et al. 2010). This mechanism can also
naturally explain the observation that slowing the rate of fork progression proportionally slows the rate of origin firing (Alvino et al. 2007; Rhind 2008; Koren et al. 2010). If a rate limiting factor required for MCM activation travels with the replication fork, such as Cdc45 (Wu and Nurse 2009; Mantiero et al. 2011; Tanaka et al. 2011; Gindin et al. 2014), new origins would be unable to fire until previously initiated forks terminate, coupling origin firing to fork progression (Rhind 2008).

Another advantage of the multiple-MCM model is that it explains how events that impact origin licensing during G1 can affect the timing of origin firing during S phase. The timing and affinity of ORC binding during G1 affects the timing of origin activation (Wu and Nurse 2009; Hoggard et al. 2013). However, since ORC is only active during G1, it has been unclear how G1 ORC activity could affect origin activation during S phase after ORC is inactivated by CDK activity. The loading of multiple MCMs onto early origins provides a memory of G1 ORC activity and stably establishes the replication timing program.

Although our results suggest that the number of MCMs loaded at origins affects origin efficiency, other factors contribute as well, in particular chromatin structure (Rhind and Gilbert 2013). The correlation we see between MCM ChIP-seq signal and replication timing is <0.5 (Fig. 1; Supplemental Fig. S1), consistent with MCM number having a significant but not exclusive role in regulating origin timing. Furthermore, we see a similar level of correlation between other published MCM ChIP-seq and replication timing data sets (Supplemental Fig. S1; De Piccoli et al. 2012; Hawkins et al. 2013; McGuffee et al. 2013; Belsky et al. 2015), suggesting the result is robust to experimental details. The lack of correlation seen with older MCM ChIP-chip data sets (Wyrick et al. 2001; Xu et al. 2006) is presumably due to the lower dynamic range of those data sets.

If we exclude origins that have been reported to be affected by the Rpd3 histone deacetylase, the Ku telomere binding protein, the Fkh1 transcription factor, or the Ctf19 kinetochore protein, we find that the correlation improves to between 0.5 and 0.6. This effect suggests that these factors, all of which affect chromatin structure, modify origin firing independent of MCM number, but also suggest that other as yet unidentified factors also play an important role in regulating origin timing. Chromatin structure could affect origin efficiency at any point in the origin licensing and firing cycle: ORC binding, MCM loading, or MCM activation. However, the fact that we observe large numbers of MCMs loaded at late-replicating telomeres (Fig. 1A) suggests that heterochromatin can delay the firing of loaded MCMs, perhaps by counteracting the activity of rate-limiting activators (Hiraga et al. 2014; Mattarocci et al. 2014).

The multiple-MCM model raises the question of where multiple MCMs may be loaded after loading. Presumably, MCM is initially loaded in the nucleosome-free region next to ORC (Bell and Kaguni 2013). For multiple MCMs to be loaded, MCMs would need to diffuse away from this loading site into surrounding chromatin. Such diffusion has been observed on chromatinized templates in frog egg extracts (Edwards et al. 2002). Furthermore, recent high-resolution ChIP mapping of MCM at budding yeast origins suggests that MCM preferentially associates with origin-flanking nucleosomes (Belsky et al. 2015). Although, on average, the strongest MCM ChIP signal tends to be either at +1 or −1 nucleosome, individual origins show signal across multiple flanking nucleosomes, allowing the possibility that multiple MCMs could associate with multiple nucleosomes in a heterogeneous manner.

The multiple-MCM model is silent as to why more MCMs are loaded at one origin than another. A simple hypothesis is that early origins have higher-affinity ORC binding sites, so that ORC spends more of G1 phase bound at those sites and can load more MCM (Fig. 4). This possibility is supported by a modest, but significant, correlation between ORC ChIP-seq and MCM ChIP-seq signal (r = 0.43) (Supplemental Fig. S1C; Eaton et al. 2010). However, the affinity of ORC for origins is likely determined by more than simply the local origin sequence, because for a significant number of origins, in vivo occupancy is affected by local chromatin structure, as well as the direct affinity of ORC for the origin sequence (Hoggard et al. 2013). Furthermore, other factors, such as trans-acting regulators of origin efficiency and chromatin structure, may also affect the number of MCMs loaded at origins.

Recent results suggest a conceptually similar mechanism may regulate origin timing in human cells (Gindin et al. 2014; Rhind 2014). In human cells, the density of DNase I hypersensitive sites is an excellent predictor of replication timing, with regions dense in DNase I hypersensitive sites replicating early (Gindin et al. 2014). Moreover, a model that uses DNase I hypersensitive sites as a proxy for licensed origins, which are fired by a hypothetical rate limiting activator that moves with replication forks, accurately predicts developmentally regulated replication timing. Importantly, the model does not require that early firing origins have a higher probability of firing than late-firing origins; the higher density of origins in early firing regions is sufficient to increase the chance of such regions replicating early. Thus, in this model, the effect of
the high density of origins in early firing regions of the human genome is conceptually similar to the effect of the high density of MCMS at early origins in the budding yeast genome in the multiple-MCM model, albeit at a much longer length scale (Rhind 2014).

Methods

Yeast strains, derivatives, growth conditions, and synchronizations

S. cerevisiae yeast strains used in this study—listed in Supplemental Table S2—were derived from W303, grown in rich (YPAD) or selective (CM–Leu–Trp) media at 30°C and manipulated using standard methods (Guthrie and Fink 2002). G1 synchronization with α-factor (Sigma) was carried out for 3 h. For bar1A strains, 0.025 μg/mL α-factor was used. For bar1A strains, 5 μg/mL α-factor was used. For release from G1 arrest, Pronase (Sigma) was added to a final concentration of 0.2 μg/μL. G2 synchronization was carried out using 15 μg/mL Nocodazole (Sigma). Flow cytometry was carried out as described (Haase 2004). yFS661 was used for ChIP-seq experiments. TALO8 strain constructions were done as follows: yFS806 contains the original TALO8 plasmid bearing eight lacO repeats with ARS1 (pFS408). Mcm2 was C-terminally tagged in yFS806 with HA using an HphMX6-HA plasmid (pFS152) and primers spd58 and spd59, resulting in yFS844. Orc2 was C-terminally tagged in yFS806 with HA using an HphMX6-HA plasmid (pFS268) and primers spd60 and spd61, forming yFS845. A double-tagged MCM2-HA Orc2-HA strain was created (yFS846) using similar strategy by sequential tagging. BAR1 was deleted in all three strains using a NatMX6 plasmid (pFS274) and primers spd74 and spd75 creating yFS847, yFS848, and yFS849, respectively. yFS850 was created by curing yFS849 of pFS408, yFS833 was used for the NanoString-based replication timing assays. An ARS1–Δ2B derivative of yFS833 (yFS842) was created using the plasmid pFS415 as described (Marahrens and Stillman 1994).

Plasmid construction

Plasmids and oligonucleotides used in this study are listed in Supplemental Tables S3 and S4, respectively. Oligonucleotide-mediated site-directed mutagenesis using primers spd69 and spd70 was used to introduce EagI and Mlu restriction sites into pFS408 to create pFS414. Two complimentary oligonucleotides, spd130 and spd133, were annealed to introduce a single Zip268 binding site in pFS414 to form pFS410. TALO8 derivatives were generated as follows: ARS1 was amplified from yFS661 using primers spd134 and spd135 and cloned into EagI-, Mlu-cut pFS410 to create pFS411. ARS316 was amplified from yFS661 using primers spd126 and spd127 and cloned into EagI-, Mlu-cut pFS410 to create pFS412. ARS305 was amplified from yFS661 using primers spd138 and spd139 and cloned into EagI-, Mlu-cut pFS410 to create pFS416. ARS1–AR2 was amplified from yFS842 using primers spd134 and spd135 and cloned into EagI-, Mlu-cut pFS410 to create pFS413.

MCM chromatin immunoprecipitation

yFS661 was synchronized at G1, formaldehyde cross-linked and subjected to MNase ChIP with rabbit anti-Mcm2-7 polyclonal antibody (UM185, gift from Steve Bell) (Bowers et al. 2004) and protein G agarose (Sigma) as described (Liu et al. 2005). Briefly, 5 × 10⁷ G1-arrested cells were cross linked with 0.1% formaldehyde for 15 min and quenched with 125 mM glycine. MNase-digested chromatin was prepared by spheroplasting cells with Zymolyase 100T (Seikagaku), recovering chromatin by centrifugation, and treating with MNase (Worthington) to digest it to mononucleosomal and sub-mononucleosomal fragments.

Sequence data analysis

The MCM ChIP-seq library was prepared from MCM-ChIPed DNA and sequenced on the Illumina GAII platform according to the manufacturer’s protocols using commercially available reagents (Epicentre). Reads were filtered for quality and aligned to the sacCer1 (R27, October 1, 2003) version of the S. cerevisiae genome using Bowtie 1.0.0 (Langmead et al. 2009), producing 1,376,249 uniquely mapped reads. Reads were output in SAM format and manipulated using SAMtools 0.1.19 (Li et al. 2009). Read density within 1 kb of every origin listed in OriDB (Siow et al. 2012) were calculated using custom Perl scripts. Origins previously identified (Yang et al. 2010) were mapped to OriDB origins by identifying reciprocal nearest neighbors using custom Perl scripts and manual curation. Pearson correlations were calculated in Igl 6.34 (Wavemetrics) and tested for significance using a two-tailed t-test.

TALO8 plasmid purification

Strains containing TALO8 derivatives were grown overnight in CM–Leu–Trp raffinose. At an OD₆₅₀ of 0.2, cells were filtered and inoculated in YP raffinose. After 2 h of growth, α-factor was added; after another 1 h, 20% galactose was added to induce Zip268-HA.
for the last 2 h of synchronization. A similar strategy was used for cells undergoing metaphase arrest except that cells were subjected to nocodazole for 2.5 h. G1 and G2 arrests were confirmed by flow cytometry. For each sample, 400 ODs of cells were collected by centrifugation, washed once with water, and suspended in 5 mL buffer H150 (25 mM HEPES KOH 7.6, 2 mM MgCl2, 0.5 mM EGTA, 0.1 mM EDTA, 10% Glycerol, 150 mM KCl, 0.02% NP40, 1 mM PMSF, and 1× Complete Protease Inhibitor cocktail [Roche]). This slurry was frozen as small pellets in liquid nitrogen and lysed at −196°C using a Retsch MM301 ball mill. Cell powder was thawed in 5 mL H150 lysis buffer on ice and cleared by centrifugation at 27,000 rpm for 90 min. As the input for Western blots, 1/40th of the supernatant was used. Dynabead Protein G (Life Technologies) coupled to FLAG antibody (Sigma) was incubated with the supernatant for 2 h at 4°C. The antibody coupled magnetic beads were washed four times with potassium glutamate buffer (HEPES KOH 7.6, 5 mM magnesium acetate, 1 mM EDTA, 1 mM EGTA, 10% Glycerol, 200 mM potassium glutamate, 0.1% Triton X-100). The protein complexes were eluted with elution buffer (50 mM ammonium bicarbonate, 0.1% SDS), run on a 0.8% polyacrylamide gel, transferred to PVDF membrane (Millipore), and probed with peroxidase conjugated HA antibody (Roche). The membrane was developed using the SuperSignal West Dura extended Duration Substrate kit (Thermo Scientific) and imaged by chemiluminescence on an LAS300 (Fujifilm). Images were quantitated using ImageJ (NIH).

Repllication timing assays
Wild-type (yFS833) and ARS1-AB2 mutant (yFS849) strains grown in rich medium (YPAD) at 30°C were synchronized at G1, filtered, and released into fresh medium in the presence of pronase. In rich medium (YPAD) at 30°C were synchronized at G1, filtered, and released into fresh medium in the presence of pronase.

Data access
The sequencing data from this study have been submitted to the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra/) under accession number SRP040498.

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